Liang-Hong Guo
Monika Mortimer *Editors*

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Preface

Rapid urbanization, increased chemical use, and subsequent accumulation of released polluted sewage and incompletely treated effluents in or near the cities are causing heightened exposures of humans and the environment to pollutants. Many of these polluting chemicals are of unknown toxicity, health hazards, and environmental risks. Thus, it is of utmost importance for the toxicology and exposure research to keep up with the vast array of chemical compounds and their transformation products released in the environment and included in the consumer products and food, to be able to assess health hazards and provide guidance for risk assessment and regulation in a timely manner.

This book addresses the advances in the toxicology of Contaminants of Emerging Concern (CEC) from three aspects: (i) methodological advances, (ii) state of the art of toxicology and mechanisms of action, and (iii) risk assessment. A critical part of assessing the impacts of CEC on the environment and human health is the detection and exposure assessment. Thus, in the first part of the book, recent developments in the methods for detection and quantification of two especially challenging CEC, engineered nanomaterials and microplastics, in environmental matrices are presented and their limitations are discussed, providing guidance for selecting suitable approaches for a specific environmental compartment. In hazard assessment, systemwide, high-throughput, and high-content methods are gaining popularity; hence, the strategies for next-generation toxicology such as omics approaches and the exposome research are discussed. Finally, the latest advancements in using zebrafish as a powerful alternative in vivo model in toxicology, including in high-throughput screening combined with artificial intelligence-assisted data analysis, are described.

The second part of the book includes an up-to-date overview of the toxicology studies and elucidated mechanisms of action of engineered nanomaterials and their co-exposures with other pollutants, micro- and nanoplastics, atmospheric particulate matter, Pharmaceuticals and Personal Care Products (PPCP), and disinfection by-products. Environmental toxicity of metallic, metal oxide, and carbon-based nanoparticles is discussed, stressing the need for increased environmental relevance in future studies, especially considering potential combined toxicity with other pollutants, reviewed in a separate chapter. To accommodate the analysis of large datasets

and bridge the gap between nanotoxicology data and nanomaterial structure-activity relationships, artificial intelligence approaches are becoming increasingly common, discussed in one of the chapters. Regarding other particulate pollutants and different environmental compartments, the effects of microplastics on soil properties and plants, as well as the human health impacts of airborne particulate matter, are critically reviewed. Also, the state of the art of the endocrine-disrupting properties of typical PPCPs, such as bisphenol A and its analogs, triclosan, triclocarban, and phthalates, is discussed, as well as emerging knowledge about the toxicity of disinfection by-products in the drinking water, highlighting the concerns about limited data about the ~700 identified disinfection by-products of which only a subset of compounds is currently regulated.

In the third part, next-generation risk assessment strategies for emerging pollutants are discussed, including adverse outcome pathway networks, in silico toxicity assessment and environmental risk assessment of emerging contaminants based on a case study of nanomaterials. As stated in the concluding chapter of the book, the experience gained during the past 15 years of nanomaterial risk analysis should be used to address potential risks of other emerging technologies and contaminants, especially as the pace of innovation is surpassing the capability of risk identification and quantification.

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Monika Mortimer
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Part I
Methodological Advances in Toxicology and Exposure Measurement

Chapter 1
Quantification and Imaging of Nanomaterials in Biological Samples

Siying Ying and Yuxiong Huang

Abstract Nanoscience and technology are rapidly emerging fields that encompass the design, production, and exploitation of novel structures, materials and devices at the scale of 1–100 nm. However, major concerns have been raised regarding the potential risks to the ecosystem and public health due to the exposure of nanomaterials. It's a great challenge to quantify and image nanomaterials in environmental matrices, particularly in the biological samples, which is essential to assess the consequential risks. This chapter provided a comprehensive review of the advanced methods for quantifying and imaging nanomaterials in biological samples with a detailed discussion on the strengths and limitations. To satisfy the increasing demands on assessing the impacts of nanomaterials at sub-cellular levels, multiple methods should be applied simultaneously in the future.

Sources and Types of Nanomaterials

Nanotechnology and nanomaterials (NMs) have developed rapidly and are widely applied in industries and consumer products. According to European Commission, NM is defined as "a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm–100 nm" (Calzolai et al. 2012). Thus, NMs can be roughly divided into three classes: natural NMs, incidental NMs and engineered NMs.

Natural NMs refer to NMs produced through the natural (bio)geochemical or mechanical processes, without direct or indirect connection to human activity or anthropogenic processes (Hochella et al. 2019). Typical natural NMs include metal oxides, viruses, clay minerals, and sulfides. Weathering and mineral formation processes in soils are the primary producers of natural NMs. There are approximately

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10^{13}–10^{14} tons of inorganic natural NMs in terrestrial environments (Hochella et al. 2019). Every year, approximately 2.36×10^8 tons of airborne natural NMs settle back onto continents.

Incidental NMs are produced unintentionally by human activity. Major amounts of incidental NMs result from the combustion of biomass and fossil fuels, as well as exhaust from industrial and agricultural operations (Hochella et al. 2019). Since the Industrial Revolution, the abundance of incidental NMs has reached similar levels as natural NMs or even exceeded their levels in certain earth compartments. Incidental NMs have complex compositions, consisting of inorganic and/or organic aggregates.

Engineered NMs refer to NMs conceived, designed, and intentionally produced by humans. The engineered NMs are used within an enormous range of commercial products. Estimates of the total global production of engineered NMs were reported to be up to several million tons, though the numbers may vary by orders of magnitude since manufacturers are reluctant to publicize the production volumes (Giese et al. 2018). Engineered NMs usually have distinct shapes or special chemical properties to achieve desired functions.

Bio-Translocation of Nanomaterials

Biological systems are continuously exposed to and affected by natural and anthropogenic NMs, which can translocate to cells, microorganisms and plants via endocytosis and direct permeation, impacting the biological systems.

When NMs are transported into the cell, the most critical barrier is the cell membrane, acting as the selective regulator to transport extracellular matters into a cell. Through many research efforts up to now, it has been found that translocation of NMs across a cell membrane can be classified into two major pathways: endocytosis and direct permeation (Beddoes et al. 2015). NMs adherence to the cell membrane and engulfment at the adhesion site require specific and nonspecific binding interactions to overcome the resistive forces (Nel et al. 2009). The surface ligands of the NMs interact with complementary molecules or receptors on the cell membrane via the specific binding interactions, which is also known as receptormediated endocytosis. On the other hand, direct permeation is a non-endocytic translocation pathway where NMs permeate across the cell membrane without being confined by the endocytic vesicles, leading to direct delivery of NMs into the cell (Nakamura and Watano 2018). It has been recognized that endocytosis is a major translocation pathway when NMs interact with the cells, while the direct permeation is a minor one (Beddoes et al. 2015). Only NMs with a particular size, shape and surface charge, such as functionalized carbon nanotubes (CNTs), cationic QDs, and other metallic NMs, have been shown to cross the cell membrane (Bhirde et al. 2011).

NMs can also translocate into microorganisms via environmental exposure and the food chain. Abdolahpur Monikh et al. (2021) investigated the number of Au NMs in algae and further trophic levels after exposure. The algal samples were analyzed

by single-cell inductively coupled plasma mass spectrometry (ICP-MS) and single-particle ICP-MS (spICP-MS) to quantify the number of cells and the number of Au NMs in and on the cells, respectively. The results showed that Au NMs would be translocated to and accumulated in algae, and the number differed as a function of particle size and shape.

Plants exhibit specific barriers against intruders, including cuticles, exudates, cell walls and pectins. A few important exceptions apply to the continuity of the barriers, which include stomata, eroded cuticles or thinned cell walls, and which can facilitate NM uptake and allow excretion (Schwab et al. 2016). Exudates are excreted into the rhizosphere via the root cap and root hairs of most plants, which can play an essential role in the translocation of NMs. Root exudates (RE) comprise many metabolites (e.g., amino acids, organic acids, sugars, and phenolic), which may impact the fate and behavior of NMs in terrestrial ecosystems. Huang et al. (2017) reported that complex interactions between nano-Cu and RE limited the translocation of nano-Cu into cucumber plants, which reduced the bioaccumulation of Cu in the plants. In addition, stomata are the key sites for transpiration in the aerial parts of phototrophic terrestrial plants, and in a few submerged water plants (Veraverbeke et al. 2003). The stomata of the plant leaves are in the microscale from 1 to 25 μm, and NMs may enter leaf tissues during the exposure (Keller et al. 2018). Keller et al. detected Cu-based nanoparticles (NPs) with a size range from 50 to 150 nm in the leaf tissues of lettuce, collard green, and kale after exposure to CuO NPs.

Nanomaterials' Physicochemical Properties Relevant for Ecotoxicity Assessment

NMs' physicochemical properties, such as chemical forms, particle size, concentration and morphology, significantly affect the NMs' environmental behavior (e.g., dissolution, aggregation and sedimentation) and the consequential ecotoxicity.

Dissolution of NMs

When released into the environment, metal NMs are prone to oxidation and reduction reactions, photochemical, and proton- and ligand-promoted reactions which may result in NM dissolution. The dissolution of NMs would affect the associated environmental mobility and bioavailability, which would further play a role in determining the consequential environmental risks. The chemical stabilities of dissolved metal ions and metallic particles are distinct (Lin et al. 2015). For instance, dissolved Cu (I) released from Cu NPs is readily oxidized to Cu (II) and then complexed in the environment. Studies have suggested that the toxicity of metallic NP suspensions is attributed to the release of metal ions (Li et al. 2011). Song et al. (2015) found that

soluble Cu was the main driver for the toxicological effect of Cu NMs for all the tested fish species. Thus, the levels of environmental standards established by some agencies have various occupational exposure limits for metal ions and metallic NMs. For example, according to the American Conference of Governmental Industrial Hygienists, the occupational exposure limit for metallic silver is 0.1 mg/m^3, while it is 0.01 mg/m^3 for dissolved silver (Drake and Hazelwood 2005). However, the standards set by other agencies have no distinct limits for different metal species. According to Occupational Safety and Health Administration (OSHA), the permissible exposure limits for the total amount of silver, including soluble silver, is 0.01 mg/m^3, while also the permissible exposure limits for copper are the same for metallic and ionic species.

Particle Size

The size of NMs is an important factor that influences the interaction with biological samples and has relevance to ecotoxicity. Kaweeteerawat et al. (2015) found that nanosized Cu caused significant ($p < 0.05$) membrane damage and a decrease in electron transport activity in *Escherichia coli*, while micron-sized particles had no effect. Also, particles with different sizes have distinct sedimentation characteristics. For example, 50-nm CuO had a faster sedimentation rate in seawater than 10-nm CuO, indicating that smaller particles have a longer residence time in suspensions (Torres-Duarte et al. 2016).

Environmental Concentrations and Exposure Levels

The biological effects of NMs highly depend on their environmental concentrations and exposure levels. In Ag NM-exposed wheat plants, there was a dose-dependent reduction in shoot and root lengths (Dimkpa et al. 2013). Similarly, Dimkpa et al. (2015) investigated the effect of CuO NMs on the growth of beans and found that both root and shoot growth was inhibited at the exposure levels of 250 and 500 mg Cu/kg, while no adverse effects were observed at the concentration of 100 mg Cu/kg. However, this does not mean that high concentrations of NMs would necessarily induce negative effects. Hernandez-Viezcas et al. (2011) grew mesquite seedlings in a hydroponic system with ZnO NMs (10 nm) at concentrations varying from 500 to 4000 mg/L. The ICP—optical emission spectrometry (ICP-OES) results showed that Zn bioaccumulation reached the highest level when exposed to 500 mg/L ZnO NMs, while Zn bioaccumulation decreased when treated with 1000–4000 mg/L ZnO NMs (Hernandez-Viezcas et al. 2011). At lower concentrations, NMs exhibited good stability, maintained small diameters and high dispersibility, leading to more NMs and ions available for plant uptake. Larger aggregates would form at a high concentration of NMs, affecting the mobility and bioavailability of NMs. Similar trends

have been found in the effects of carbonaceous nanomaterials (CNMs) on soybean nodulation, dinitrogen fixation potential, and growth in soil (Wang et al. 2017). Nodulation and N_2 fixation potential appeared negatively impacted by CNMs, with inverse dose–response relationships attributed to reduced bioavailability due to the CNMs' aggregation.

Morphology and Shape

Recent experimental evidence has shown that the morphology and shape of NMs play an important role in the associated toxicity. Samei et al. (2019) found that compared to rod-shaped ZnO NMs, spherical ZnO NMs were more destructive to microalgal cells. The addition of 0.7 mg/L of ZnO nano-rods to microalgal samples caused 30% cell death, while 50% cell death was observed by adding the same concentration of spherical ZnO NPs (Samei et al. 2019). Also, it was found that Au NMs with spherical shapes interacted more readily with algae than rod-shaped NMs (Abdolahpur Monikh et al. 2021). When algae were exposed to spherical and rod-shaped Au NMs at the same concentration, 68% of algal cells internalized spherical 10-nm Au NMs, whereas only 34% of cells contained rod-shaped (10 × 45 nm) Au NMs.

In summary, the ecotoxicity of NMs can be caused by several factors, including dissolution, size, concentration and morphology. Due to the complexity and wide array of NM properties, the establishment of environmental standards for the safe levels of NMs is complicated. With the development of visualization and quantification techniques of NMs, it is possible to generate more data that would support and provide evidence for the environmental standards regarding NMs.

Advanced Methods for Quantifying and Imaging Nanomaterials

It is an enormous challenge to conduct qualitative and quantitative analyses of NMs in the environment. For comprehensive characterization of NMs, both quantitative data, including mass-based (or molar) and number concentrations, and qualitative information such as chemical (elemental composition, ionic or metallic species) and physical (e.g., size, shape, and aggregation state) parameters need to be measured. The need for qualitative and quantitative analysis of NMs is driven by the aspect that physicochemical properties of NMs are closely tied to their occurrence, fate and toxicity in the environment (Laborda et al. 2016). The current challenge for analytical scientists is to develop accurate and fast approaches for the detection, characterization and quantification of NMs in complex environmental samples at realistic concentrations.

Imaging Methods

Imaging methods can provide qualitative information about NMs, including size, morphology, localization, and surface coating. Imaging methods can achieve high spatial resolution, sometimes at sub-nanometer resolution. However, imaging methods require careful sample preparations, as imaging is commonly performed in vacuum conditions, which is a great challenge for environmental samples with high moisture content.

Hyperspectral imaging (HSI) provides point by point measurement and analysis of the spectral characteristics, which holds a proven record of success in environmental monitoring. HSI yields a hyperdata cube with continuous spectral and spatial information in one measurement, engaging non-contact and remote sensing (Zamora-Perez et al. 2018). Moreover, HSI is sensitive to subtle spectral changes ensuring thorough discrimination of chemical or biological entities, or tracking agents' changes over time (Gao and Smith 2015). Combining the spatial-scanning HSI with dark-field microscopy is considered highly advantageous for optical studies of nanoscale materials. Hyperspectral-enhanced dark field microscopy (HEDFM) has a powerful resolving capability and is designed to provide in situ optical observation and spectral characterization of a wide range of NMs during their interactions with biological matrixes. The unique interaction between light and matter generates characteristic profiles of scattered light, serving as spectral signatures for each target. The main advantages include the nondestructive nature of the method and the direct sample testing without pretreatment.

Raman microspectroscopy is a label-free technique, which can provide a holistic, real-time representation of the biochemistry of the whole cell, at subcellular levels (Byrne et al. 2020). Based on inelastic scattering of light due to coupling of energy of incident photons with vibrations of target samples, Raman spectroscopy is well established for chemically fingerprinting materials, with applications in, for example, forensics and the pharmacological industry (Vankeirsbilck et al. 2002; Vandenabeele 2004). Similar to infrared vibrational spectroscopic analysis and imaging, Raman microspectroscopy has the advantage of being label-free, producing a spectrum that comprises contributions from each molecular bond, and is a "signature" or "fingerprint" which is characteristic of a material, or changes associated with a physical or chemical process (Byrne et al. 2020). Raman microspectroscopy offers a label-free high content probe of NMs within cells, which can potentially analyze the local environment, fate, and ultimately changes in their cellular metabolism, which can be correlated with cytotoxic responses and oxidative stress or inflammation (Byrne et al. 2020).

Atomic force microscope (AFM) provides images by quantifying the forces between the probe and the sample surface (Delvallée et al. 2013). It can provide information such as size, morphology, and surface characterization at the sub-nanometer resolution in the Z direction. Sample preparation for AFM is a critical step to guarantee the reliability of analysis results, while sample preparation methods can lead

to divergent results. According to Baalousha and Lead (2012), the ultracentrifugation method could be used to study NMs at environmentally relevant concentrations, performing better than the adsorption and drop deposition methods. By combining mathematical calculation and correlation between mass and number concentrations, AFM has been successfully used to detect the number concentration of NPs (Baalousha et al. 2014). However, AFM has a limitation of nonelement specific measurement. And due to the size and geometry of the tip, AFM may broaden lateral dimensions (Delvallée et al. 2013), resulting in poor resolutions in the X and Y direction. In comparison, transmission electron microscopy (TEM) can obtain 0.1 nm resolution in the X and Y directions, so the two techniques can be complementary in measuring the size of NMs (Delvallée et al. 2013).

Transmission electron microscopy (TEM) can be used to visualize NMs and obtain information on the size, number, shape, and aggregation state of NMs. The advantage of TEM over other methods is that it provides accurate particle size measurements compared to optical and centrifugal methods as the latter only provide hydrodynamic sizes. Another advantage of TEM is that it can be used to analyze samples containing a mixture of particles with different shapes, which is not measurable by most other methods. Specifically, high-resolution TEM (HRTEM), which enables visualization of material properties at the atomic scale, allows differentiating engineered NMs (such as carbon nanotubes and metal NPs) from natural particles (Edgington et al. 2014; Maher et al. 2016). NMs can be identified by coupling TEM with spectroscopic analysis, such as energy-dispersive X-ray spectroscopy for metallic NMs (Mielke et al. 2013) and electron energy loss spectroscopy for CNTs (Edgington et al. 2014). However, TEM cannot be considered an ideal approach for NM quantification, as a large number of particles needs to be counted and measured to obtain statistically significant and representative results for average NM size. Walczyk et al. (2010) evaluated about 500 images of nanoparticles to obtain meaningful statistical results for the particle size, which is a time-consuming and low-throughput technique for quantification. Additionally, the high expense and expertise required to operate TEM are potential limitations.

Scanning electron microscopy (SEM) can be used to image the sample surface by scanning it with a high-energy beam of electrons (Sadik et al. 2009). Traditionally, the specimen is required to be completely dry, which is difficult for environmental samples. The drying process may aggregate NMs or change their surface properties in environmental samples. To image NMs in their ambient conditions, environmental SEM (ESEM) was developed. In ESEM, NMs can be imaged under relatively high moisture conditions, which provides a new approach to investigate NMs in natural matrices (Tiede et al. 2009). In addition to ESEM, which allows only the top layer of the wet sample surface to be studied (Bogner et al. 2005), there is a more recently developed method called WETSEM which differs from ESEM mainly in that it can be applied to any SEM and enables observation of liquid samples that are a few microns deep (Dyab and Paunov 2010).

Spectroscopic and Optical Methods

Based on light scattering and light absorption principles, spectroscopic and optical methods can offer quantitative information about NP samples such as number concentration and size distribution.

Ultraviolet–visible (UV–VIS) spectroscopy can be used to identify the concentration of homogeneously dispersed NMs in a solvent. Additionally, it can be adopted to estimate the bandgap energy of NMs and identify specific NPs. Bao et al. (2011) adopted time-dependent UV–VIS spectroscopy to confirm the formation of Ag NPs on graphene oxide (GO) nanosheets. The absorption spectrum of the Ag NP/GO composite suspension showed an absorption band with a maximum at 420 nm, indicating the presence of single spherical or roughly spherical Ag NPs.

Dynamic light scattering (DLS) is the most commonly employed technique to measure nanoparticle size in aqueous suspensions. While commonly conducted in batch mode in a cuvette requiring sample volumes in an mL range, DLS in high-throughput microwell-plate format allows to analyze a 4-μL sample volume. The instrument measures the diffusion coefficient of particles moving under Brownian diffusion conditions. The diffusion coefficient can be extracted by analyzing fluctuations of the scattered light intensity over time. The particle size can then be calculated by applying the Stokes–Einstein equation (Baalousha et al. 2011). However, DLS can provide average hydrodynamic diameter rather than single particle size. Moreover, the NMs are considered spherical by DLS techniques, which may underestimate the actual size of the particles in the dispersion (Brar and Verma 2011).

Nanoparticle tracking analysis (NTA) is a recently developed method to measure NM size and number concentration in liquid dispersions accurately. It relates the rate of Brownian motion to particle size. In the NTA system, NMs are visualized by light scattering using a light microscope. The NTA software tracks the Brownian motion of individual NPs and calculates their size and total concentration (Dragovic et al. 2011). A range of parameters can be adjusted in video capture and analysis, thus allowing the user to optimize particle identification and tracking for a particular sample. NTA successfully addresses one of the key problems with DLS, in that it can resolve and accurately measure particles with different sizes simultaneously within the same solution (Dragovic et al. 2011). NTA also shows high sensitivity in terms of particle number concentration and hydrodynamic size distributions since it detects NPs individually. Compared to electron microscopy, NTA can measure a large number of NMs in much less time, and NMs are not subject to shrinkage artifacts due to fixation. However, since NTA determines particle size based on Brownian motion, it depends on the refractive index. It has a limited capacity to detect weakly scattering NMs due to the low amount of scattered light.

Hyphenated Techniques

Environmental samples are often composed of populations of polydispersed NPs in complex matrices. Separation techniques such as flow field-flow fractionation, capillary electrophoresis and hydrodynamic chromatography have been used to separate sub-populations of NPs or resolve them from complex environmental matrices. Combining separation techniques with detectors can provide a sound foundation for the resolution of complex NP systems.

Flow field-flow fractionation (FLFFF) is a family of chromatography-like separation techniques used for particle and macromolecule sizing and separation (Baalousha et al. 2011). The separation is based on hydrodynamic principles, in which particles are separated due to their interaction with a cross-flow of carrier liquid, applied over the cross-section of a thin, flat channel. Due to the opposed movements of field transport and Brownian motion, a group of particles with the same diffusion coefficient would have a similar retention time to separate the particles (Baalousha et al. 2011).

The FLFFF method can separate NMs continuously, non-destructively, at high resolution with a size ranging from 1 nm to 100 μm (Baalousha et al. 2011). Meanwhile, FLFFF could separate NMs in their native conditions since the carrier liquid can be adapted to the dispersed nanoparticle system, as well as the possibility to couple either on-line or off-line to a wide range of detectors such as UV, ICP-MS, TEM and AFM (Meermann 2015). FLFFF has been applied to analyze NMs (e.g., Ag, Au, Se, SiO_2, TiO_2 and ZnO) in biological samples (Schmidt et al. 2011; Bolea et al. 2014; Poda et al. 2011; Hawkins et al. 2014). Although FLFFF has several advantages compared to other separation and fractionation techniques, it still suffers from certain limitations, such as material losses, particle–membrane interaction, sample dilution, washing of sample components and overloading (Baalousha et al. 2011).

Given the grade of the complexity of the samples analyzed, FLFFF is usually hyphenated to different detectors (Baalousha et al. 2011). ICP-MS is commonly used as an online elemental detector due to its high sensitivity and elemental selectivity (M-M and Siripinyanond 2014). Poda et al. (2011) investigated the use of FLFFF interfaced with ICP-MS as a sensitive and selective method for detecting and characterizing Ag NMs. The fractograms of different sizes of Ag NMs obtained under the standardized processing conditions by FFF–ICP-MS agreed well with the DLS size measurement results (Poda et al. 2011).

Electrophoretic techniques, which are based on the migration of charged species under the influence of an applied electric field, are available in different formats. Capillary electrophoresis (CE) is one of the most commonly used electrophoretic techniques for separating and characterizing NMs (Surugau and Urban 2009). CE only requires a minimal amount (nanoliters) of sample for analysis with high resolution, making it suitable for analyzing precious samples with limited amounts available. However, special attention must be paid to the surface characteristics of the NMs, since distinct surfaces can lead to inconsistent migration behaviors. By adding ionic surfactants, the capability for CE to separate different sizes of metal NPs

enhanced. For instance, Liu et al. (2005) demonstrated that the addition of sodium dodecyl sulfate (SDS) to the background electrolyte improved the size separation of Au NPs as the charge of the NPs was related to the number of molecules of surfactant adsorbed.

Hydrodynamic chromatography (HDC) can separate NMs based on their hydrodynamic diameters (Striegel and Brewer 2012). Like FLFFF, HDC separates NMs based on the particle diffusion coefficients, which are inversely related to their hydrodynamic diameters through the Stokes–Einstein equation (Proulx and Wilkinson 2014). The column void volume can be considered to be a network of flow channels or capillaries in which a parabolic flow profile is established, where larger particles that cannot reach the surface of the beads are eluted faster. Coupling HDC with various detectors enables to gain further knowledge on the size, shape and concentration of NMs. For example, Ag NPs with a radius of 20 nm could be detected in a river water sample at the concentration as low as 4 μg/L by coupling HDC to ICP-MS (Proulx and Wilkinson 2014).

Single-Particle ICP-MS (spICP-MS)

The emerging spICP-MS technology was designed to separate individual NMs at environmentally realistic concentrations from the background, including ionic components (Laborda et al. 2011). The principle is to detect one NP as a single pulse in each reading cycle. spICP–MS operates in a time-resolved mode, and the frequency of signals is directly related to the number concentration of NPs while the intensity of signals is related to NM size (Degueldre and Favarger 2003). spICP-MS can provide information about the number concentration of NP suspensions, as well as about the elemental mass content per NP. With additional information on NP physicochemical properties (shape, composition and density), the core size of the NPs can be calculated, and number size distributions can be obtained. The dynamic range of spICP-MS may be extended up to the micrometer region; also polydispersed systems, aggregation, or agglomeration processes may be studied. In addition, dissolved ions from NPs can also be detected and determined. spICP-MS has been used for screening purposes to detect the presence of NMs and their dissolved ions in biological tissues (Peters et al. 2015) and blood (Jenkins et al. 2015).

spICP-MS has been coupled to different separation techniques. The first online coupling of spICP-MS to hydrodynamic chromatography (HDC) was presented by Pergantis et al. (2012), where Au NPs were separated by their size. In 2016, spICP-MS was coupled online to asymmetric field flow fractionation (AF_4) to fractionate the NMs by their size and also core–shell NPs from mono-component NPs (Huynh et al. 2016). The coupling of separation techniques online to spICP-MS has the potential to answer non-trivial questions in the analysis of mixtures of different NPs, where spICP-MS alone does not provide sufficient information.

Challenges and Future Perspectives

Biological Samples

Most of the environmental and biological samples require pretreatment (e.g., matrix cleanup) prior to the quantification of NMs. Particularly, it is essential to extract NMs from biological samples, however, conventional acid digestion would dissolve and change the physicochemical properties of NMs. To overcome this issue, alkaline reagents have been applied as alternatives to acid digestion. For example, Gray et al. (2013) extracted NMs from tissues via alkaline digestion using tetramethylammonium hydroxide (TMAH). The results proved that TMAH was a highly efficient extraction reagent that didn't cause NM size distribution alterations and the procedure had a high recovery rate. Similarly, enzymatic digestion has been used to degrade proteins in the biological samples (Loeschner et al. 2014) and digest plant cell walls (Dan et al. 2015). When enzymatic or alkaline digestions are used for complex matrices (e.g., animal tissues and plants), care must be taken not to alter the NM properties. Another NM extraction method is cloud point extraction (CPE), which involves the addition of a surfactant at a concentration above its critical micelle concentration (CMC). If the solution temperature is increased above the cloud point temperature of the surfactant, micelles are formed, and hydrophobic compounds are extracted with the surfactant micelles (Duester et al. 2016). CPE has been optimized for metallic NPs (e.g., AuNPs) in complex media such as soil matrix (El Hadri and Hackley 2017). The ultimate goal of any sample preparation step must be a high particle recovery rate and little to no NM transformation (Mozhayeva and Engelhard 2020).

On the other hand, microscopy techniques provide the opportunity to visualize and measure NM size and size distribution, structure and morphology, and the interaction of NMs with environmental compounds without the extraction of NMs from the environmental sample matrix. However, microscopy techniques (AFM, TEM and SEM) have not been effectively applied to analyze the number concentration of NMs in suspension due to the inherent sample preparation issues. Traditional sample preparation protocol based on drop deposition of the sample solution on microscopy substrates provides an inaccurate representation of the NM size distribution and concentration (Baalousha and Lead 2012). For example, evaporating a sample drop into dryness could increase particle and solute concentrations, with the consequent aggregation of particles and the possible precipitation of salts. Multiple approaches are used to preserve the hydrated state of particles, namely by cryofixation or by embedding the particles in a water-soluble resin to fix the water (Hassellöv et al. 2008).

Detection Limits

Due to the development of techniques to image and quantify NMs, the detection limits of NMs have been significantly improved compared to conventional methods. However, there are several challenges to the further improvements of detection limits. For example, the detection limits of DLS depend on the presence of interfering particles such as dust or large-sized particles. The dust would affect diffusion coefficients, and the large size particles increase the light scattering intensity. To eliminate the interference of dust and large-sized particles, pretreatment such as filtration is needed.

The detection limit of spICP-MS is related to the transmission efficiency of the packet of ions generated from each NP through the spectrometer (Laborda et al. 2011). The loss of transmission efficiency may come from adsorption to the nebulizer, NMs surface charges and adsorption to spray chamber walls (Mozhayeva and Engelhard 2020). As for the challenges on the detection limits of NM number concentrations, the data acquisition frequency and background signals should be considered. The acquisition frequency should be set appropriately to avoid multiple NM events, and the signal to background ratio ought to be high enough to extract data produced by NMs rather than natural background.

Future Perspectives

To enhance the performance of quantification and imaging of NMs in biological samples, more attention should be paid to the sample preparation, particularly the recovery of NMs from environmental samples. In addition, the detection limit of NMs should be further improved to meet the requirements of environmental sample analysis, i.e., detection of NMs at low concentrations in complex matrices. For example, one approach taken towards achieving this goal is the optimization of dwell time and/or acquisition frequency to enhance the resolution of NMs in the spICP-MS analysis.

The accuracy of NM analysis could be enhanced by applying multiple methods simultaneously. Laborda et al. (2016) divided the levels of complexity on NM quantification and imaging into three categories. The first level includes NM detection in commercial products, where the concentration of NMs is high. The second level includes ecotoxicology and fate study of NMs in the laboratory, where control experiments can be done and the composition of detected samples is always known. The third level concerns NM detection in complex environmental matrices at realistic concentrations, where the NM type and concentration are unknown. The studies involving the first two levels can lay a solid foundation for the complex third-level application. It is necessary to apply a combination of different techniques to successfully detect and characterize engineered NMs in the environmental samples. As the realistic environmental concentrations of engineered NMs are low, methods like spICP-MS are needed for size and concentration determination. Imaging methods

(e.g., TEM and SEM) can be performed to visualize NMs and approximately confirm the composition of NMs. Additionally, UV–VIS spectroscopy can be applied as a supplementary technique to identify and characterize metallic NMs. DLS can also provide supplementary information about hydrodynamic diameters. Thus, a multi-method technique should be developed in the future to satisfy the urgent need for detecting NMs in real environmental samples with low concentrations and unknown compositions.

References

Abdolahpur Monikh F, Chupani L, Arenas-Lago D et al (2021) Particle number-based trophic transfer of gold nanomaterials in an aquatic food chain. Nat Commun 12(1):899. https://doi.org/10.1038/s41467-021-21164-w

Baalousha M, Lead JR (2012) Rationalizing nanomaterial sizes measured by atomic force microscopy, flow field-flow fractionation, and dynamic light scattering: sample preparation, polydispersity, and particle structure. Environ Sci Technol 46(11):6134–6142. https://doi.org/10.1021/es301167x

Baalousha M, Prasad A, Lead JR (2014) Quantitative measurement of the nanoparticle size and number concentration from liquid suspensions by atomic force microscopy. Env Sci Process Impacts 16(6):1338–1347. https://doi.org/10.1039/C3EM00712J

Baalousha M, Stolpe B, Lead JR (2011) Flow field-flow fractionation for the analysis and characterization of natural colloids and manufactured nanoparticles in environmental systems: a critical review. J Chromatogr A 1218(27):4078–4103. https://doi.org/10.1016/j.chroma.2011.04.063

Bao Q, Zhang D, Qi P (2011) Synthesis and characterization of silver nanoparticle and graphene oxide nanosheet composites as a bactericidal agent for water disinfection. J Colloid Interface Sci 360(2):463–470. https://doi.org/10.1016/j.jcis.2011.05.009

Beddoes CM, Case CP, Briscoe WH (2015) Understanding nanoparticle cellular entry: a physicochemical perspective. Adv Colloid Interface Sci 218:48–68. https://doi.org/10.1016/j.cis.2015.01.007

Bhirde A, Xie J, Swierczewska M et al (2011) Nanoparticles for cell labeling. Nanoscale 3(1):142–153. https://doi.org/10.1039/C0NR00493F

Bogner A, Thollet G, Basset D et al (2005) Wet STEM: a new development in environmental SEM for imaging nano-objects included in a liquid phase. Ultramicroscopy 104(3–4):290–301. https://doi.org/10.1016/j.ultramic.2005.05.005

Bolea E, Jiménez-Lamana J, Laborda F et al (2014) Detection and characterization of silver nanoparticles and dissolved species of silver in culture medium and cells by AsFlFFF-UV-Vis-ICPMS: application to nanotoxicity tests. Analyst 139(5):914–922. https://doi.org/10.1039/C3AN01443F

Brar SK, Verma M (2011) Measurement of nanoparticles by light-scattering techniques. TrAC Trends Anal Chem 30(1):4–17. https://doi.org/10.1016/j.trac.2010.08.008

Byrne HJ, Bonnier F, Efeoglu E et al (2020) In vitro label free Raman microspectroscopic analysis to monitor the uptake, fate and impacts of nanoparticle based materials. Front Bioeng Biotechnol 8:544311. https://doi.org/10.3389/fbioe.2020.544311

Calzolai L, Gilliland D, Rossi F (2012) Measuring nanoparticles size distribution in food and consumer products: a review. Food Addit Contam Part A 29(8):1183–1193. https://doi.org/10.1080/19440049.2012.689777

Dan Y, Zhang W, Xue R et al (2015) Characterization of gold nanoparticle uptake by tomato plants using enzymatic extraction followed by single-particle inductively coupled plasma-mass spectrometry analysis. Environ Sci Technol 49(5):3007–3014. https://doi.org/10.1021/es506179e

Degueldre C, Favarger P-Y (2003) Colloid analysis by single particle inductively coupled plasma-mass spectroscopy: a feasibility study. Colloids Surf Physicochem Eng Asp 217(1–3):137–142. https://doi.org/10.1016/S0927-7757(02)00568-X

Delvallée A, Feltin N, Ducourtieux S et al (2013) Comparison of nanoparticle diameter measurements by Atomic Force Microscopy and Scanning Electron Microscopy. In: Filtz J-R, Larquier B, Claudel P, Favreau J-O (eds) 16th International Congress of Metrology. EDP Sciences, Paris, France, p 06007

Dimkpa CO, McLean JE, Britt DW et al (2015) Nano-CuO and interaction with nano-ZnO or soil bacterium provide evidence for the interference of nanoparticles in metal nutrition of plants. Ecotoxicology 24(1):119–129. https://doi.org/10.1007/s10646-014-1364-x

Dimkpa CO, McLean JE, Martineau N et al (2013) Silver nanoparticles disrupt wheat (*Triticum aestivum* L.) growth in a sand matrix. Environ Sci Technol 47(2):1082–1090. https://doi.org/10.1021/es302973y

Dragovic RA, Gardiner C, Brooks AS et al (2011) Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis. Nanomedicine Nanotechnol Biol Med 7(6):780–788. https://doi.org/10.1016/j.nano.2011.04.003

Drake P, Hazelwood K (2005) Exposure-related health effects of silver and silver compounds: a review. Ann Occup Hyg. https://doi.org/10.1093/annhyg/mei019

Duester L, Fabricius A-L, Jakobtorweihen S et al (2016) Can cloud point-based enrichment, preservation, and detection methods help to bridge gaps in aquatic nanometrology? Anal Bioanal Chem 408(27):7551–7557. https://doi.org/10.1007/s00216-016-9873-5

Dyab AKF, Paunov VN (2010) Particle stabilised emulsions studied by WETSEM technique. Soft Matter 6(12):2613. https://doi.org/10.1039/c0sm00032a

Edgington AJ, Petersen EJ, Herzing AA et al (2014) Microscopic investigation of single-wall carbon nanotube uptake by *Daphnia magna*. Nanotoxicology 8(sup1):2–10. https://doi.org/10.3109/17435390.2013.847504

El Hadri H, Hackley VA (2017) Investigation of cloud point extraction for the analysis of metallic nanoparticles in a soil matrix. Environ Sci Nano 4(1):105–116. https://doi.org/10.1039/C6EN00322B

Gao L, Smith RT (2015) Optical hyperspectral imaging in microscopy and spectroscopy—a review of data acquisition. J Biophotonics 8(6):441–456. https://doi.org/10.1002/jbio.201400051

Giese B, Klaessig F, Park B et al (2018) Risks, release and concentrations of engineered nanomaterial in the environment. Sci Rep 8(1):1565. https://doi.org/10.1038/s41598-018-19275-4

Gray EP, Coleman JG, Bednar AJ et al (2013) Extraction and analysis of silver and gold nanoparticles from biological tissues using single particle inductively coupled plasma mass spectrometry. Environ Sci Technol 47(24):14315–14323. https://doi.org/10.1021/es403558c

Hassellöv M, Readman JW, Ranville JF et al (2008) Nanoparticle analysis and characterization methodologies in environmental risk assessment of engineered nanoparticles. Ecotoxicology 17(5):344–361. https://doi.org/10.1007/s10646-008-0225-x

Hawkins AD, Bednar AJ, Cizdziel JV et al (2014) Identification of silver nanoparticles in Pimephales promelas gastrointestinal tract and gill tissues using flow field flow fractionation ICP-MS. RSC Adv 4(78):41277–41280. https://doi.org/10.1039/C4RA08630A

Hernandez-Viezcas JA, Castillo-Michel H, Servin AD et al (2011) Spectroscopic verification of zinc absorption and distribution in the desert plant Prosopis juliflora-velutina (velvet mesquite) treated with ZnO nanoparticles. Chem Eng J 170(2–3):346–352. https://doi.org/10.1016/j.cej.2010.12.021

Hochella MF, Mogk DW, Ranville J et al (2019) Natural, incidental, and engineered nanomaterials and their impacts on the Earth system. Sci 363(6434):eaau8299. https://doi.org/10.1126/science.aau8299

Huang Y, Zhao L, Keller AA (2017) Interactions, transformations, and bioavailability of nano-copper exposed to root exudates. Environ Sci Technol 51(17):9774–9783. https://doi.org/10.1021/acs.est.7b02523

Huynh KA, Siska E, Heithmar E et al (2016) Detection and quantification of silver nanoparticles at environmentally relevant concentrations using asymmetric flow field-flow fractionation online with single particle inductively coupled plasma mass spectrometry. Anal Chem 88(9):4909–4916. https://doi.org/10.1021/acs.analchem.6b00764

Jenkins SV, Qu H, Mudalige T et al (2015) Rapid determination of plasmonic nanoparticle agglomeration status in blood. Biomaterials 51:226–237. https://doi.org/10.1016/j.biomaterials.2015.01.072

Kaweeteerawat C, Chang CH, Roy KR et al (2015) Cu nanoparticles have different impacts in *Escherichia coli* and *Lactobacillus brevis* than their microsized and ionic analogues. ACS Nano 9(7):7215–7225. https://doi.org/10.1021/acsnano.5b02021

Keller AA, Huang Y, Nelson J (2018) Detection of nanoparticles in edible plant tissues exposed to nano-copper using single-particle ICP-MS. J Nanoparticle Res 20(4):101. https://doi.org/10.1007/s11051-018-4192-8

Laborda F, Bolea E, Cepriá G et al (2016) Detection, characterization and quantification of inorganic engineered nanomaterials: a review of techniques and methodological approaches for the analysis of complex samples. Anal Chim Acta 904:10–32. https://doi.org/10.1016/j.aca.2015.11.008

Laborda F, Jiménez-Lamana J, Bolea E et al (2011) Selective identification, characterization and determination of dissolved silver(i) and silver nanoparticles based on single particle detection by inductively coupled plasma mass spectrometry. J Anal at Spectrom 26(7):1362. https://doi.org/10.1039/c0ja00098a

Li M, Zhu L, Lin D (2011) Toxicity of ZnO nanoparticles to *Escherichia coli*: mechanism and the influence of medium components. Environ Sci Technol 45(5):1977–1983. https://doi.org/10.1021/es102624t

Lin S, Taylor AA, Ji Z et al (2015) Understanding the transformation, speciation, and hazard potential of copper particles in a model septic tank system using zebrafish to monitor the effluent. ACS Nano 9(2):2038–2048. https://doi.org/10.1021/nn507216f

Liu F-K, Lin Y-Y, Wu C-H (2005) Highly efficient approach for characterizing nanometer-sized gold particles by capillary electrophoresis. Anal Chim Acta 528(2):249–254. https://doi.org/10.1016/j.aca.2004.08.052

Loeschner K, Brabrand MSJ, Sloth JJ et al (2014) Use of alkaline or enzymatic sample pretreatment prior to characterization of gold nanoparticles in animal tissue by single-particle ICPMS. Anal Bioanal Chem 406(16):3845–3851. https://doi.org/10.1007/s00216-013-7431-y

Maher BA, Ahmed IAM, Karloukovski V et al (2016) Magnetite pollution nanoparticles in the human brain. Proc Natl Acad Sci 113(39):10797–10801. https://doi.org/10.1073/pnas.1605941113

Meermann B (2015) Field-flow fractionation coupled to ICP–MS: separation at the nanoscale, previous and recent application trends. Anal Bioanal Chem 407(10):2665–2674. https://doi.org/10.1007/s00216-014-8416-1

Mielke RE, Priester JH, Werlin RA et al (2013) Differential growth of and nanoscale TiO_2 accumulation in Tetrahymena thermophila by direct feeding versus trophic transfer from Pseudomonas aeruginosa. Appl Environ Microbiol 79(18):5616–5624. https://doi.org/10.1128/AEM.01680-13

M-M P, Siripinyanond A (2014) Field-flow fractionation with inductively coupled plasma mass spectrometry: past, present, and future. J Anal Spectrom 29(10):1739–1752. https://doi.org/10.1039/C4JA00207E

Mozhayeva D, Engelhard C (2020) A critical review of single particle inductively coupled plasma mass spectrometry—a step towards an ideal method for nanomaterial characterization. J Anal at Spectrom 35(9):1740–1783. https://doi.org/10.1039/C9JA00206E

Nakamura H, Watano S (2018) Direct permeation of nanoparticles across cell membrane: a review. KONA Powder Part J 35(0):49–65. https://doi.org/10.14356/kona.2018011

Nel AE, Mädler L, Velegol D et al (2009) Understanding biophysicochemical interactions at the nano–bio interface. Nat Mater 8(7):543–557. https://doi.org/10.1038/nmat2442

Pergantis SA, Jones-Lepp TL, Heithmar EM (2012) Hydrodynamic chromatography online with single particle-inductively coupled plasma mass spectrometry for ultratrace detection of metal-containing nanoparticles. Anal Chem 84(15):6454–6462. https://doi.org/10.1021/ac300302j

Peters R, Herrera-Rivera Z, Undas A et al (2015) Single particle ICP-MS combined with a data evaluation tool as a routine technique for the analysis of nanoparticles in complex matrices. J Anal at Spectrom 30(6):1274–1285. https://doi.org/10.1039/C4JA00357H

Poda AR, Bednar AJ, Kennedy AJ et al (2011) Characterization of silver nanoparticles using flow-field flow fractionation interfaced to inductively coupled plasma mass spectrometry. J Chromatogr A 1218(27):4219–4225. https://doi.org/10.1016/j.chroma.2010.12.076

Proulx K, Wilkinson KJ (2014) Separation, detection and characterisation of engineered nanoparticles in natural waters using hydrodynamic chromatography and multi-method detection (light scattering, analytical ultracentrifugation and single particle ICP-MS). Environ Chem 11(4):392. https://doi.org/10.1071/EN13232

Sadik OA, Zhou AL, Kikandi S et al (2009) Sensors as tools for quantitation, nanotoxicity and nanomonitoring assessment of engineered nanomaterials. J Environ Monit 11(10):1782. https://doi.org/10.1039/b912860c

Samei M, Sarrafzadeh M-H, Faramarzi MA (2019) The impact of morphology and size of zinc oxide nanoparticles on its toxicity to the freshwater microalga. Raphidocelis Subcapitata. Environ Sci Pollut Res 26(3):2409–2420. https://doi.org/10.1007/s11356-018-3787-z

Schmidt B, Loeschner K, Hadrup N et al (2011) Quantitative characterization of gold nanoparticles by field-flow fractionation coupled online with light scattering detection and inductively coupled plasma mass spectrometry. Anal Chem 83(7):2461–2468. https://doi.org/10.1021/ac102545e

Schwab F, Zhai G, Kern M et al (2016) Barriers, pathways and processes for uptake, translocation and accumulation of nanomaterials in plants—critical review. Nanotoxicology 10(3):257–278. https://doi.org/10.3109/17435390.2015.1048326

Song L, Vijver MG, Peijnenburg WJGM et al (2015) A comparative analysis on the in vivo toxicity of copper nanoparticles in three species of freshwater fish. Chemosphere 139:181–189. https://doi.org/10.1016/j.chemosphere.2015.06.021

Striegel AM, Brewer AK (2012) Hydrodynamic chromatography. Annu Rev Anal Chem 5(1):15–34. https://doi.org/10.1146/annurev-anchem-062011-143107

Surugau N, Urban PL (2009) Electrophoretic methods for separation of nanoparticles. J Sep Sci 32(11):1889–1906. https://doi.org/10.1002/jssc.200900071

Tiede K, Tear SP, David H et al (2009) Imaging of engineered nanoparticles and their aggregates under fully liquid conditions in environmental matrices. Water Res 43(13):3335–3343. https://doi.org/10.1016/j.watres.2009.04.045

Torres-Duarte C, Adeleye AS, Pokhrel S et al (2016) Developmental effects of two different copper oxide nanomaterials in sea urchin (*Lytechinus pictus*) embryos. Nanotoxicology 10(6):671–679. https://doi.org/10.3109/17435390.2015.1107145

Vandenabeele P (2004) Raman spectroscopy in art and archaeology. J Raman Spectrosc 35(89):607–609. https://doi.org/10.1002/jrs.1217

Vankeirsbilck T, Vercauteren A, Baeyens W et al (2002) Applications of Raman spectroscopy in pharmaceutical analysis. TrAC Trends Anal Chem 21(12):869–877. https://doi.org/10.1016/S0165-9936(02)01208-6

Veraverbeke EA, Verboven P, Scheerlinck N et al (2003) Determination of the diffusion coefficient of tissue, cuticle, cutin and wax of apple. J Food Eng 58(3):285–294. https://doi.org/10.1016/S0260-8774(02)00387-4

Walczyk D, Bombelli FB, Monopoli MP et al (2010) What the cell "sees" in bionanoscience. J Am Chem Soc 132(16):5761–5768. https://doi.org/10.1021/ja910675v

Wang Y, Chang CH, Ji Z et al (2017) Agglomeration determines effects of carbonaceous nanomaterials on soybean nodulation, dinitrogen fixation potential, and growth in soil. ACS Nano 11(6):5753–5765. https://doi.org/10.1021/acsnano.7b01337

Zamora-Perez P, Tsoutsi D, Xu R et al (2018) Hyperspectral-enhanced dark field microscopy for single and collective nanoparticle characterization in biological environments. Materials 11(2):243. https://doi.org/10.3390/ma11020243

Chapter 2
An Overview of Methodologies for Tracing and Quantifying Microplastics in Environmental Samples

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Abstract Microplastics in the environment are a serious environmental problem. The absence of standard methods for sampling and sample preparation and techniques for characterization and quantification of microplastics, particularly, in the complex matrices of environmental samples hinder assessing the fate and the risk of these emerging contaminants. In this chapter, we provide an overview of the approaches used for sampling and sample preparation of microplastics in different environmental compartments and the techniques used to characterize and quantify microplastics. The chapter also provides a comprehensive summary of the challenges associated with each approach and the limitations of the techniques. Finally, the chapter highlights the need to establish standard approaches in future research.

Introduction

The increase in plastics production and application inevitably leads to the generation of a considerable amount of plastic waste around the globe. Plastic pollution is highly debated in society as well as in the scientific and legislative communities. Plastics represent a type of pollution of which we are fully accountable, and that represents

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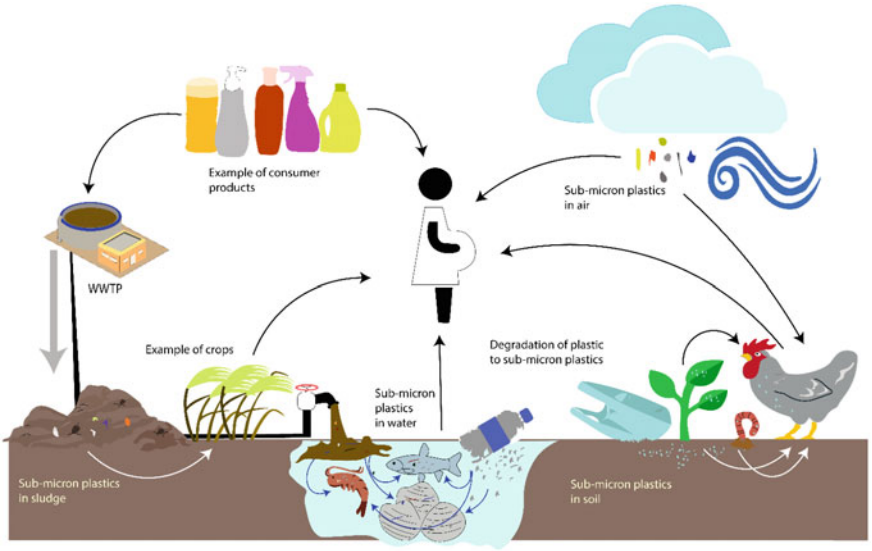

Fig. 2.1 Origin of microplastics and their distribution in different ecosystems and food chains (*Note* WWTP—wastewater treatment plant)

the nature and extent we pollute our planet. Poor management and unintentional release of plastic waste in the environment have raised concerns about their possible environmental adverse effects, as most of the applied plastics in our daily life are non-biodegradable and may persist in the environment for a long time. From 1950 to 2015, approximately 6,300 Mt of plastic have been produced worldwide and only 9% has been recycled (Geyer et al. 2017). Plastic waste will eventually end up and accumulate in the environment. Plastics in the environment are exposed to various environmental physicochemical factors that can break down plastics, e.g., ultraviolet (UV) radiation, varying temperatures, and the activities of microorganisms. This will lead to the formation of small-scale plastic pieces, so-called secondary microplastics (Fig. 2.1) (Jahnke et al. 2017). Consensus exists that microplastics consist of plastic pieces with particle sizes between 1 μm and 5 mm (Gagné et al. 2019). Other size definitions have also been proposed, e.g., <10 mm, <2 mm and <1 mm (Pinto da Costa et al. 2019). Primary microplastics directly enter the environment in the microscale size, which is fabricated to be used in certain consumer products such as cosmetics, air-blasting media paints, textile fibers, etc. These products often end up in wastewater treatment plants (Fig. 2.1) and finally can find their way into the environment. Wastewater treatment plants could, thus, accumulate a considerable amount of microplastics (Kazour et al. 2019), which can end up in the agricultural soils upon application of sewage sludge as biosolid (Crossman et al. 2020). In addition, it was estimated that approximately 4,594–94,500 microplastic particles are released from a single use of exfoliants into the environment (Napper et al. 2015) and personal care products are the source of 0.1–1.5% of microplastics in the North Sea (Gouin et al. 2015).

Microplastics in the environment are distributed in different ecosystems. Research performed in the last decades has documented the presence of microplastics in different environmental compartments, ranging from soil and sediment to snow and air (Fig. 2.1) (Wright et al. 2020), and even in remote areas such as the deep sea (Woodall et al. 2014) and the Arctic polar waters (Lusher et al. 2015). As a result, organisms from different ecosystems may be exposed to microplastics. Uptake of microplastics has been reported in some biota including fish, crabs, and bivalves (Granek et al. 2020). Whether these microplastics can induce adverse effects in organisms is under investigation. Most of the documented assessments of adverse effects of microplastics were performed under controlled conditions by using a high concentration of standard particles, mostly polystyrene (PS). This may raise the question of whether the findings are representative of real-life scenarios. To mimic the real-life environmental scenarios, it is required to quantify the concentration of microplastics in the environment and to understand their transformation as the actual exposure concentration can significantly influence the adverse effects of these materials.

Microplastics are present as a complex mixture of different polymer types, shapes, and sizes in both the environment and in organisms (Fig. 2.2). Plastics also contain different chemical additives such as metals as pigments and phthalates to soften plastics. There are already more than 5,000 different types of plastic on the market and the number of chemicals used to make plastics is likely even larger. If all these materials end up in the environment, it results in a very broad range of microplastics with various chemical compositions. The detection and subsequent identification of materials with this diversity are rather complex and cannot be done based on a single method.

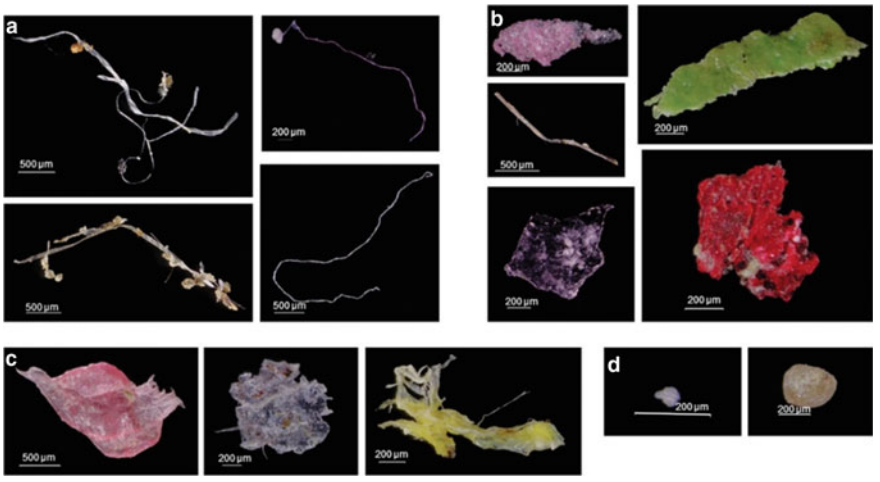

Fig. 2.2 Examples of plastic **a** fibers, **b** fragments, **c** films, and **d** spherical particles reprinted from Brandon et al. (2019) (Copyright © 2019; exclusive licensee American Association for the Advancement of Science)

Analytical methods have been improved for measuring microplastics in the environment (Renner et al. 2018). A large amount of research has been carried out on the identification, quantification, and characterization of microplastics in the environment and organisms. The complete microplastics analysis involves different stages, including sampling, sample preparation, identification, characterization, and quantification. A standardized protocol for these stages is still missing. Consequently, analyzing microplastics in different environmental samples may be challenging, and comparing the results generated from different laboratories is not yet possible. The objective of this chapter is to present the current knowledge on common approaches for sampling, sample preparation, identification, characterization, and quantification of microplastics in the environment. The limitations of these approaches and techniques are also discussed. This chapter provides a structured overview of the analytical techniques and methods currently applied for the analysis of microplastics in environmental samples including samples as complex as whole organisms.

Methods

A systematic review was carried out of publications published from the year 2000 up to 2020, which were identified by the search engines Scopus, Web of Science and Science Direct. We applied the search term "microplastic" in any topic, title, or text category. The keyword string was microplastics, methods, and the environment. The search results identified 2,669 peer-reviewed research articles. By reading the abstracts of all articles that we found, we were able to identify the relevant studies reporting on methods for microplastics in environmental samples. Articles focusing on ecotoxicity and microplastics in consumer products and wastewater treatment plants were excluded from the scope of this chapter.

Sampling Microplastics in Different Environmental Matrices

Herein, we provide a brief overview of the methods, the limitations of the methods, and the knowledge gaps in microplastics measurements. The selection of a sampling approach is largely dependent on the environmental matrices, e.g., soil, sediment, water, biota, or air, and the type and size of microplastics. The sampling procedures for microplastics in water, sediment, beach sand (Ferreira et al. 2020), organisms, and tissues (Avio et al. 2015) have been reported in some of the previous studies. Despite this, the absence of a standard protocol for sampling microplastics and the complexity of sampling organisms and soil has shifted most of the focus toward sampling of water and sediment of aquatic ecosystems.

Sampling Water

Water samples are mostly collected from the surface or close to the surface using manta trawls and neuston nets (Cabernard et al. 2018), plankton nets, planktonic circular tows (Cutroneo et al. 2020; Ferreira et al. 2020), bongo nets (Crawford and Quinn 2017), bottles, and water intake pumps (Ding et al. 2019). For instance, Rodrigues et al. (2018), applied a motor water pump with a 0.055-mm mesh net (nylon with 0.01 m^2) for sampling water, recovering all particles with a size $\geq$0.055 mm. Although these sampling procedures are not developed specifically for microplastics, they are straightforward to be used for sampling microplastics in water. There are some disadvantages associated with these approaches. For example, the main disadvantage is the bias towards the larger microplastics due to the mesh size of the sampling devices which easily ignores microplastics with a smaller size than the mesh size (Dris et al. 2018). Another disadvantage is the non-selective sampling of microplastics because the microplastics might be mixed with other debris. This necessitates an extra step of sample purification and complicates the direct identification of microplastics. Moreover, nets and sieves with a small mesh size can get clogged fast (Kazour et al. 2019). Collecting water samples using a bottle, so-called bulk sampling (Wang and Wang 2018), can in principle provide a sample without any influence on the size distribution of the collected microplastics. This approach, however, allows collecting a relatively small amount of water samples, which may not only influence the representativeness of the sample (Hidalgo-Ruz et al. 2012) but also challenges the identification of microplastics, as the amount of microplastics in samples is expected to be low.

Sampling Sediment

Regarding sampling large microplastics in beach sediment, which mostly are collected from the top 5 cm, devices such as tweezers (Ashton et al. 2010) and metal shovels (Wang and Wang 2018) are used. These methods are not suitable if an assessment of size distribution and abundance of the microplastics in sediment is the intended purpose, as these methods exclude microplastics of smaller sizes than what can be observed with a naked eye. To address this issue, a stepwise sampling method for microplastics in the sand has been developed and validated by Besley et al. (2017). In this method, a 50 $\times$ 50 cm quadrat is placed into the sand, using a ruler to measure the top 5 cm sand. The sand from the 5 cm upper layer in the quadrat is carefully removed with a metal trowel, sieved by a 5 mm sieve and transported to the lab. It has been shown that the sampling method is robust and also suitable for sample collection by non-professional volunteers (Lots et al. 2017).

Metallic grabs are often used to sample sediment from deeper underwater ecosystems. To provide a representative sample, collecting several replicates of samples from the same area is recommended (Wang and Wang 2018). This method is not

applicable in some cases, e.g., when the area for collecting sediment samples contains coral reefs. For example, Ferreira et al. (2020) used scuba diving from the seafloor and they used a metal scoop as an alternative method for using the grab to collect samples from sediment due to the presence of coral reefs.

Sampling Organisms

There is no specific sampling method for organisms that is tailored to microplastics. Most of the tested organisms to date are aquatic organisms, particularly fish, and the specimen collection procedures are the same as the procedures used for other contaminants, e.g., purchasing organisms from the market (Yu et al. 2019), or using fishermen to capture fish or plankton nets to harvest zooplankton. For example, Ferreira et al. (2020) captured 120 fish from Laucala Bay and Vanua Navakavu using local fishermen to investigate microplastics in the organisms. Bessa et al. (2018) collected 120 individual fish from the Mondego estuary using a 2 × 0.5 m beam trawl with one tickler chain and a 5 mm mesh size in the cod end. Fernández Severini et al. (2020) collected shrimp from Canal Vieja at a mean depth of 6.5 m using a shrimp trawl. Zooplankton sample was also collected from Kenya's marine environment using horizontally towing of a zooplankton net with a 500-μm mesh (Kosore et al. 2018). Standardized sampling procedures for microplastics in plants and mammals are not available, although laboratory-based studies showed that plants may take up microplastics (Goss et al. 2018; Li et al. 2019, 2020a). Like aquatic organisms, plants and mammals could also be sampled following the sampling procedures developed for other chemicals.

Sampling Other Environmental Compartments

Very few studies reported sampling methods for microplastics in soil and air. Crossman et al. (2020) used a cylindrical stainless-steel corer, 5 × 8 cm, to collect soil samples from three depths, 0–5 cm, 5–10 cm and 10–15 cm. Huang et al. (2020) applied bulk sampling to collect soil samples from cotton fields in China. Scheurer and Bigalke (2018) collected soil samples using simple steel tools and packed the samples into an aluminum box before transporting them to the laboratory. Similarly, Li et al. (2020b) collected soil samples randomly using a narrow stainless-steel spade from a 0.1 × 0.1 m quadrat with 0.2 m depth.

There is also no specific sampling method tailored to microplastics in the air and atmospheric depositions. Wright et al. (2020) collected total atmospheric deposition samples from a nine-story-high roof at a riverside urban site in Central London using an aluminum rain gauge with a 0.03 m^2 (200 mm diameter) orifice. O'Brien et al. (2020) collected all airborne microplastics, indiscriminate of size, using a high-volume air sampler of total suspended particles with a sampling volume of

55 m^3/h. Airborne microplastics samples were collected indoors, in the living room, and outdoors, on a second floor, using a portable air sampler equipped with a PM_{10} inlet in quartz fiber filters for 48 h at a flow rate of 5 L/min (Prata et al. 2020). Wang et al. (2020) collected samples at stations along a ship track using the KB-120 F type intelligent middle-flow sampler for total suspended atmospheric particulate matter with a sampling flow rate of 100 ± 0.1 L/min.

There are many studies in which no sampling method was reported. During sample collection, one must consider the possible errors that may occur and that might affect the representativeness of the samples due to relatively low concentrations of microplastics and their uneven spatial distribution in environmental samples and organisms. Generic sampling protocols for microplastics without considerable biases towards a specific class of size must be developed to facilitate an informed choice on the appropriate sampling mode and the inter-laboratory comparisons (Sun et al. 2019).

Sample Preparation

After sampling, microplastics are present in a complex sample matrix containing many different materials ranging from biological molecules in organisms to sand and clay minerals in the soil which can diminish the analytical accuracy. The existing analytical challenges are especially pronounced in the case of the smaller-sized microplastics because fit-for-purpose analytical techniques that can be used to analyze these materials in complex matrices of environmental samples are not available so far. Most of the available analytical techniques can measure only one physicochemical property of microplastics and in most cases only in pure and simple media. Environmental samples such as soil, sediment, water, and organisms are complex matrices. Detection, identification, and characterization of microplastics in such a complex matrix are inherently difficult because of the small size and the composition of the microplastics, which is similar to the composition of the biogenic polymers present in the environment e.g., proteins, cellulose, and chitin. Most of the available analytical techniques cannot directly measure microplastics in environmental matrices. Sample preparation is thus required to purify the microplastics by removing the background matrices. The sample preparation method of choice depends on the matrices of interest (Fig. 2.3) and commonly includes density separation, sieving, digestion, and filtration (Wang and Wang 2018). For example, according to the protocol developed by Besley et al. (2017), after bringing sand samples to the laboratory, approximately 350 g of wet sand was placed in a glass Petri dish, covered with aluminum foil and dried at 60 °C for 48 h. Then an aliquot of the sand sample was used for microplastic extraction.

Table 2.1 summarizes some of the reported sample preparation methods for microplastics in different environmental samples. For the separation of microplastics from sediment and water, sieving is usually followed by density separation (Eerkes-Medrano et al. 2015). The density differentiation and flotation method, which is

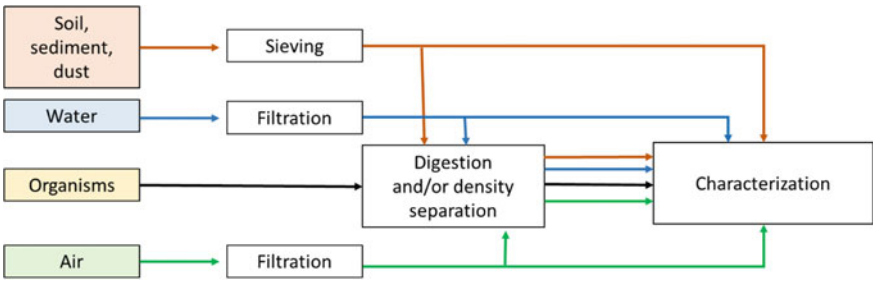

Fig. 2.3 Commonly used methods for the separation of microplastics from different environmental samples

the most applied method, is based on the principle that microplastics float on the surface of a liquid due to buoyancy while heavier materials settle in the liquid. This facilitates the separation of low-density microplastics (Eerkes-Medrano et al. 2015). Many different salt solutions have been used for the density separation process such as saturated sodium chloride (NaCl), sodium iodide (NaI), zinc chloride ($ZnCl_2$), and, to a lower extent, sodium polytungstate ($Na_6[H_2W_{12}O_{40}]$) (Wang and Wang 2018). Nevertheless, sieving and filtration as well as the density separation method cannot isolate microplastics that are attached to organic materials in the samples. In aquatic ecosystems, for example, microplastics may be covered by biofouling (Rummel et al. 2017) which can enhance the density of the low-density microplastics and challenge the filtration and density-based separation. For example, following the protocol developed by Besley et al. (2017) for sediment, 50 g of dried sediment sample was put into Erlenmeyer flask, and mixed with 200 mL of fully saturated salt solution. The mixtures were stirred on a magnetic stirrer for 2 min at 600 rounds per minute (rpm), followed by a settling period of a minimum of 8 h. Approximately 100–150 mL supernatant was filtered through a gridded 0.45-μm pore-size and 47-mm diameter Millipore filter paper. Hereafter, the sand was resuspended in the starting volume of 200 mL of saturated salt solution and stirred for a second round of extraction.

Different digestion methods have been successfully used to remove the background of biological materials and isolate microplastics from the samples (Table 2.1). For waters and sediments, the National Oceanic and Atmospheric Administration (NOAA) in the United States and the European Union Marine Strategy Framework Directive (MSFD) have suggested the application of hydrogen peroxide (H_2O_2) as a suitable agent for removing organic materials and extracting microplastics from environmental samples (Rocha-Santos and Duarte 2015). Aggressive digesting agents such as nitric acid (HNO_3), perchloric acid ($HClO_4$), and hydrochloric acid (HCl) have also been applied to extract microplastics (Wang and Wang 2018). Other alternative agents that have been used, include potassium hydroxide (KOH), sodium hydroxide (NaOH), and sulfuric acid (H_2SO_4) (Huppertsberg and Knepper 2018). Enzymatic digestion procedures (Cole et al. 2015) and the use of alkaline solutions such as tetramethylammonium hydroxide (TMAH) were also applied for removing

Table 2.1 Summary of some sample preparation methods and analytical techniques used for microplastics in different environmental samples

Sample	Microplastics	Sample preparation method	Characterization technique	References
Water	Polypropylene Polyvinylchloride Polyester Polyethylene Polystyrene	The samples were filtered and digested using a mixture of 50% H_2O_2 and sodium dodecyl sulfate (SDS) for 2 days. Microplastics were removed from the filters using the ultrasonic treatment and collected into SDS solution. The residuals were digested using enzymes Cellubrix and Viscozyme at 50 °C for 3 days, upon which Alcalase was added and the solution further incubated for another 3 days at 50 °C. 50% H_2O_2, 0.1 M $FeSO_4$, and 0.1 M NaOH (Fenton reaction) was added to further remove organic matter	Micro- Fourier-transform infrared (FTIR) spectroscopy	Liu et al. (2019)
Water Sediment	Polyethylene Polyvinyl chloride Polystyrene	Water: 30% H_2O_2 was added to the sample at 65 °C and 100 rpm for 12 h to remove the natural organic matter. The samples were treated with NaCl for density separation of microplastics. The suspension was stirred for 2 min and allowed to settle for 24 h. The supernatant was filtered through 0.45 μm filter paper. The filter papers were placed in a clean culture dish and dried at 50 °C for 24 h Sediment: the samples were dried at 70 °C for 24 h to a constant weight. A saturated salt solution was added to 100 g of dried sediment. The samples were stirred for 2 min and settled for 24 h. 30% H_2O_2 was added to the suspension and kept at 100 rmp, 65 °C for 24 h to remove organic matter. The obtained solution was filtered and then rinsed with Milli Q water. Finally, the remaining procedure was the same as used for the water samples	Metallographic microscopy Scanning electron microscopy (SEM)	Ding et al. (2019)

(continued)

Table 2.1 (continued)

Sample	Microplastics	Sample preparation method	Characterization technique	References
Water Sediment	Polyethylene Polypropylene Polystyrene Polyethylene terephthalate Polyvinyl acetate Ethylene–vinyl acetate Polytetrafluorethylene Poly(methyl methacrylate) Poly(ethyl acrylate) Cellulose acetate Styrene butadiene rubber	Water: the water sample was digested using H_2O_2 (30%) with 0.05 M Fe(II) catalyst at approximately 75 °C for 5/10 min. The reaction continued at room temperature for 15 h. After that, zinc chloride (933.3 g/L; density 1.6 g/cm^3) was added for density separation for at least 4 h. The samples were filtered using vacuum filtration (0.45 μm) and the filters were carefully transferred to the oven to dry at 40 °C for 3–5 days Sediment: after stirring 500 g of the sediment, sub-samples were dried at 90 °C for 2 days. 400 mL of sodium polyphosphate (5.5 g/L) was added to the sediment sample and stirred for 1 h at high rpm. The sediments were sieved (5 and 0.055 mm), mixed with 300 mL of zinc chloride solution (933.3 g/L) and stirred with a stainless-steel spoon. After 1 h of settling, the supernatant was filtered through a 0.055 mm sieve and rinsed with Milli-Q water. The following procedure was the same as the one described above for water processing	Attenuated total reflection—Fourier-transform infrared (ATR–FTIR) spectroscopy	Rodrigues et al. (2018)
Water Sediment	Polyethylene Polypropylene	Water: the water samples were filtered (GF/F; 0.75 μm; 47 mm) in the laboratory. The filter papers were dried at 60 °C and stored in Petri dishes. The microplastics on the filter paper were identified and counted using a stereomicroscope Sediment: the sediment sample was well mixed with 330 mL of saturated NaCl solution, vigorously shaken by hand for 1 min and then kept stationary for 10 min. The supernatant was filtered (polycarbonate membrane filter, 1.2 μm; 47 mm). The filters were placed in Petri dishes and dried at 60 °C. The microplastics on the filter paper were identified and counted using a stereomicroscope	Stereomicroscopy FTIR microscopy	Song et al. (2015)

(continued)

Table 2.1 (continued)

Sample	Microplastics	Sample preparation method	Characterization technique	References
Water Sediments Fish	In water polyethylene, polypropylene, polyethylene terephthalate, polystyrene, latex and nitrile were most identified In sediment polyethylene followed by nylon, polytetrafluoroethylene, polyethylene terephthalate and polypropylene were most identified In fish, the polymer types were similar to those in sediment	Water: samples were filtered using stacked 500-μm and 300-μm stainless steel mesh sieves. The filtered sample was dried at 90 °C and 20 mL of 0.05 M Fe(II) solution and 20 ml of 30% H_2O_2 were added to the dried samples. The suspension was heated at 75 °C. H_2O_2 was added until no organic matter was visible in the sample. 6 g of NaCl were added per 20 mL of sample and heated to dissolve NaCl for density separation Sediment: 400 g (wet weight) of sediments were dried at 90 °C. 400 mL of potassium metaphosphate solution (5.5 g/L) were added to the dried sediment and stirred for 1 h. The sample was filtered through a 300-μm sieve and the retained solids were dried at 90 °C. 400 mL of aqueous lithium metatungstate (1.6 g/L) were added to the dried samples. The suspension was stirred, the floating solids were transferred to a 300-μm sieve and dried at 90 °C. To the dried sample, 20 mL of 0.05 M Fe(II) solution and 20 ml of 30% H_2O_2 were added and heated at 75 °C. H_2O_2 was added until no organic matter was visible in the sample. After cooling the sample, 6 g of NaCl were added per 20 mL of sample and heated to dissolve NaCl for density separation Fish: 250 mL of NaCl (1.2 g/cm^3) solution was added to the fish tissues. The sample was stirred for 10 min and decanted, followed by filtration through a 63-μm sieve. 20–30 mL of 15% H_2O_2 were added to the sample to allow for partial digestion of residual organic matter and dried at 60 °C overnight	Optical microscopy ATR-FTIR spectroscopy	Ferreira et al. (2020)

(continued)

Table 2.1 (continued)

Sample	Microplastics	Sample preparation method	Characterization technique	References
Mussels Water Sediment	Polystyrene Polyvinylchloride	Mussels: 1 g of the tissue samples were put in 1 M NaOH and heated at 50 °C for 15 min with mixing using a stirring bar. 17.5 mL 16 M HNO_3 and 2.5 mL ultrapure water were added, and the samples were heated at 50 °C for another 15 min followed by heating at 80 °C for 15 min. The samples were diluted with ultrapure water at a 1:2 ratio (v:v) and filtered through a 0.45-μm filter. Each filter was then dissolved using 1 M NaOH. 20 g of NaI was added (after 5 min of resting) and 15 mL of the solution was drained and discarded. The supernatant was diluted 1:2 (v:v) with ultrapure water at 80 °C, and filtered on 25 mm quartz filters Water: the samples were directly filtered and then the filter was dried at room temperature Sediment: the samples were dried overnight at 50 °C and then analyzed	Thermal gravimetric (TGA)-FTIR	Yu et al. (2019)
Water Sediments Oysters	Polyethylene, polypropylene, polystyrene, polyethylene terephthalate, polyamide, and polyvinylchloride were identified in the seawater	Seawater: 1 M NaOH solution was added and allowed to stand for 24 h. After centrifugation at 3,000 × g for 10 min, the supernatant was run through a series of steel sieves with a diminishing pore size (2 mm, 1 mm, 0.5 mm, 0.1 mm, and 0.05 mm). The retained fractions were heated at 60 °C overnight to dry the particles	FTIR spectroscopy Optical microscopy SEM and energy dispersive X-ray spectroscopy	Wang et al. (2019)

(continued)

Table 2.1 (continued)

Sample	Microplastics	Sample preparation method	Characterization technique	References
		Sediment: samples were heated at 60 °C overnight and treated with 30% H_2O_2 to digest the organic matter. NaCl solution (density: 1.2 g/cm^3) was added for density separation. The supernatant was filtered through a 30-μm steel sieve. The particles retained on the sieve were rinsed with an aqueous solution of NaI (density: 1.8 g/cm^3). After centrifugation at 5,000 × g for 10 min the supernatant was sieved again Oysters: the samples were mixed with a mixture of 30% H_2O_2 and 65% HNO_3 (1:3, v/v). Saturated NaCl solution (500 mL) was added for density separation		
Water Zooplankton	Polypropylene was predominant in the surface water Low-density polyethylene in zooplankton	Water: microplastics were extracted by filtering the samples through 0.7-μm Whatman Filter paper Zooplankton: the organisms were digested using 20 mL 1 M NaOH on a shaker at room temperature for 24 h. Then the samples were heated at 60 °C for 2 h, and at 100 °C for 30 min. After digestion, the residual was filtered through a 0.7-μm Whatman filter paper. The filtered samples were then dried	Dissecting microscopy ATR-FTIR spectroscopy	Kosore et al. (2018)
Water Mussel	The most common microplastics were polyester, rayon, polyethylene, polyvinylchloride and polypropylene in water and mussels	Water: the samples were filtered (20-μm pore-sized filter). 100 mL of 30% H_2O_2 (v/v) was added to the filtered samples to digest organic matter. The samples were digested at 65 °C and 80 rpm for approximately 72 h. The obtained suspension was filtered again through a 5-μm pore-size filter Mussel: approximately 200 mL of 30% H_2O_2 was added to the tissues of the mussel and digested. Saturated saline solution (1.2 g/cm^3) was added to the suspension for density separation of microplastics. Approximately 800 mL NaCl solution was added to each digested tissue and mixed overnight. The overlying water was directly filtered through a 5-μm pore-size filter	Stereomicroscopy LUMOS microscopy	Qu et al. (2018)

(continued)

Table 2.1 (continued)

Sample	Microplastics	Sample preparation method	Characterization technique	References
Fish	Polyester Polypropylene Rayon	The samples were digested using 10% KOH solution at 60 °C for 5 days (to ensure the complete digestion of the organic matter). The residual was vacuum filtered through 1.2 -μm filter and oven-dried at 60 °C	Stereomicroscopy, Micro-FTIR spectroscopy	Bessa et al. (2018)
Shrimp	Polyethylene Polypropylene Cellulose	Two digestion methods were used – 10 mL of 10% KOH were added to each abdominal muscle and the samples were heated on a hot plate at 40 °C for 48 h – 5 mL of H_2O_2 were added and heated at 60 °C for 72 h After digestions, samples were filtered through 0.70 μm glass fiber filters	Stereomicroscopy Raman spectroscopy coupled to microscope BWTEC Inc SEM equipped with energy dispersive spectroscopy	Fernández Severini et al. (2020)
Sand Sediment	Polystyrene Polyethylene Polypropylene	Microplastics were separated from the thoroughly mixed beach sample using density separation	ATR-FTIR	Zhang et al. (2018)
Soil	Not identified	The samples were immersed in a 30% H_2O_2 and incubated for 72 h at 50 °C to digest any organic matter. The residuals were filtered through a 5-mm sieve, the films remaining on the sieve were directly collected using tweezers, and dried at room temperature. Density separation was conducted with saturated NaI solution (1.85 g/cm^3) to extract microplastics from the samples	Stereomicroscopy micro-FTIR SEM	Huang et al. (2020)
Soil	Polyethylene Polypropylene Polystyrene Polyamide Polyvinyl chloride	The samples were air-dried and sieved through a 0.150-mm sieve. Then the samples were dried at 65 °C in an oven to constant weight. The samples were treated with a mixture of 30% KOH: NaClO to digest organic matter at 50 °C for 48 h. The suspension was centrifuged at 4,000 × g for 5 min and the supernatants were collected. Saturated NaCl solution (1.19 g/cm^3) was added to the samples and centrifuged again at 4000 × g for 5 min. $ZnCl_2$ (1.55 g/cm^3) was added to the samples for further density separation of microplastics	Stereomicroscopy Raman microscopy SEM	Zhou et al. (2019)

(continued)

Table 2.1 (continued)

Sample	Microplastics	Sample preparation method	Characterization technique	References
Soil	Polyethylene Polypropylene Polyethylene terephthalate	Organic material was removed from complex solid matrices using a solution of: – 30% (v/v) H_2O_2 at 60 °C and at 70 °C – Fenton's reagent (30% (v/v) H_2O_2 with an iron catalyst) – 1 M NaOH at 60 °C and a 10 M NaOH at 60 °C – 10% KOH solution at 60 °C	Stereomicroscopy FTIR	Hurley et al. (2018)
Soil	Polyvinyl chloride, polystyrene and styrene butadiene were mostly found in the samples	The sample was dried at 65 °C and passed through a 1-mm sieve. An aliquot of the sieved sample was added to a 27% NaCl solution (1.2 g/cm^3) and stirred for 10 min. The suspension was centrifuged at 3450 g for 30 min and the supernatant was filtered (0.45 μm pore size). The particles were washed from the filter and then treated with 40–80 mL of 65% HNO_3 at 90 °C for 48 h. The supernatant was filtered (0.2 μm pore size)	FTIR	Scheurer and Bigalke (2018)
Leachate samples from solid waste landfills	Polyethylene Polypropylene	The sample was filtered and digested with peroxide oxidation to remove natural organic materials. 40 mL 0.05 M Fe(II) solution and 40 mL 30% H_2O_2 were added to the samples. The mixture was filtered through a 0.45-μm filter membrane and a dual-density separation was conducted using NaI solution (~1.7 g/cm^3) for 2 h. The supernatant was filtered through a 0.45-μm filter membrane and the particles on the filter were rinsed with ethanol (~0.79 g/cm^3). The residual (after 2 h settling) was separated using a vacuum filter with a 0.45-μm filter membrane and rinsed	micro-FTIR	He et al. (2019)

organic matter. For example, Abdolahpur Monikh et al. (2020) successfully extracted small microplastics from algae and daphnids using TMAH (5%). In many cases, however, extraction methods using strong acids such as HNO_3 could lead to significant degradation of some polymers, such as polyamide (PA), and this may cause significant changes in the resulting weight, size, and color of microplastics (Löder and Gerdts 2015). Therefore, caution must be used when selecting and applying digestion agents or a mixture of agents. This can, for instance, be done by optimizing the ratio of digestion agents to the samples, the time of digestion, and/or the temperature at which the digestion takes place. This will ensure that successful removal of biological materials from samples is achieved with minimum influences on the microplastics of interest. It is also important to note that the selected approaches do not result in the accidental generation of additional microplastics. Proper method validation is essential before using a method on environmental samples.

Characterization and Quantification

Analyzing the physical and chemical properties of microplastics such as size distribution, shape, polymer composition, and surface chemistry is essential to assess their fate and possible risk in the environment. The most commonly identified microplastics in the marine environment are polyethylene (PE), polypropylene (PP), PS, polyvinyl chloride (PVC), polyurethane (PUR), and polyethylene terephthalate (PET) with some variations including copolymer compositions (Fahrenfeld et al. 2019) which may have different behavior and adverse effects in the environments. Different techniques have been proposed to be used for the characterization and quantification of microplastics (Fu et al. 2020). They range from simple techniques such as optical microscopy after sieving and/or density separation to a sophisticated combination of techniques such as thermo-extraction and desorption coupled with gas chromatography-mass spectroscopy (TDS-GC/MS). As shown in Table 2.1, visual inspection and molecular vibrational techniques such as Fourier-transform infrared (FTIR) and Raman spectroscopy are mostly used to identify microplastics extracted from environmental samples. For example, in a review paper published in 2019, Prata et al. (2019) reported that visual inspection is almost always carried out for microplastics quantification. The authors reported that 50% of the reviewed studies used FTIR-based methods and 10% used Raman spectroscopy when investigating microplastics in water and sediment. In the next sections, we describe the reasons for the extensive application of these techniques and the challenges associated with each technique.

Stereomicroscopy

The application of stereomicroscopy-based methods for microplastics inspection is a simple method for the classification of microplastics based on different physicochemical properties such as shape, size and color (Renner et al. 2018). Due to the availability of stereomicroscopy in many laboratories, this is one of the main techniques for investigating microplastics and for pre-selection of microplastics for chemical analysis. The data provided by this method made a considerable contribution to understanding the presence and distribution of microplastics over the last years. However, this method is subjective and suffers from some limitations. The main challenges associated with this method are (a) high detection limit of stereomicroscopy which biases the observation toward small microplastics, (b) the misclassification of different materials as microplastics which may lead to overestimation of microplastics in the investigated samples, and (c) variation between the data obtained by different observers, which could be due to observer-related errors (Lavers et al. 2016). Thus, this method might be used as a complementary method in the workflow developed for a comprehensive analysis of microplastics in the environment. Nile red staining and subsequent photographing of the sample in a UV light Photobox were used as a complementary method for stereomicroscopy for microplastics observation (Hengstmann and Fischer 2019). In contrast to a fluorescence microscope, the Nile red staining approach was faster, cost-effective, and more accurate for microplastics. However, the approach was suitable for large microplastics >0.63 mm and, therefore, may be applied when large sample volumes need to be analyzed (Hengstmann and Fischer 2019).

Vibrational Spectroscopy

Raman and FTIR spectroscopy are nondestructive vibrational spectroscopy techniques that produce a spectrum based on the interaction of light with molecules. Several comprehensive review papers have been published so far describing the application, advantages and disadvantages of these two techniques for microplastics analysis in the environment (Xu et al. 2019; Chen et al. 2020; Lee and Chae 2021). FTIR generates an infrared spectrum as a result of changes in the dipole moment of molecules and Raman generates a molecular fingerprint spectrum resulting from the polarizability of chemical bonds (Käppler et al. 2016). The last decades' research showed that Raman spectroscopy offers a better size resolution and is capable of detecting particles down to a size of 1 μm. The presence of fluorescence (resulting from biological materials, some organic materials, clay minerals, and some crystals structures), however, often interferes with the Raman spectra of interest (Xu et al. 2019). On the other hand, FTIR, which is not influenced by fluorescent materials, has a lower size resolution with the capability of detecting particles as small as 10–20 μm. The presence of water in the samples, however, impairs the FTIR

spectra (Cabernard et al. 2018). Over the last years, both techniques have progressed dramatically regarding microplastics identification.

Attenuated total reflection FTIR (ATR-FTIR) (Ferreira et al. 2020), which improves the information on irregular microplastics, transmission FTIR, which is applicable to thinner samples compared to ATR, and micro-FTIR, which produces a high-resolution map of the sample, are the most common modes of FTIR used for microplastics in the environment (Xu et al. 2019). As reported by da Silva et al. (2020), they developed an automated analytical method for the characterization of microplastics (<100 μm) using micro-FTIR hyperspectral imaging and partial least squares discriminant analysis. The authors reported that the approach could successfully characterize microplastics in a sediment sample and offer information about the number and size distribution (da Silva et al. 2020). The application of different FTIR techniques for microplastics analysis can challenge inter-laboratory comparisons. Xu et al. (2019) reported that the comparison between ATR and transmission data is not straightforward due to the optical path for transmitted spectra and ATR. The authors reported that these differences have not been considered adequately when comparing the obtained unknown spectra to previously reported spectra in literature or spectral library.

In comparison to FTIR, particularly in transmission mode, where a thin sample is required, Raman enables the analysis of both dark and thick samples (Käppler et al. 2016). Surface-enhanced Raman spectroscopy was applied for the analysis of PS, PE and PP microplastics in pure water and seawater (Lv et al. 2020). The authors concluded that surface-enhanced Raman spectroscopy offers a good enhancement efficiency, overcomes the lower size detection limit of microplastics in liquids and can detect microplastics of 100 nm in size down to the concentration of 40 μg/mL (Lv et al. 2020). Raman Tweezers, namely optical tweezers combined with Raman spectroscopy, were recently applied for the identification of microplastics in seawater (Gillibert et al. 2019). The authors reported that optical trapping and chemical identification of sub-20-μm microplastics, down to the 50 nm range, allowed unambiguous discrimination of microplastics from organic matter and mineral sediments. They claimed that this technique could overcome the capacities of standard Raman spectroscopy in liquid, intrinsically limited to ensemble measurements (Gillibert et al. 2019). Levermore et al. (2020) optimized Raman spectral imaging for the identification of microplastics smaller than 2 μm in airborne particulate matter. They showed that Raman spectral images as analyzed by using chemometric statistical approaches were appropriate for the identification of pristine and aged microplastics smaller than 2 μm (Levermore et al. 2020). Nevertheless, molecule vibrational spectroscopy is still limited by the time and effort required to analyze the signals and sample preparation process. FTIR and Raman spectroscopy could be complementary techniques, where a strong Raman intensity can have weak IR intensities and vice versa.

Thermoanalytical and Gas Chromatography-Mass Spectrometry

Coupling thermogravimetric analysis (TGA) and gas chromatography-mass spectrometry (GC/MS) is another family of techniques that have been used for microplastics chemical analysis. In comparison to spectroscopic techniques, TGA techniques are destructive, where the samples are thermally degraded using pyrolysis (Py) or other thermogravimetric systems. The generated products are transferred to the GC/MS for analysis of their composition. Accordingly, the polymer chemical composition can be identified. The combination of TGA and GC/MS received increasing attention recently for analyzing microplastics in environmental samples. Currently, Py-GC/MS and thermal extraction and desorption (TED)-GC/MS are the major TGA techniques used for microplastics analysis. Both techniques are suited for the detection and quantification of the amount of microplastics present in a sample (Primpke et al. 2020). Fabbri et al. (2000) successfully used Py-GC/MS to analyze synthetic polymers in sediment cores and suspended particulate matter of the coastal lagoon Pialassa Baiona (Adriatic Sea). The authors reported that upon direct pyrolysis, sediments generated high levels of styrene and benzene and a series of aromatic hydrocarbons (α-methyl styrene, indene, naphthalene, biphenyl, diphenyl propane), which indicated the presence of PVC and PS. Markers for rubbers (butadiene dimer, styrene-butadiene) were identified in pyrograms of suspended particulate matter. In 2011, Antić et al. (2011) developed a method using Py-GC/MS to identify and quantify poly(vinylpyrrolidone) in surface water samples. Good recovery of the spiked surface water samples (94.6 ± 1.6%) was reported. This indicated the successful application of the method for the intended purpose in complex matrices of environmental samples. Py-GC/MS was applied successfully to investigate the composition of synthetic polymers in sediments of a coastal lagoon located north-east the city of Ravenna (Italy), where some selected markers (e.g., chlorobenzene for PVC, acetic acid for poly(vinyl acetate) and cyclohexenylbenzene for styrene–butadiene rubbers) were detected (Fabbri 2001). Dekiff et al. (2014) investigated the spatial distribution of microplastics in beach sediments of the North Sea island of Norderney using Py-GC/MS. They could successfully identify PP, PE, PET, PVC, PS and PA microplastics. Fischer and Scholz-Böttcher claimed that Curie-Point Py-GC/MS combined with thermochemolysis was an excellent analytical tool for simultaneous identification and optional quantification of microplastics in environmental samples at polymer-specific mass-related trace level (Fischer and Scholz-Böttcher 2017). Dierkes et al. (2019) recently combined pressurized liquid extraction and Py-GC/MS to investigate microplastics in the environmental samples (sediment, suspended matter and soil). The limit of quantification of 0.007 mg/g was achieved for PE, PP, and PS. The authors reported that PE and PP were detected at concentrations ranging from 0.03 to 3.3 mg/g in the samples.

It is reported that TED-GC/MS has an advantage over Py-GC/MS due to the application of relatively high sample masses allowing identification and quantification of polymers in environmental samples without preselection (Dümichen et al. 2017).

Coupling TGA with solid-phase extraction (TGA-SPE) followed by thermodesorption (TDS)-GC/MS, and also coupling TGA with differential scanning calorimetry (TGA–DSC) have been reported in the literature as successful methods for identification and characterization of microplastics in the environments (Käppler et al. 2018). For example, Majewsky et al. (2016) used TGA–DSC for the simultaneous detection of PE and PP in wastewater samples. The combination of TGA-SPE and TDS-GC/MS was initially used to quantify PE in spiked soil, suspended solids, and mussels (Dümichen et al. 2015) and for identification of PP and PS in aquatic and terrestrial samples (Dümichen et al. 2017). Recently online coupling of TGA with FTIR and GC/MS to identify and quantify microplastics in mussels was reported (Liu et al. 2021). Using this technique, samples were pyrolyzed in TGA and the pyrolysis gases were transferred to FTIR to obtain the absorption spectra of different combusted products. The gas was then transferred online to GC/MS for the quantification and identification of microplastics. The authors concluded that the technique is powerful to quantify masses of microplastics in biological samples (Liu et al. 2021).

Size, Number, Shape and Color of Microplastics

The information provided by the above-mentioned techniques is mostly limited to chemical composition. Quantification of the microplastic number and size and determining their shape (e.g., fiber, foam, fragment, or film) poses a challenge in many respects, with analytical determination as one major issue. The size distribution of microplastics in environmental samples has been mostly obtained using sieving or filtration after some pre-treatments as described in the previous sections. For example, Uurasjärvi et al. (2020) used filters with pore sizes of 300, 100, and 20 μm to determine the size distribution of microplastics in the surface waters of a northern European lake. Some analytical techniques can be used to measure physical parameters of microplastics as small as several nanometers, but this fraction is commonly disregarded when using filtration and sieving methods. For example, laser diffraction and dynamic light scattering (DLS) are used to determine the hydrodynamic size of microplastics (Rocha-Santos and Duarte 2015). Nanoparticle tracking analysis (NTA) is a size measurement technique suitable for accurate measurement of size distribution and the number of microplastics with sizes as low as 70 nm in pure water (Abdolahpur Monikh et al. 2019a). Although these techniques are relatively fast, they can only be applied to microplastics in pure water. Also electron microscopy, such as transmission electron microscopy (TEM) and scanning electron microscopy (SEM), is used to measure the size of microplastics and determine their shapes. For example, SEM with an energy-dispersive X-ray microanalyzer has been applied to determine the shape and obtain the chemical composition of microplastics (Fries et al. 2013). Abdolahpur Monikh et al. (2019b) determined the size and shape of microplastics of 60, 200 and 600 nm in size in a mixture of natural organic matter using TEM and measured the hydrodynamic size using DLS. They also applied NTA to obtain the size distribution and number of the microplastics in the samples.

Color is an important property of microplastics that could be used to determine the origin and the possible adverse effects of microplastics, e.g., due to the color of microplastics they may be mistaken as food by some organisms (Rochman et al. 2019). Different colors have been reported for microplastics, including red, orange, yellow, brown, tan, white, grey, blue and green (Zhang et al. 2020). Dris et al. (2015) reported that the reason for highlighting the blue and red color of microplastics compared to other colors, which could lead to overestimating the two colors, is that these colors can be easily recognized. An optical microscope is a commonly applied technique for determining the color of microplastics. As described before, this technique has some limitations regarding the size detection limit. It is also possible that sample preparation for extraction of microplastics from complex matrices of environmental samples leads to discoloration of microplastics, which should be reported and interpreted (Zhang et al. 2020).

Summary

The applied analytical methods differed among different studies due to the absence of standard protocols for sampling, sample preparation and characterization of microplastics. This makes inter-laboratory comparison very challenging and hinders the risk assessment of microplastics. Currently, there is a gap of knowledge on microplastics in soil, air and organisms and most efforts on analytical developments have been conducted with aquatic samples. This could be due to the simplicity of extracting microplastics from water samples and sediment compared to soil and organisms where microplastics are present in more complex matrices. There is a lack of fit-for-purpose analytical techniques and standardized methods for microplastic characterization and quantification in complex matrices of environmental samples. Although FTIR and Raman spectroscopy can shed light on the presence of microplastics in different ecosystems, obtaining detailed information on microplastics characteristics and quantity, particularly for small-scale microplastics, requires novel techniques capable of detecting low concentrations of small microplastics in complex matrices. The combination of TGA with GC/MS is a promising setup to measure the mass of small microplastics even at trace levels. More research and innovation are required to optimize TGA-GC/MS in combination with an ensemble measurement technique to see the full picture of microplastics pollution in the environment.

References

Abdolahpur Monikh F, Chupani L, Vijver MG et al (2019a) Analytical approaches for characterizing and quantifying engineered nanoparticles in biological matrices from an (eco)toxicological perspective: old challenges, new methods and techniques. Sci Total Environ 660:1283–1293

Abdolahpur Monikh F, Chupani L, Vijver MG et al (2020) Parental and trophic transfer of nanoscale plastic debris in an assembled aquatic food chain as a function of particle size. Environ Pollut 269:116066. https://doi.org/10.1016/j.envpol.2020.116066

Abdolahpur Monikh F, Grundschober N, Romeijn S et al (2019b) Development of methods for extraction and analytical characterization of carbon-based nanomaterials (nanoplastics and carbon nanotubes) in biological and environmental matrices by asymmetrical flow field-flow fractionation. Environ Pollut 255:113304. https://doi.org/10.1016/j.envpol.2019.113304

Antić VV, Antić MP, Kronimus A et al (2011) Quantitative determination of poly(vinylpyrrolidone) by continuous-flow off-line pyrolysis-GC/MS. J Anal Appl Pyrolysis 90:93–99. https://doi.org/10.1016/j.jaap.2010.10.011

Ashton K, Holmes L, Turner A (2010) Association of metals with plastic production pellets in the marine environment. Mar Pollut Bull 60:2050–2055. https://doi.org/10.1016/j.marpolbul.2010.07.014

Avio CG, Gorbi S, Regoli F (2015) Experimental development of a new protocol for extraction and characterization of microplastics in fish tissues: first observations in commercial species from Adriatic Sea. Mar Environ Res 111:18–26. https://doi.org/10.1016/j.marenvres.2015.06.014

Besley A, Vijver MG, Behrens P et al (2017) A standardized method for sampling and extraction methods for quantifying microplastics in beach sand. Mar Pollut Bull 114:77–83. https://doi.org/10.1016/j.marpolbul.2016.08.055

Bessa F, Barría P, Neto JM et al (2018) Occurrence of microplastics in commercial fish from a natural estuarine environment. Mar Pollut Bull 128:575–584. https://doi.org/10.1016/j.marpolbul.2018.01.044

Brandon JA, Jones W, Ohman MD (2019) Multidecadal increase in plastic particles in coastal ocean sediments. Sci Adv 5:eaax0587. https://doi.org/10.1126/sciadv.aax0587

Cabernard L, Roscher L, Lorenz C et al (2018) Comparison of Raman and Fourier transform infrared spectroscopy for the quantification of microplastics in the aquatic environment. Environ Sci Technol 52:13279–13288. https://doi.org/10.1021/acs.est.8b03438

Chen Y, Wen D, Pei J et al (2020) Identification and quantification of microplastics using Fourier-transform infrared spectroscopy: current status and future prospects. Curr Opin Environ Sci Heal 18:14–19. https://doi.org/10.1016/j.coesh.2020.05.004

Cole M, Webb H, Lindeque PK et al (2015) Isolation of microplastics in biota-rich seawater samples and marine organisms. Sci Rep 4:4528. https://doi.org/10.1038/srep04528

Crawford CB, Quinn B (2017) Microplastic collection techniques. In: Microplastic pollutants. Elsevier, pp 179–202

Crossman J, Hurley RR, Futter M et al (2020) Transfer and transport of microplastics from biosolids to agricultural soils and the wider environment. Sci Total Environ 724:138334. https://doi.org/10.1016/j.scitotenv.2020.138334

Cutroneo L, Reboa A, Besio G et al (2020) Microplastics in seawater: sampling strategies, laboratory methodologies, and identification techniques applied to port environment. Environ Sci Pollut Res 27:8938–8952. https://doi.org/10.1007/s11356-020-07783-8

da Silva VH, Murphy F, Amigo JM et al (2020) Classification and quantification of microplastics (<100 μm) using a Focal Plane Array-Fourier transform infrared imaging system and machine learning. Anal Chem 92:13724–13733. https://doi.org/10.1021/acs.analchem.0c01324

Dekiff JH, Remy D, Klasmeier J et al (2014) Occurrence and spatial distribution of microplastics in sediments from Norderney. Environ Pollut 186:248–256. https://doi.org/10.1016/j.envpol.2013.11.019

Dierkes G, Lauschke T, Becher S et al (2019) Quantification of microplastics in environmental samples via pressurized liquid extraction and pyrolysis-gas chromatography. Anal Bioanal Chem 411:6959–6968. https://doi.org/10.1007/s00216-019-02066-9

Ding L, Mao R, Guo X et al (2019) Microplastics in surface waters and sediments of the Wei River, in the northwest of China. Sci Total Environ 667:427–434. https://doi.org/10.1016/j.scitotenv.2019.02.332

Dris R, Gasperi J, Rocher V et al (2015) Microplastic contamination in an urban area: a case study in Greater Paris. Environ Chem 12:592. https://doi.org/10.1071/EN14167

Dris R, Gasperi J, Rocher V et al (2018) Synthetic and non-synthetic anthropogenic fibers in a river under the impact of Paris Megacity: sampling methodological aspects and flux estimations. Sci Total Environ 618:157–164. https://doi.org/10.1016/j.scitotenv.2017.11.009

Dümichen E, Barthel A-K, Braun U et al (2015) Analysis of polyethylene microplastics in environmental samples, using a thermal decomposition method. Water Res 85:451–457. https://doi.org/10.1016/j.watres.2015.09.002

Dümichen E, Eisentraut P, Bannick CG et al (2017) Fast identification of microplastics in complex environmental samples by a thermal degradation method. Chemosphere 174:572–584. https://doi.org/10.1016/j.chemosphere.2017.02.010

Eerkes-Medrano D, Thompson RC, Aldridge DC (2015) Microplastics in freshwater systems: a review of the emerging threats, identification of knowledge gaps and prioritisation of research needs. Water Res 75:63–82

Fabbri D (2001) Use of pyrolysis-gas chromatography/mass spectrometry to study environmental pollution caused by synthetic polymers: a case study: the Ravenna Lagoon. J Anal Appl Pyrolysis 58–59:361–370. https://doi.org/10.1016/S0165-2370(00)00170-4

Fabbri D, Tartari D, Trombini C (2000) Analysis of poly(vinyl chloride) and other polymers in sediments and suspended matter of a coastal lagoon by pyrolysis-gas chromatography-mass spectrometry. Anal Chim Acta 413:3–11. https://doi.org/10.1016/S0003-2670(00)00766-2

Fahrenfeld NL, Arbuckle-Keil G, Naderi Beni N et al (2019) Source tracking microplastics in the freshwater environment. TrAC Trends Anal Chem 112:248–254. https://doi.org/10.1016/j.trac.2018.11.030

Fernández Severini MD, Buzzi NS, Forero López AD et al (2020) Chemical composition and abundance of microplastics in the muscle of commercial shrimp Pleoticus muelleri at an impacted coastal environment (Southwestern Atlantic). Mar Pollut Bull 161:111700. https://doi.org/10.1016/j.marpolbul.2020.111700

Ferreira M, Thompson J, Paris A et al (2020) Presence of microplastics in water, sediments and fish species in an urban coastal environment of Fiji, a Pacific small island developing state. Mar Pollut Bull 153:110991. https://doi.org/10.1016/j.marpolbul.2020.110991

Fischer M, Scholz-Böttcher BM (2017) Simultaneous trace identification and quantification of common types of microplastics in environmental samples by pyrolysis-gas chromatography-mass spectrometry. Environ Sci Technol 51:5052–5060. https://doi.org/10.1021/acs.est.6b06362

Fries E, Dekiff JH, Willmeyer J et al (2013) Identification of polymer types and additives in marine microplastic particles using pyrolysis-GC/MS and scanning electron microscopy. Environ Sci Process Impacts 15:1949. https://doi.org/10.1039/c3em00214d

Fu W, Min J, Jiang W et al (2020) Separation, characterization and identification of microplastics and nanoplastics in the environment. Sci Total Environ 721:137561. https://doi.org/10.1016/j.scitotenv.2020.137561

Gagné F, Auclair J, Quinn B (2019) Detection of polystyrene nanoplastics in biological samples based on the solvatochromic properties of Nile red: application in Hydra attenuata exposed to nanoplastics. Environ Sci Pollut Res 26:33524–33531. https://doi.org/10.1007/s11356-019-06501-3

Geyer R, Jambeck JR, Law KL (2017) Production, use, and fate of all plastics ever made. Sci Adv 3:e1700782. https://doi.org/10.1126/sciadv.1700782

Gillibert R, Balakrishnan G, Deshoules Q et al (2019) Raman Tweezers for small microplastics and nanoplastics identification in seawater. Environ Sci Technol 53:9003–9013. https://doi.org/10.1021/acs.est.9b03105

Goss H, Jaskiel J, Rotjan R (2018) Thalassia testudinum as a potential vector for incorporating microplastics into benthic marine food webs. Mar Pollut Bull 135:1085–1089. https://doi.org/10.1016/j.marpolbul.2018.08.024

Gouin T, Avalos J, Brunning I et al (2015) Use of micro-plastic beads in cosmetic products in Europe and their estimated emissions to the north sea environment. SOFW J 141:40–46

Granek EF, Brander SM, Holland EB (2020) Microplastics in aquatic organisms: improving understanding and identifying research directions for the next decade. Limnol Oceanogr Lett 5:1–4. https://doi.org/10.1002/lol2.10145

He P, Chen L, Shao L et al (2019) Municipal solid waste (MSW) landfill: a source of microplastics? -Evidence of microplastics in landfill leachate. Water Res 159:38–45. https://doi.org/10.1016/j.watres.2019.04.060

Hengstmann E, Fischer EK (2019) Nile red staining in microplastic analysis—proposal for a reliable and fast identification approach for large microplastics. Environ Monit Assess 191:612. https://doi.org/10.1007/s10661-019-7786-4

Hidalgo-Ruz V, Gutow L, Thompson RC et al (2012) Microplastics in the marine environment: a review of the methods used for identification and quantification. Environ Sci Technol 46:3060–3075. https://doi.org/10.1021/es2031505

Huang Y, Liu Q, Jia W et al (2020) Agricultural plastic mulching as a source of microplastics in the terrestrial environment. Environ Pollut 260:114096. https://doi.org/10.1016/j.envpol.2020.114096

Huppertsberg S, Knepper TP (2018) Instrumental analysis of microplastics—benefits and challenges. Anal Bioanal Chem 410:6343–6352. https://doi.org/10.1007/s00216-018-1210-8

Hurley RR, Lusher AL, Olsen M et al (2018) Validation of a method for extracting microplastics from complex, organic-rich, environmental matrices. Environ Sci Technol 52:7409–7417. https://doi.org/10.1021/acs.est.8b01517

Jahnke A, Arp HPH, Escher BI et al (2017) Reducing uncertainty and confronting ignorance about the possible impacts of weathering plastic in the marine environment. Environ Sci Technol Lett 4:85–90

Käppler A, Fischer D, Oberbeckmann S et al (2016) Analysis of environmental microplastics by vibrational microspectroscopy: FTIR, Raman or both? Anal Bioanal Chem 408:8377–8391. https://doi.org/10.1007/s00216-016-9956-3

Käppler A, Fischer M, Scholz-Böttcher BM et al (2018) Comparison of μ-ATR-FTIR spectroscopy and py-GCMS as identification tools for microplastic particles and fibers isolated from river sediments. Anal Bioanal Chem 410:5313–5327. https://doi.org/10.1007/s00216-018-1185-5

Kazour M, Terki S, Rabhi K et al (2019) Sources of microplastics pollution in the marine environment: importance of wastewater treatment plant and coastal landfill. Mar Pollut Bull 146:608–618. https://doi.org/10.1016/j.marpolbul.2019.06.066

Kosore C, Ojwang L, Maghanga J et al (2018) Occurrence and ingestion of microplastics by zooplankton in Kenya's marine environment: first documented evidence. African J Mar Sci 40:225–234. https://doi.org/10.2989/1814232X.2018.1492969

Lavers JL, Oppel S, Bond AL (2016) Factors influencing the detection of beach plastic debris. Mar Environ Res 119:245–251. https://doi.org/10.1016/j.marenvres.2016.06.009

Lee J, Chae K-J (2021) A systematic protocol of microplastics analysis from their identification to quantification in water environment: a comprehensive review. J Hazard Mater 403:124049. https://doi.org/10.1016/j.jhazmat.2020.124049

Levermore JM, Smith TEL, Kelly FJ et al (2020) Detection of microplastics in ambient particulate matter using Raman spectral imaging and chemometric analysis. Anal Chem 92:8732–8740. https://doi.org/10.1021/acs.analchem.9b05445

Li L, Luo Y, Li R et al (2020a) Effective uptake of submicrometre plastics by crop plants via a crack-entry mode. Nat Sustain 3:929–937. https://doi.org/10.1038/s41893-020-0567-9

Li L, Zhou Q, Yin N et al (2019) Uptake and accumulation of microplastics in an edible plant. Chinese Sci Bull 64:928–934. https://doi.org/10.1360/N972018-00845

Li W, Wufuer R, Duo J et al (2020b) Microplastics in agricultural soils: extraction and characterization after different periods of polythene film mulching in an arid region. Sci Total Environ 749:141420. https://doi.org/10.1016/j.scitotenv.2020.141420

Liu F, Olesen KB, Borregaard AR et al (2019) Microplastics in urban and highway stormwater retention ponds. Sci Total Environ 671:992–1000. https://doi.org/10.1016/j.scitotenv.2019.03.416

Liu Y, Li R, Yu J et al (2021) Separation and identification of microplastics in marine organisms by TGA-FTIR-GC/MS: a case study of mussels from coastal China. Environ Pollut 272:115946. https://doi.org/10.1016/j.envpol.2020.115946

Löder MGJ, Gerdts G (2015) Methodology used for the detection and identification of microplastics—a critical appraisal. In: Marine anthropogenic litter. Springer International Publishing, Cham, pp 201–227

Lots FAE, Behrens P, Vijver MG et al (2017) A large-scale investigation of microplastic contamination: abundance and characteristics of microplastics in European beach sediment. Mar Pollut Bull 123:219–226. https://doi.org/10.1016/j.marpolbul.2017.08.057

Lusher AL, Tirelli V, O'Connor I et al (2015) Microplastics in Arctic polar waters: the first reported values of particles in surface and sub-surface samples. Sci Rep 5:14947. https://doi.org/10.1038/srep14947

Lv L, He L, Jiang S et al (2020) In situ surface-enhanced Raman spectroscopy for detecting microplastics and nanoplastics in aquatic environments. Sci Total Environ 728:138449. https://doi.org/10.1016/j.scitotenv.2020.138449

Majewsky M, Bitter H, Eiche E et al (2016) Determination of microplastic polyethylene (PE) and polypropylene (PP) in environmental samples using thermal analysis (TGA-DSC). Sci Total Environ 568:507–511. https://doi.org/10.1016/j.scitotenv.2016.06.017

Napper IE, Bakir A, Rowland SJ et al (2015) Characterisation, quantity and sorptive properties of microplastics extracted from cosmetics. Mar Pollut Bull 99:178–185. https://doi.org/10.1016/j.marpolbul.2015.07.029

O'Brien S, Okoffo ED, O'Brien JW et al (2020) Airborne emissions of microplastic fibres from domestic laundry dryers. Sci Total Environ 747:141175. https://doi.org/10.1016/j.scitotenv.2020.141175

Pinto da Costa J, Reis V, Paço A et al (2019) Micro(nano)plastics—analytical challenges towards risk evaluation. TrAC Trends Anal Chem 111:173–184. https://doi.org/10.1016/j.trac.2018.12.013

Prata JC, Castro JL, da Costa JP et al (2020) The importance of contamination control in airborne fibers and microplastic sampling: experiences from indoor and outdoor air sampling in Aveiro. Portugal. Mar Pollut Bull 159:111522. https://doi.org/10.1016/j.marpolbul.2020.111522

Prata JC, da Costa JP, Duarte AC et al (2019) Methods for sampling and detection of microplastics in water and sediment: a critical review. TrAC Trends Anal Chem 110:150–159. https://doi.org/10.1016/j.trac.2018.10.029

Primpke S, Fischer M, Lorenz C et al (2020) Comparison of pyrolysis gas chromatography/mass spectrometry and hyperspectral FTIR imaging spectroscopy for the analysis of microplastics. Anal Bioanal Chem 412:8283–8298. https://doi.org/10.1007/s00216-020-02979-w

Qu X, Su L, Li H et al (2018) Assessing the relationship between the abundance and properties of microplastics in water and in mussels. Sci Total Environ 621:679–686. https://doi.org/10.1016/j.scitotenv.2017.11.284

Renner G, Schmidt TC, Schram J (2018) Analytical methodologies for monitoring micro(nano)plastics: which are fit for purpose? Curr Opin Environ Sci Heal 1:55–61. https://doi.org/10.1016/j.coesh.2017.11.001

Rocha-Santos T, Duarte AC (2015) A critical overview of the analytical approaches to the occurrence, the fate and the behavior of microplastics in the environment. TrAC Trends Anal Chem 65:47–53. https://doi.org/10.1016/j.trac.2014.10.011

Rochman CM, Brookson C, Bikker J et al (2019) Rethinking microplastics as a diverse contaminant suite. Environ Toxicol Chem 38:703–711. https://doi.org/10.1002/etc.4371

Rodrigues MO, Abrantes N, Gonçalves FJM et al (2018) Spatial and temporal distribution of microplastics in water and sediments of a freshwater system (Antuã River, Portugal). Sci Total Environ 633:1549–1559. https://doi.org/10.1016/j.scitotenv.2018.03.233

Rummel CD, Jahnke A, Gorokhova E et al (2017) Impacts of biofilm formation on the fate and potential effects of microplastic in the aquatic environment. Environ Sci Technol Lett 4:258–267. https://doi.org/10.1021/acs.estlett.7b00164

Scheurer M, Bigalke M (2018) Microplastics in Swiss floodplain soils. Environ Sci Technol 52:3591–3598. https://doi.org/10.1021/acs.est.7b06003

Song YK, Hong SH, Jang M et al (2015) A comparison of microscopic and spectroscopic identification methods for analysis of microplastics in environmental samples. Mar Pollut Bull 93:202–209. https://doi.org/10.1016/j.marpolbul.2015.01.015

Sun J, Dai X, Wang Q et al (2019) Microplastics in wastewater treatment plants: detection, occurrence and removal. Water Res 152:21–37. https://doi.org/10.1016/j.watres.2018.12.050

Uurasjärvi E, Hartikainen S, Setälä O et al (2020) Microplastic concentrations, size distribution, and polymer types in the surface waters of a northern European lake. Water Environ Res 92:149–156. https://doi.org/10.1002/wer.1229

Wang J, Lu L, Wang M et al (2019) Typhoons increase the abundance of microplastics in the marine environment and cultured organisms: a case study in Sanggou Bay, China. Sci Total Environ 667:1–8. https://doi.org/10.1016/j.scitotenv.2019.02.367

Wang W, Wang J (2018) Investigation of microplastics in aquatic environments: an overview of the methods used, from field sampling to laboratory analysis. TrAC Trends Anal Chem 108:195–202. https://doi.org/10.1016/j.trac.2018.08.026

Wang X, Li C, Liu K et al (2020) Atmospheric microplastic over the South China Sea and East Indian Ocean: abundance, distribution and source. J Hazard Mater 389:121846. https://doi.org/10.1016/j.jhazmat.2019.121846

Woodall LC, Sanchez-Vidal A, Canals M et al (2014) The deep sea is a major sink for microplastic debris. R Soc Open Sci 1:140317. https://doi.org/10.1098/rsos.140317

Wright SL, Ulke J, Font A et al (2020) Atmospheric microplastic deposition in an urban environment and an evaluation of transport. Environ Int 136:105411. https://doi.org/10.1016/j.envint.2019.105411

Xu J-L, Thomas KV, Luo Z et al (2019) FTIR and Raman imaging for microplastics analysis: state of the art, challenges and prospects. TrAC Trends Anal Chem 119:115629. https://doi.org/10.1016/j.trac.2019.115629

Yu J, Wang P, Ni F et al (2019) Characterization of microplastics in environment by thermal gravimetric analysis coupled with Fourier transform infrared spectroscopy. Mar Pollut Bull 145:153–160. https://doi.org/10.1016/j.marpolbul.2019.05.037

Zhang H, Zhou Q, Xie Z et al (2018) Occurrences of organophosphorus esters and phthalates in the microplastics from the coastal beaches in north China. Sci Total Environ 616–617:1505–1512. https://doi.org/10.1016/j.scitotenv.2017.10.163

Zhang Y, Kang S, Allen S et al (2020) Atmospheric microplastics: a review on current status and perspectives. Earth-Science Rev 203:103118. https://doi.org/10.1016/j.earscirev.2020.103118

Zhou Y, Liu X, Wang J (2019) Characterization of microplastics and the association of heavy metals with microplastics in suburban soil of central China. Sci Total Environ 694:133798. https://doi.org/10.1016/j.scitotenv.2019.133798

Chapter 3
Advances in Exposome

Hongli Tan and Da Chen

Abstract Exposome has emerged and developed as a novel research concept in many fields over the past decade. In the environmental health research, the exposome represents "the cumulative measure of environmental influences and associated biological responses throughout the lifespan including exposures from the environment, diet, behavior, and endogenous processes," which has driven the integration of both internal and external factors into the study of adverse health outcomes in humans. Existing approaches to characterize and measure the exposome mainly include targeted chemical and biological monitoring, non-targeted omics (e.g., metabolomics, genomics, proteomics, transcriptomics, and epigenomics), and other related techniques. Considering the importance of the exposome concept to human health evaluations, several government and public sectors in the European Union (EU) and the United States (U.S.) have launched large-scale exposome-related research projects in order to integrate the exposome into human health studies and advance the development of exposome databases and platforms. The exposome and related techniques have also been increasingly employed in environmental and human health risk assessment. However, broader applications of the exposome concept still require researchers to overcome several major challenges, including the measurements of the variability and complexity of exposures, data integration and analysis, and the difficulty to untangle the cause-effect relationships.

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The Concept of the Exposome

Development of the Exposome Concept

Over the past decade, "exposome" has emerged as a novel research paradigm in many fields including epidemiology, biotechnology, clinical research, and other environmental health sciences. The concept of "human exposome", originally conceived by an epidemiologist Dr. Christopher Wild in 2005, encompasses the totality of a person's environmental exposures from conception onwards, which complements the genome (Wild 2005). Subsequently, Wild also proposed three broad exposome categories that often overlap with each other to refine the definition of the "exposome" (Wild 2012; Wild et al. 2013). The refined definition of the exposome includes every exposure to which the individual is subjected from conception to death. Three exposome categories are: internal, i.e., the within the body measures, such as metabolism, endogenous circulating hormones, physical activity, gut microflora, inflammation, lipid peroxidation, and oxidative stress; specific external, such as environmental chemicals, radiation, infectious agents, medical interventions, diet, lifestyle factors, and occupation; and general external, such as social capital, education, financial status, psychological and mental stress, urban–rural environment, and climate (Wild 2012). Wild's idea was insightful and timely, receiving widespread attention from scientists across the disciplines. Later, some scholars further refined and improved the definition of "exposome" based on Wild's original definition (Vineis et al. 2017; Miller and Jones 2014; Dagnino 2019; Rappaport 2011; Miller 2020; Vermeulen et al. 2020). An even broader definition proposed by Gary W. Miller and Dean P. Jones was described as "The cumulative measure of environmental influences and associated biological responses throughout the lifespan including exposures from the environment, diet, behavior, and endogenous processes" (Miller and Jones 2014; Miller 2020). This extended definition provides more detail and makes it more inclusive, making it more adaptable to different research fields and purposes (Fig. 3.1). Thus, the concept of "exposome" has driven the integration of both internal and external factors into the study of adverse health outcomes in humans to achieve a comprehensive assessment of the causes and mechanisms associated with health outcomes (Cui et al. 2016). In particular, the exposome concept has strongly influenced environmental toxicology and environmental health research, stimulating the expansion from traditional environmental research to mechanism-driven, more environmentally relevant investigations on the effects of environmental chemicals on human health.

The Importance of Understanding the Exposome

A growing body of evidence shows that genetic variation explains a limited part of the variability in the risk of human diseases, leaving a potentially large role for environmental exposures and interaction between environmental and genetic factors. For

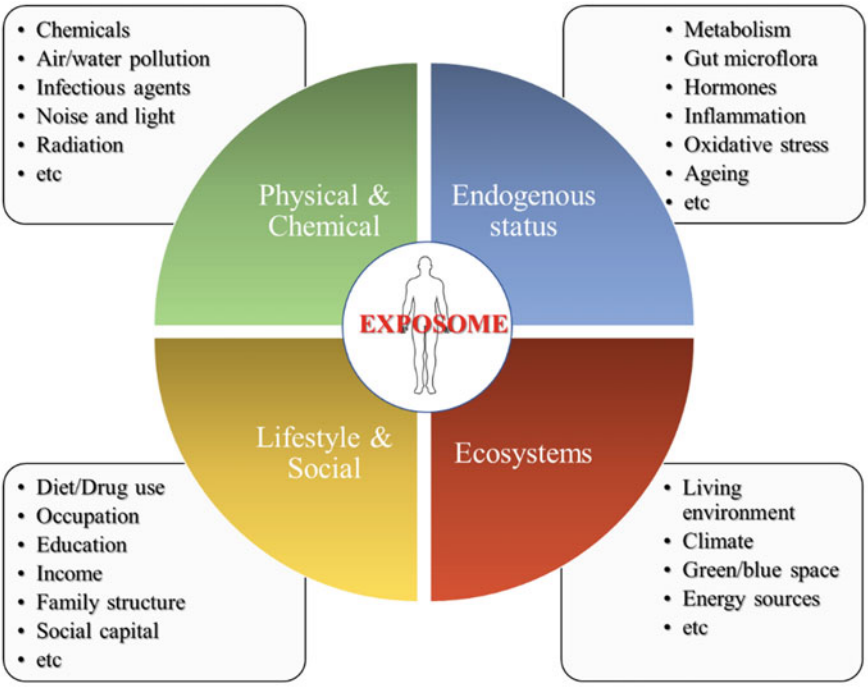

Fig. 3.1 The exposome concept

example, although genetic studies provided insights into the pathways and mechanisms by which autism occurs, they could only explain a small fraction of the variability in disease risk nor account for it systematically (Chaste and Leboyer 2012). Environmental factors are thought to be an important cause of autism induction but their roles in the disease remain unclear (Chaste and Leboyer 2012). The same issue is associated with other diseases, such as allergies, reproductive diseases, type II diabetes, and obesity (Locke et al. 2015; Portelli et al. 2015), which have been related to environmental factors (e.g., urbanization, contaminants, and occupational exposure), lifestyle (smoking, diet, and pets) and other risks factors (Beasley et al. 2015). However, the interactions between internal and external risk factors and the subsequent impact on health outcomes have not been well characterized in most cases.

The concept of exposome considers lifetime exposure history and therefore requires taking into account changes in exposure over time and the many accompanying risk factors. Unlike most previous environmental health evaluations, which have generally focused on "one exposure—one outcome" associations at a single or limited number of time points (which might not cover the highest risk exposure window), the exposome goes one step further by examining the multitude of environmental exposures that dynamically evolve over time and space for individuals or

populations. This deepens our insight into the dynamics and complexity of nature in the development of adverse health outcomes, related causes and mechanisms.

In the study of human exposure to toxic chemicals and related health effects, traditional research generally focusses on a single or a limited number of environmental factors but largely overlooks the co-existence of many different types of chemicals and the complex interactions between chemical exposure and metabolism, genetics, or other internal factors, as well as subsequent impact on toxic endpoints. Hundreds of chemicals may be present in the environment, resulting in human exposure to the complexity of anthropogenic chemicals via various pathways such as dietary intake, inhalation, and dermal contact (Thakur and Pathania 2020). The co-exposure to the variety of chemicals could lead to a "cocktail" effect different from what can be predicted from the exposure to individual chemicals. Environmental exposure to toxic chemicals may interfere or interact with endogenous metabolism and genetic responses, synergistically inducing toxic effects or adverse health outcomes. Other internal or external factors, such as sociodemographic factors, lifestyle, occupation, and nutrition, could also contribute to the synergistic impact. Therefore, exposome plays a very important role in the characterization of human exposure to environmental stressors and subsequent health impacts. Exposome research complements the traditional research framework and contributes to a more comprehensive understanding of human exposure to environmental contamination.

Considering the importance of the exposome concept to human health evaluations, several governments/public sectors from the European Union (EU) and the United States (U.S.) have launched large-scale exposome-related research projects. For example, the Human Early-Life Exposome (HELIX) project was launched to develop novel tools for integrating assessments of early-life environmental exposures and child health across Europe (Siroux et al. 2016). The HELIX "early-life exposome" approach involves combining all environmental hazards that mothers and children are exposed to and linking this to the health, growth and development of children (Vrijheid et al. 2014). The EXPOsOMICS project defines its main aim as predicting individual disease risk related to the environment by characterizing external and internal exposome for common exposures during critical periods of life and using the new tools in risk assessment and the estimation of the burden of environmental disease (Vineis et al. 2017). The "Health and Environment-wide Associations based on Large Population Surveys" (HEALS) project aims at identifying the complex links among the environment, genes, and human disease, such as allergies, asthma, neurodevelopmental and metabolic disorders, based on individual exposome characterization (Sarigiannis 2019). Other major exposome projects include the "Sustainable Futures for Europe's Heritage in Cultural Landscapes" (HERCULES) project, focusing on developing and refining new tools and techniques for the exposome (Siroux et al. 2016). These exposome-related projects greatly facilitate exposome research and broaden its applications in human health studies.

Methodological Development for Characterizing the Exposome

Although the exposome concept is fascinating, the methodological approach to "exposome" is complicated and challenging. The concept of considering both external (e.g., environmental chemicals) and internal factors (e.g., endogenous metabolic balances) throughout life and relating them to health effects requires specific research tools and techniques. Using highly informative exposure assessment tools, techniques and metrics enables efficient and accurate measurement of the exposome. Existing approaches and tools mainly include targeted chemical and biological monitoring, non-targeted omics techniques (e.g., metabolomics, genomics, proteomics, transcriptomics, and epigenomics), sensor technologies, imaging, portable computerized devices, and modeling technologies including deep learning and artificial intelligence (Wild 2012; Haddad et al. 2019). These technologies or methods cover the fields of chemistry, biology, physics, mathematics, medicine, computer and environmental sciences, and many other disciplines. Using a combination of approaches, for instance, the environment-wide association study (EWAS) approach or the metabolome-wide association study (MWAS) approach that are usually combined with the more typical genome-wide association study (GWAS) approach as well as other advanced methods or tools, are necessary for exposome research (Haddad et al. 2019; Gangler et al. 2019).

Around the concept of the exposome, relevant quantitative and qualitative methods and techniques are constantly being created, developed, and updated. Despite of massive challenges (e.g., technical difficulties, significant expenses, and requirement for professional training), scientists and engineers have made some breakthrough achievements in the development of exposome research approaches. In particular, the fast-developing mass spectrometry techniques and instruments allow highly sensitive and high-throughput characterization of small and large molecules in environmental and biological specimens and the development of various "omics" technologies. Below we briefly review the development of selected approaches for characterizing exogenous and endogenous molecules for exposome research.

Targeted Measurements

Targeted measurement technologies are widely used in environmental and biological monitoring, including the determination of selected pollutants, genetic material (such as DNA and RNA), proteins, and other human endogenous substances (such as lipids, hormones, and small carbohydrate molecules). Targeted measurements are characterized by accuracy and relatively simple analysis, but lack globality and can only target specific subsets of related molecules. Nevertheless, targeted measurements add valuable data to the overall exposome characterization.

Using targeted measurement techniques, selected environmental contaminants have been characterized in various environmental matrices (e.g., soil, dust, airborne particles, and water), food (e.g., fish, cereals, meat, and vegetables), consumer products (e.g., cosmetics), and biological specimens (e.g., urine and blood) (Rasheed et al. 2020; Tan et al. 2018; Tan et al. 2021; Chen et al. 2016; Tang et al. 2021a; Eqani et al. 2012; Careghini et al. 2015). Gas/liquid chromatography coupled to single quadrupole or tandem mass spectrometry has been broadly employed to identify and quantify hundreds of environmental contaminants, such as plasticizers, pesticides, flame retardants, and per- and poly-alkylated fluorinated substances in global environmental and human samples, demonstrating ubiquitous human exposure risks. They have also been employed to quantify endogenous chemicals (e.g., fatty acids, lipid molecules, hormones, organic acids, and bile acids) in biological samples. These techniques allow the accurate determination of target chemicals and quantitative characterization of biomarkers associated with environmental exposure and adverse health outcomes.

Although targeted measurements are highly accurate, they often require the operators to have prior knowledge of the subject or research project to better select the "target". In most cases, reference standards are required to achieve quantitative and accurate measurements. This limits the capacity of targeted exposome research in the elucidation of complicated environmental exposures and associated health risks. For example, in many cases, environmental chemicals may be subjected to biotransformation or metabolism, producing major metabolites as more appropriate exposure markers than the parent chemicals. However, these metabolites may not be among the target analytes list or may even be unknown without prior in vitro or in vivo studies. In other cases, the endogenous biomarkers for a specific health effect are not known, limiting the use of target exposome techniques in exposure studies. Nevertheless, targeted exposome techniques have found broad applications in environmental exposure and health effect studies.

Non-targeted Measurements

The Chemical Abstracts Service (CAS) Registry (CAS 2016) lists over 120 million unique organic and inorganic compounds. However, only about 85,000 manufactured or processed chemicals are currently registered under the Toxic Substances Control Act (TSCA) with the United States Environmental Protection Agency (US EPA) (EPA 2016), and about 30,000 of these chemicals are widely used in consumer products (Muir and Howard 2006; Howard and Muir 2010). Humans could be exposed to hundreds or even thousands of anthropogenic chemicals via different pathways and many of them can probably reach into human bodies with a proportion accumulated into tissues or organs. When facing such a large number of chemicals, targeted approaches can only deal with a limited number of them, unlikely meeting the requirements of exposome characterization. For example, a recent report by the Centers for Disease Control and Prevention (CDC) indicated that only about 250 chemicals are

frequently monitored through targeted analysis methods (CDC 2015). In addition to a large number of chemical substances, thousands of endogenous substances in human bodies also require more sophisticated techniques beyond targeted approaches.

Compared with targeted measurements, the non-targeted methods are more complex and require more advanced analytical platforms. The main advantages of non-targeted measurements include a relatively complete, systematic, and high-throughput characterization of tens of thousands of objects, covering both known, suspected, and unknown substances. Such advantages lead to broad applications of non-targeted approaches in many research fields, such as epidemiology, food safety, metabolomics, biochemical and environmental analyses (Andra et al. 2017; Pourchet et al. 2020; Wild 2012).

Non-targeted measurements rely on high-resolution mass spectrometers (MS) such as Fourier transform ion cyclotron resonance (FT-ICR), orbitrap, and time-of-flight (TOF) MS. These mass spectrometers can be combined with tandem analyzers such as hybrid ion trap/orbitrap (LTQ-Orbitrap) and quadrupole time-of-flight (QqTOF), which provide both full-scan MS and MS/MS to obtain an accurate mass of both precursor and product ions, leading to more confident and accurate compound identification (Andra et al. 2017). Identification and measurement of suspected or unknown proteins, environmental contaminants or their degradation products, and endogenous substances are often performed using the instruments and techniques described above. For example, a recent exposome study reported a non-targeted analysis of plasma samples from women with pre-eclampsia, revealing polar and apolar changes in the metabolome (Sander et al. 2019). Another common non-targeted technology is high-throughput sequencing, which can sequence millions of gene units and screen biomarkers and is widely used in multi-level studies of genomics, transcriptomics and epigenomics (Zhu et al. 2014).

Non-targeted and targeted techniques have their advantages and disadvantages, depending on the analytical aims and scientific questions. They are often employed together to achieve both quantitative and qualitative screening of exogenous or endogenous substances. For example, in the case–control studies, screening and identification of potential biomarkers are often conducted with non-targeted analysis, followed by quantitative determination of the target analytes with targeted analysis (Johnson et al. 2021). The and complemental use of both non-targeted and targeted exposome techniques can maximize what exposome research can achieve. As a result, assimilation of the known and unknown is becoming the main goal of the new generation of methods for assessing the exposome.

Application of Exposome Research in Environmental and Human Health Impact Studies

The exposome overhauls the traditional paradigms of environmental health impact research and provides a novel methodological framework that comprehensively addresses the complex, highly dynamic interplay between human internal, external,

and modifiable factors. Holistic assessments of the adverse health effects and systematic elucidation of the mechanisms underlying a multitude of environmental exposures have widespread implications. In this part of the chapter, we will review the application of exposome research in environmental and human health assessment.

Rappaport et al. (2011) has proposed two complementary approaches (bottom-up and top-down) to characterize the causal human exposome. In the bottom-up approach, the sources of important exogenous exposures are identified and the level of exposure is assessed through environmental media (e.g., air, water, food, and the built environment). In the top-down approach, the circulating levels of parent compounds and/or their metabolites and by-products are assessed individually through biological assays in blood, urine or other tissues. Rappaport's idea perfectly combines the environment with humans and reflects the importance of environmental studies in the exposome research. They can provide valuable information on the sources and environmental levels of exposure that can be associated with the top-down exposure research approach (i.e., identifying disease triggers and toxicity). In addition, the techniques and concepts of exposome can make environment-related research more systematic, comprehensive and efficient, and thus have become increasingly popular in recent years.

In one of our previous studies, an indoor exposome database through comprehensive data mining on the occurrence of identified contaminants in house dust was established (Dong et al. 2019). Chemicals of health concern were prioritized by using the ToxCast database developed by the US EPA. Applying the concept and techniques of the exposome research, 511 chemicals, including heavy metals and organic contaminants, were determined with their concentration ranges in indoor dust, which were then employed to prioritize their toxicity based on 16 toxicological endpoints. The study found that indoor dust contained higher levels of heavy metals than organic pollutants, while organic pollutants such as phthalates, plasticizers, flame retardants, organotins, and phenols were significantly associated with the bioassays which indicated endocrine disruption (Dong et al. 2019). This investigation provides one of the most comprehensive analyses on chemical occurrence in indoor dust and the prioritization of their toxicity. The database lays the ground for future exposome studies of the indoor environment and provides guidance for indoor risk assessments.

Using the exposome-based advanced analysis tools, Sarigiannis et al. (2018) designed a systematic study of landfill-human health exposome. Briefly, the study recruited 325 children near Europe's second-largest landfill site and used a combination of environmental monitoring of metal and organic pollutants, human biomonitoring, advanced-omics analysis technologies, and other approaches for adverse health impact tracking and risk assessment (Sarigiannis et al. 2018). The study confirmed that proximity to a landfill and the consequent soil contamination with metals were critical to children's neurodevelopment, and provided a novel tool (exposome paradigm) for holistic health risk assessment and management of industrially contaminated sites. Similar approaches have also been undertaken to evaluate e-waste contamination and air pollution (Garg et al. 2020; Vineis et al. 2020; Tang et al. 2021b). Overall, the concept of exposome research and associated techniques have

greatly expanded the understanding of the complexity of environmental pollution and its subsequent impact on human health.

The exposome approach in epidemiological research involves measurement and description of multiple environmental exposures from the different exposome domains during whole life including early life (pregnancy and childhood) and adulthood and association of these with biomarkers and various health outcomes. A well-established "exposome-health" example is the innovative work of Yu and his colleagues who used tooth biomarkers to characterize the temporal dynamics of the fetal and early-life exposome (Yu et al. 2021). In their study, untargeted metabolomics was conducted on micro-dissected layers from naturally shed deciduous teeth. Applying four liquid-chromatography high-resolution mass spectrometry analytical modes, they profiled small molecules (<1000 Da) from prenatal and postnatal tooth fractions and identified several hundred substances including exogenous exposures and endogenous metabolites, many of which were biologically relevant and have been linked to adverse health outcomes (Yu et al. 2021). This study provides a direct window into fetal exposures that is not possible by using maternal biomarkers, thus indicating that identification of early chemical and molecular changes can offer the possibility of identifying novel response biomarkers which can be employed much earlier in the population monitoring for hazard identification and human risk assessment.

The impacts of air pollution on health are multi-faceted, and there is an urgent need to identify appropriate ways to integrate data generated by different disciplines to address the complex combination of environmental, health, economic, and sociodemographic issues. Tang et al. (2021b) has simultaneously and comprehensively measured airborne pollutant exposures and explored the associated biomarkers in susceptible healthy elderly subjects. In this study, advanced tools for personal airborne exposure monitoring (external exposures), characteristics of biological samples for exogenous and endogenous compounds (e.g., targeted and untargeted monitoring), and multi-omics measurements to explore potential biomarkers and putative toxicity pathways were all considered. In addition, the work systematically evaluated the relationships between personal exposure to air pollutants and novel biomarkers of exposures and effects using exposome-wide association study approaches (Tang et al. 2021b). Finally, the study established a most extensive exposome database of biomarkers of air pollutant exposure in Chinese people aged 60–69 years, which included 76 healthy residents from a representative community in Jinan City, Shandong province (Tang et al. 2021b). This is a very typical example of employing the exposome concept in exposure-based epidemiological studies, by combining personal monitoring for airborne pollutants, extensive human biomonitoring, advanced omics analysis, confounding information, and statistical methods (Tang et al. 2021b). The study contributed to the understanding of the mechanisms underlying the adverse health impacts of air pollution exposures and identifying adverse clinical outcomes that can facilitate the development of effective prevention and targeted intervention techniques.

Nevertheless, studies employing the exposome concept to address environmental exposure and health outcomes have remained limited to date. Broader applications

of the exposome concept require researchers to overcome several major challenges which we summarize below.

Challenges in Exposome Research

The Challenges of Exposure Variability and Measurement

The exposome approach presents opportunities in many fields but there remain several major challenges. The exposome varies throughout life, presenting challenges for relevant research due to the temporal, spatial, environmental, and genetic variability in both exposures and individuals' responses to the exposures. Even when exposome studies cover lifelong exposures, they generally do not place enough emphasis on defining and measuring windows of sensitivity to accurately capture the most important exposures, especially biological exposures. Existing studies are still insufficient to fully track the variability in the biological distribution of chemicals in the human body (e.g., the toxicokinetic variability) (Tsaioun et al. 2016). A similar problem also exists in the external environment, where industrial chemicals are constantly updated and social, economic and personal life are constantly changing.

In addition to the challenges of exposure variability, issues such as sample availability and quality, identification of unknown or suspected analytes, and capture of environmental chemicals remain challenges that must be continually addressed. To date, the ability to identify and quantify low-abundance environmental contaminants is still limited, leading to low-level chemicals often being undetected because of the inability to define reference or baseline values. Similarly, biomarkers of environmental exposures are not well defined. While untargeted measurements are promising for the discovery of such biomarkers, it also highlights the challenges of feature identification, quantification, variability, and repeatability (Sill´e et al. 2020). Despite the fast-developing analytical techniques and instruments, there remain vast challenges in a comprehensive and cost-effective characterization of the substances essential to the "totality" of environmental exposures. The advancement of exposome research requires simpler sample treatment strategies and more sophisticated and highly selective and sensitive mass spectrometry techniques compared with traditional approaches, as well as the development of other techniques for the determination of large molecules and genetic information.

The Challenge of Data Integration in Exposome Research

Another challenge originates from the integration of massive quantities of data collected from multiple sources using different technologies over time, as well as the

prediction of adverse health outcomes based on the patterns derived. Traditional modeling or data analyses are unlikely to meet such demands. Therefore, deep learning or artificial intelligence (AI) techniques may find important applications in exposome research. However, the studies that have employed deep learning or AI in exposome research remain scarce.

To facilitate the generation, sharing, and integration of large omics data from different laboratories, the Human Toxome Project has created and developed the Human Toxome Collaboratorium, a shared cloud-based data storage platform with metadata analyses and their documentation. Similarly, the HELIX, EXPOsOMICS, Heals and Hercules projects have contributed greatly to the research and method integration of the exposome.

Other Challenges

Admittedly, the exposome is in its infancy, with many challenges that need to be overcome. In terms of assessment, hundreds of time-varying exposures need to be accurately assessed using various technologies. Estimating the association between a large set of exposures and health outcomes leads to statistical challenges, including the difficulty to efficiently untangle the exposures truly affecting the health outcome from correlated exposures and the difficulty in identifying synergistic effects between exposures (Siroux et al. 2016). In addition, heterogeneity, experimental variation, and statistical bias are inevitable between laboratories, which pose great challenges to the cooperation among researchers of the exposome. The quality assurance and quality control challenges, bioinformatic challenges, database challenges, chemical mixture challenges, and the challenges of social and economic impact also continue to plague the researchers. However, all of these challenges should not hinder research relying on the exposome paradigm, and instead, we hope that the ongoing projects will greatly advance the exposome concept and analysis tools.

References

Andra SS, Austin C, Patel D et al (2017) Trends in the application of high-resolution mass spectrometry for human biomonitoring: an analytical primer to studying the environmental chemical space of the human exposome. Environ Int 100:32–61

Beasley R, Semprini A, Mitchell EA (2015) Risk factors for asthma: is prevention possible? Lancet 386:1075–1085

Careghini A, Mastorgio AF, Saponaro S et al (2015) Bisphenol A, nonylphenols, benzophenones, and benzotriazoles in soils, groundwater, surface water, sediments, and food: a review. Environ Sci Pollut Res 22:5711–5741

Chemical Abstracts Service (2016) CAS REGISTRY - The gold standard for chemical substance information. American Chemical Society, Columbus, OH. https://www.cas.org/cas-data/cas-registry. Accessed on 26 Oct 2021

Centers for Disease Control and Prevention (2015) Fourth National Report on Human exposure to environmental chemicals updated tables. Atlanta, GA. https://www.cdc.gov/exposurereport/index.html. Accessed on 26 Oct 2021
Chaste P, Leboyer M (2012) Autism risk factors: genes, environment, and gene-environment interactions. Dialogues Clin Neurosci 14:281–292
Chen D, Kannan K, Tan H et al (2016) Bisphenol analogues other than BPA: Environmental occurrence, human exposure, and toxicity-a review. Environ Sci Technol 50:5438–5453
Cui Y, Balshaw DM, Kwok RK et al (2016) The exposome: embracing the complexity for discovery in environmental health. Environ Health Perspect 124:A137–A140
Dagnino S (2019) Unravelling the exposome: conclusions and thoughts for the future. Unraveling the exposome: a practical view. Springer, New York, NY, pp 425–437
Dong T, Zhang Y, Jia S et al (2019) Human indoor exposome of chemicals in dust and risk prioritization using EPA's ToxCast database. Environ Sci Technol 53:7045–7054
Eqani SAMAS, Malik RN, Alamdar A et al (2012) Status of organochlorine contaminants in the different environmental compartments of Pakistan: a review on occurrence and levels. Bull Environ Contam Toxicol 88:303–310
Environmental Protection Agency (2016) TSCA Chemical Substance Inventory. Washington, DC. https://www.epa.gov/tsca-inventory. Accessed on 26 Oct 2021
Gangler S, Waldenberger M, Artati A et al (2019) Exposure to disinfection byproducts and risk of type 2 diabetes: a nested case-control study in the HUNT and Lifelines cohorts. Metabolomics 15:60
Garg S, Kumar P, Mishra V et al (2020) A review on the sources, occurrence and health risks of per-/poly-fluoroalkyl substances (PFAS) arising from the manufacture and disposal of electric and electronic products. J Water Process Eng 38:101683
Haddad N, Andrianou XD, Makris KC (2019) A scoping review on the characteristics of human exposome studies. Curr Pollut Rep 5:378–393
Howard PH, Muir DCG (2010) Identifying new persistent and bioaccumulative organics among chemicals in commerce. Environ Sci Technol 44:2277–2285
Johnson CL, Jazan E, Kong SW et al (2021) A two-step gas chromatography-tandem mass spectrometry method for measurement of multiple environmental pollutants in human plasma. Environ Sci Pollut Res 28:3266–3279
Locke AE, Kahali B, Berndt SI et al (2015) Genetic studies of body mass index yield new insights for obesity biology. Nature 518:197–206
Miller Gary W, Jones Dean P (2014) The nature of nurture: refining the definition of the exposome. Toxicol Sci 137:1–2
Miller Gary (2020) The exposome (Second Edition). The exposome: purpose, definitions, and scope. Elsevier, San Diego, CA, pp 1−26
Muir DCG, Howard PH (2006) Are there other persistent organic pollutants? a challenge for environmental chemists. Environ Sci Technol 40:7157–7166
Portelli MA, Hodge E, Sayers I (2015) Genetic risk factors for the development of allergic disease identified by genome-wide association. Clin Exp Allergy 45:21–31
Pourchet M, Debrauwer L, Klanova J et al (2020) Suspect and non-targeted screening of chemicals of emerging concern for human biomonitoring, environmental health studies and support to risk assessment: from promises to challenges and harmonisation issues. Environ Int 139:105545
Rappaport SM (2011) Implications of the exposome for exposure science. J Exposure Sci Environ Epidemiol 21:5–9
Rasheed T, Hassan AA, Bilal M et al (2020) Metal-organic frameworks based adsorbents: a review from removal perspective of various environmental contaminants from wastewater. Chemosphere 259:127369
Sarigiannis DA, Karakitsios SP (2018) Addressing complexity of health impact assessment in industrially contaminated sites via the exposome paradigm. Epidemiol Prev 42:37–48
Sarigiannis DA (2019) The HEALS project. Unraveling the exposome - a practical view. Springer, New York, NY, pp 405–422

Sander KN, Kim DH, Ortori CA et al (2019) Untargeted analysis of plasma samples from pre-eclamptic women reveals polar and apolar changes in the metabolome. Metabolomics 15:157
Sill´e F, Karakitsios S, Kleensang A et al (2020) The exposome: a new approach for risk assessment. ALTEX 37:3–23
Siroux V, Agier L, Slama R (2016) The exposome concept: a challenge and a potentialdriver for environmental health research. Eur Respir Rev 25:124–129
Tan H, Chen D, Peng C et al (2018) Novel and traditional organophosphate esters in house dust from south China: association with hand wipes and exposure estimation. Environ Sci Technol 52:11017–11026
Tan H, Yang L, Huang Y et al (2021) "Novel" synthetic antioxidants in house dust from multiple locations in the Asia-Pacific Region and the United States. Environ Sci Technol 55:8675–8682
Tang S, Chen Y, Song G et al (2021a) A cocktail of industrial chemicals in lipstick and nail polish: profiles and health implications. Environ Sci Technol Lett 8:760–765
Tang S, Li T, Fang J et al (2021b) The exposome in practice: an exploratory panel study of biomarkers of air pollutant exposure in Chinese people aged 60–69 years (China BAPE Study). Environ Int 157:106866
Thakur M, Pathania D (2020) Chapter 12 - environmental fate of organic pollutants and effect on human health. In: Abatement of Environmental Pollutants, Editor(s): Pardeep Singh. Elsevier, San Diego, CA, pp 245–262
Tsaioun K, Blaauboer BJ, Hartung T (2016) Evidence- based absorption, distribution, metabolism, excretion and toxicity (ADMET) and the role of alternative methods. Altex 33:343–358
Vermeulen R, Schymanski EL, Barabási AL et al (2020) The exposome and health: where chemistry meets biology. Science 367:392–396
Vineis P, Chadeau-Hyam M, Gmuender H et al (2017) The exposome in practice: design of the EXPOsOMICS project. Int J Hyg Environ Health 220:142–151
Vineis P, Demetriou CA, Probst-Hensch N (2020) Long-term effects of air pollution: an exposome meet-in-the-middle approach. Int J Public Health 65:125–127
Vrijheid M, Slama R, Robinson O et al (2014) The human early-life exposome (HELIX): project rationale and design. Environ Health Perspect 122:535–544
Wild CP (2005) Complementing the genome with an "exposome": the outstanding challenge of environmental exposure measurement in molecular epidemiology. Cancer Epidemiol Biomarkers Prev 14:1847–1850
Wild CP (2012) The exposome: from concept to utility. Int J Epidemiol 41:24–32
Wild CP, Scalbert A, Herceg Z (2013) Measuring the exposome: a powerful basis for evaluating environmental exposures and cancer risk. Environ Mol Mutagen 54:480–499
Yu M, Tu P, Dolios G et al (2021) Tooth biomarkers to characterize the temporal dynamics of the fetal and early-life exposome. Environ Int 157:106849
Zhu QL, Liu S, Gao P et al (2014) High-throughput sequencing technology and its application. J Northeast Agri Univ 21:84–96

Chapter 4
Omics Approaches in Toxicological Studies

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Abstract As elucidating molecular mechanisms of toxicity and establishing adverse outcome pathways (AOPs) have become the focus of chemical toxicology and risk assessment, there is an urgent need for high throughput and high content data to enable these goals. To comply with these strategies of the next generation toxicology, omics (e.g., transcriptomics, proteomics, and metabolomics) methods have been increasingly employed for analyses of chemical effects. This chapter gives an overview of the recent methodological advances in the main omics approaches applied in toxicology. Molecular fingerprinting, biomarker discovery and molecular mechanism elucidation using transcriptomics are discussed, advantages and limitations of various label-free and isotope labeling methods in proteomics are reviewed, and mass spectrometry- and nuclear magnetic resonance spectroscopy-based techniques in targeted and untargeted metabolomics are discussed. The main limitations of the omics methods in toxicology, including the relatively high cost, lack of comprehensive and comparable databases and the need for standardized analysis pipelines are outlined. Future directions of omics in toxicology are envisioned to include the application of novel omics methods such as translatomics, integration of multiple omics approaches (multi-omics) and extending chemical risk assessment to microbial communities (host-microbiota metabolite interactions) using metatranscriptomics and metaproteomics.

Introduction

According to the "central dogma" of molecular biology, postulated by Francis Crick in 1957, the genetic information is transferred from DNA to RNA to proteins, and

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never the other way around (e.g., when the information from DNA has been translated into the protein, it cannot be turned into a genetic code again) (Cobb 2017). The concept of the one-directional flow of genetic information and the knowledge of the components of this molecular biology machinery is the basis for toxicogenomics—a discipline that explores the toxicity mechanisms using a range of "omics" technologies (Fig. 4.1). During recent years, omics methods and bioinformatics have increasingly been employed in toxicology. Omics approaches are applied in toxicology to find biomarkers for pollution biomonitoring, for the elucidation of toxicity mechanisms, and development of adverse outcome pathways (AOPs). Among omics approaches, the best-developed methods which have been used in toxicology, include transcriptomics, proteomics and metabolomics. In this chapter, the focus will be on these methods, outlining the recent methodological advances and examples of their applications in toxicity studies of chemicals of emerging concern. Two or more omics methods are often used in parallel to gain better insight into the affected pathways upon toxicant exposure. These, so-called multi-omics or integrated omics approaches will be discussed from the aspect of opportunities but also challenges in integrating the data sets from different omics analyses. In addition to the abovementioned three main omics approaches, methods such as translatomics, which more accurately reflect the gene expression and protein synthesis processes, are emerging and may soon find applications in toxicology as the technology advances. Thus, the chapter will also give a brief overview of emerging omics methods yet to be widely used in toxicology.

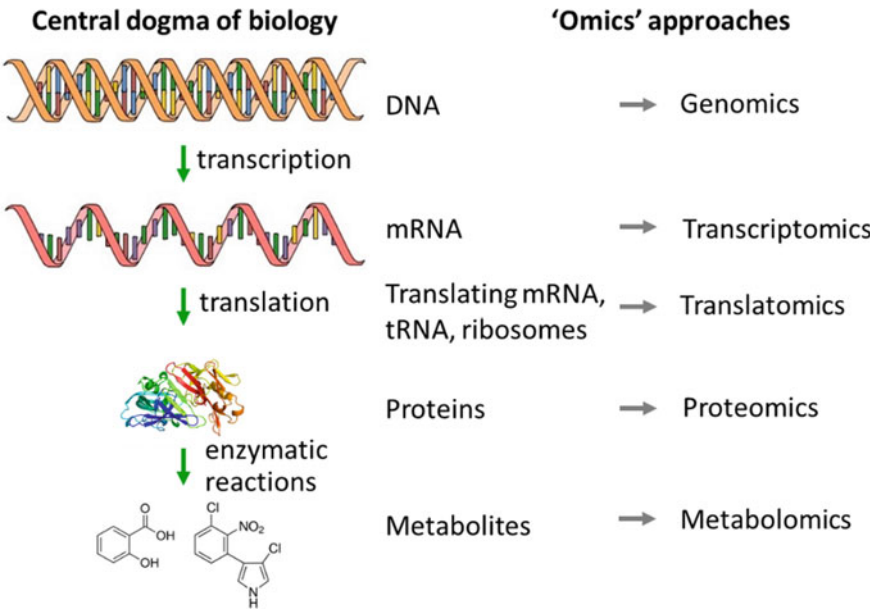

Fig. 4.1 The central dogma of biology and associated omics approaches

Also, the limitations and future perspectives of omics technologies in toxicology will be outlined.

Genomics and Transcriptomics

Developments in sequencing technologies have allowed researchers to gather genomic and transcriptomic data with much higher coverage and more cost-effectively than ever, leading to an increase in the application of these technologies in various fields, including (eco)toxicology. While genomics aims to analyze the complete DNA sequence of a (micro)organism, transcriptomics (in a wide sense) refers to the set of an entire repertoire of transcripts in the cell, including mRNA, noncoding RNA and small RNA. The expression of these molecules is dynamic and reflects a cellular response to environmental conditions, which makes transcriptome analysis a suitable candidate to study cellular responses to potentially hazardous compounds. Transcriptomics has so far been the most widely used high-throughput approach in toxicology research (Shin et al. 2018). This is associated with relatively lower cost, well-established computational pipelines and higher amounts of data one can obtain compared to proteomics or metabolomics (Liang et al. 2020). In toxicogenomics, gene expression analysis methods are mainly used in three areas: (1) obtaining the "molecular fingerprint" of the chemicals, (2) identifying new biomarkers for chemicals, and (3) elucidating the molecular mechanism of chemicals. The methodological approaches of applying gene expression analysis in toxicogenomics have been recently reviewed (Alexander-Dann et al. 2018) (Fig. 4.2), thus a brief overview will be given of the three main application areas in the next sections.

Molecular Fingerprinting of Toxicants

For "molecular fingerprinting" of toxicants or pollutants, genome-wide expression profiling provides unprecedented resolution and can comprehensively characterize the molecular biological pathway effects of chemicals (Su et al. 2014). Considering that the number of genes in organisms is large (e.g., humans have about 20,000 genes and mice about 21,000 genes), numerous changes induced by chemical exposures occur simultaneously, which need to be detected. Transcriptomics provides the means to describe the molecular pathways affected by toxicants by simultaneously detecting changes in the expression profiles of tens of thousands of genes. This overall picture contains complex regulation and network relationships among genes across the genome and is a "molecular fingerprint" with extremely rich biological information about chemicals. So, researchers can effectively distinguish chemicals with different toxicity mechanisms by comparing genome-wide expression profiles (Shi et al. 2006) and discover potential toxicity mechanisms of new chemicals based on the

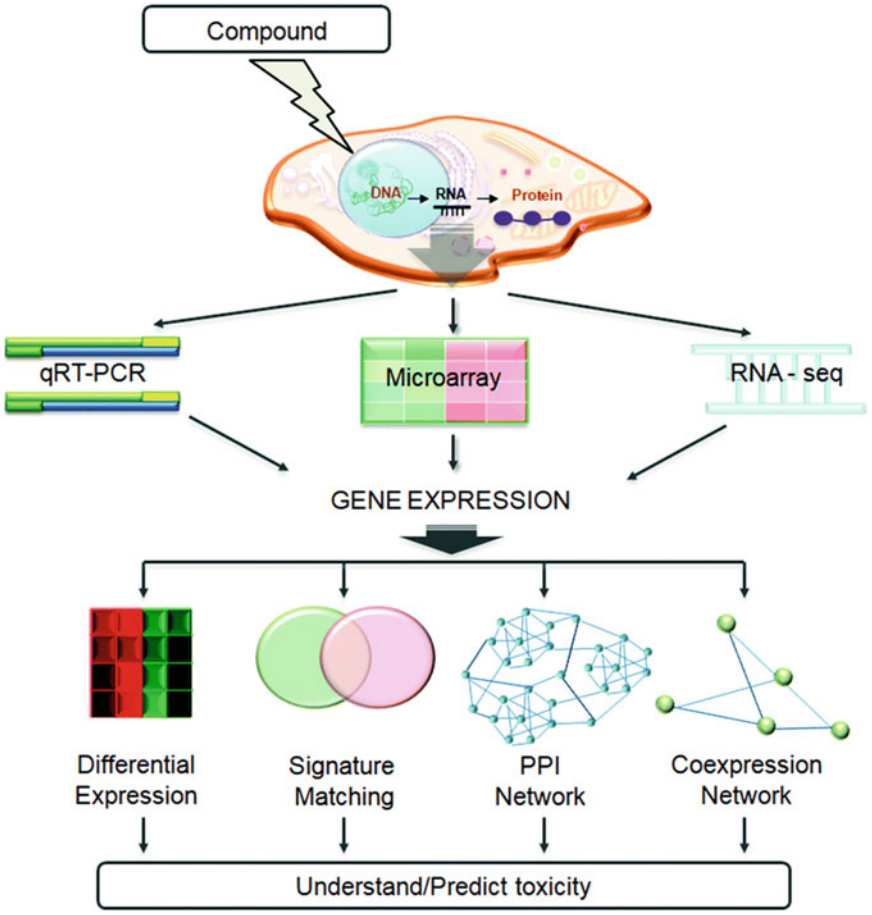

Fig. 4.2 Overview of the methodological approaches and application fields of gene expression analysis in toxicogenomics. qRT-PCR—quantitative reverse transcription-polymerase chain reaction; RNA-seq—RNA sequencing; PPI—protein–protein interaction. Reproduced from (Alexander-Dann et al. 2018) with permission from the Royal Society of Chemistry

similarity of their genome-wide expression profiles with the profiles of other known toxic chemicals. This kind of chemical classification based on whole-genome expression profiles relies on a standardized whole-genome expression profile database, to enable accurate and reliable comparison of the profiles of chemicals. However, due to the wide range of chemicals and high cost of omics technology, currently, no institution or research group can independently test the whole genome expression profiles of all chemicals. Although some public databases, that collect and integrate published whole-genome expression profile data, exist, such as the Gene Expression Omnibus (GEO) database of the National Center for Biotechnology Information (NCBI), these

data are usually derived from different laboratories and have been obtained by experiments with different designs, sequencing platforms, and data processing methods (Wang et al. 2009), which makes the collected data not readily comparable.

Biomarker Identification

After obtaining a set of genome-wide expression profiles for a chemical, biomarkers of the chemical can be identified based on its classification characteristics (Hamadeh et al. 2002; Hayes and Bradfield 2005). The identification of biomarkers is usually conducted using bioinformatics methods which enable to find the marker genes that are similarly regulated in the whole genome expression profiles of the same type of chemicals. These biomarkers can help optimize or simplify the classification of chemicals. For example, Li et al. compared the transcriptome profiles of 14 genotoxic and 14 non-genotoxic chemicals and detected 65 marker genes that could accurately distinguish genotoxic chemicals (Li et al. 2015). In addition, the selected biomarkers can help to predict potential molecular mechanisms of unknown toxic chemicals (Minowa et al. 2012). Furthermore, the genome-wide expression profile of chemicals can indirectly explain the toxic mechanism of chemicals at the level of molecular biology pathways based on existing gene function databases such as Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO). These databases enable the annotation of the genes identified by the genome-wide expression profile. The "gene-function" annotation information can reflect the influence of chemicals on molecular biology pathways to a certain extent, and help indirectly explain the toxic effects of chemicals (Moffat et al. 2015). However, relying solely on toxicogenomics for finding biomarkers is not sufficiently accurate to assess the toxic effects of chemicals, which need to be further verified by conducting traditional phenotyping experiments (Waters et al. 2010). It is worth noting that the results must be treated with caution because the impact of chemicals on biological pathways does not necessarily manifest physiologically. Thus, genome-wide expression profiles can serve as one of several indicators when elucidating the chemical's toxicity mechanism (Currie 2012).

Elucidating the Molecular Mechanisms

Transcriptomics is the best-developed technology among the omics approaches used in toxicogenomics, with the main methods including microarray (Lobenhofer et al. 2001) and RNA-sequencing (RNA-seq) (Su et al. 2014). The microarray relies on the known genome sequence of the species and detects the changes in mRNA expression of the whole genome through the hybridization of a nucleic acid sample to oligonucleotide probes attached to a solid support. The hybridization-based approach measures the abundance of a known set of genes (in the form of cDNA molecules

immobilized on a glass slide) using an array of complementary probes (Bumgarner 2013). Commercial microarrays are available for over 30 organisms including plants, animals and humans. However, the technology depends on existing genome data or annotation which makes the method inapplicable for the identification and quantification of novel transcripts (Mezencev and Subramaniam 2019). Also, for species with incomplete genome information, microarrays cannot be designed to cover the entire genome. In addition, the method has low reproducibility and, due to background interference of the fluorescence signal, does not enable the detection of low-abundant transcripts. It is also unsuitable for the analysis of noncoding RNA and the identification of isoforms over a wide dynamic range. However, most of the abovementioned limitations related to microarray technology have been solved by the alternative approach that relies on deep sequencing of the complete set of isolated, reverse-transcribed and amplified RNA transcripts, RNA-seq (Lowe et al. 2017). RNA-seq is a next-generation sequencing (NGS) technology that has been designed for the analysis of total mRNA samples, yielding quantitative data about mRNA expression levels as well as providing mRNA sequence information. The RNA sequence information obtained by RNA-seq can be used to assemble (de novo) reference transcriptomes for annotation as well as quantify mRNA expression (Wang et al. 2009). The Sequencing Quality Control Alliance led by the US FDA specifically compared the chemical test performance of RNA-seq and microarrays and found that RNA-seq had greater advantages, mainly due to its higher sensitivity and accuracy to low-expressed genes, however, gene expression-based predictive models generated from RNA-seq and microarray data were similar (Wang et al. 2014). In general, though proteomics and metabolomics compared with transcriptomics more intuitively reflect the characteristics of the biological phenotype, current proteomics and metabolomics technologies do not allow for an exhaustive analysis of all proteins and metabolites in an organism, leaving their application scope inferior to that of transcriptomics technologies.

Limitations of Current Transcriptomics Methods in Toxicogenomics

Although RNA-seq technology is improving, there are still challenges associated with RNA-seq applications in toxicology studies. Firstly, there is a lack of public databases of transcriptomics data that would allow comprehensive chemical classification analysis. At present, the cost of transcriptomic analysis is still a limiting factor in testing the whole genome expression profiles induced by all chemicals. Therefore, lowering the cost of obtaining the information of genome-wide expression profiles upon toxicant exposures is the key to the successful construction of the transcriptome databases. Recently, reduced transcriptomics technology was proposed to comprehensively, but with reduced budget and labor, evaluate the toxic effects of chemicals and identify key toxicity pathways that lead to key biological effects (Dai 2018).

The reduced transcriptome approach is based on the principle that a small number of genes can represent the expression of whole gene networks (Lamb et al. 2006; Judson et al. 2012). Targeted sequencing technology allows detection of a small number of key genes, avoiding redundant gene information and allowing to gain information on the entire transcriptome efficiently and economically (Zhang et al. 2018). The method is based on the design of multiple PCR primers for the cDNA sequence of the mRNA of the target gene. Then the target sequence in the sample is amplified through high-throughput sequencing and the expression abundance of the target gene mRNA and the biological pathway changes are analyzed (Bodi et al. 2013). Zhang and Zhao demonstrated the application of reduced transcriptomics in the detection of neurotoxic properties of chemicals by using a reduced transcriptome atlas (RTA) approach which integrated transcriptomic data sets and a set of genes associated with neurogenesis and the early neurodevelopment of zebrafish (Zhang and Zhao 2018). Integration of transcriptomics data sets of 74 chemicals and 736 gene expression profiles resulted in 135 exposure signatures. Gene expression index (GEI) analysis of the data identified a gene panel with 300 genes that was sufficient to assess the neurotoxic potential and the biological pathways affected by each neurotoxicant. Also, reduced transcriptomics projects are being developed in the United States with support from the government agencies, including the Library of Integrated Network-based Cellular Signatures (LINCS) project funded by the National Institutes of Health (NIH) and the S1500 test plan supported by the US Environmental Protection Agency (EPA). The LINCS project is based on the L1000 test platform which measures about 1000 human genes in each experiment while the rest of the transcriptome (~22 000 genes) are estimated by a model built from thousands of gene expression datasets in Gene Expression Omnibus (GEO) (Duan et al. 2014). In addition to integrating 1,000 genes in the L1000 project, the S1500 project has generated a set of more than 1500 "sentinel genes" which represent all known canonical pathways from the Molecular Signature Database (Mav et al. 2018). Currently, the number of human genes tested by S1500 is between 1500 and 3000. However, both the LINCS project and the S1500 project lack the complete dose–effect relationship characterization of the test samples. At present, the LINCS project includes the transcriptome profiles of chemicals tested at a single exposure concentration (De Wolf et al. 2016) and the S1500 project data has been obtained from testing at up to three chemical concentrations (Grimm et al. 2016; House et al. 2017).

Thus, some of the recent studies have focused on developing a cost-effective approach for chemical concentration–response modeling and quantitative characterization of gene expression profiles and pathways. For example, a concentration-dependent reduced transcriptome analysis method was developed using Ion Ampliseq sequencing to obtain the expression levels of thousands of genes upon exposure to single chemicals or mixtures at 7–8 different concentrations (Xia et al. 2017; Wang et al. 2018). The method uses a transcription dose–response model (S-, L- or U-type model) based on the expression levels of each gene. The model enables the calculation of each gene's point of departure (POD), also known as PODDEG. Then the POD of the biological pathway can be obtained based on the results of PODDEG. In this method, all genes from existing databases of biological pathways such as

the KEGG or GO, AOP-Wiki (https://aopwiki.org/aops) and hallmark gene set from Molecular Signatures Database (MSigDB) were included. To date, concentration-dependent reduced transcriptome-based methods have been established for human cells (Reduced Human Transcriptome, RHT) (Xia et al. 2017) and zebrafish embryos (Reduced Zebrafish Transcriptome, RZT) (Wang et al. 2018). RHT and RZT have been applied to evaluate water samples with different degrees of pollution. The analysis of the number of affected genes and the distribution of biological pathway sensitivity curves allowed the authors to distinguish contaminated and clean water samples. In addition, the results showed that the biological activity profile obtained by the concentration-dependent RHT and RZT tests was similar to the results of 103 in vitro biological tests (Xia et al. 2017; Wang et al. 2018).

Additional challenges in the application of transcriptomics in toxicology include the lack of standardization and difficulties in data interpretation. One of the complications involves the aspect that the changes in gene expression do not always translate into physiological impairment. Biological interpretations of the transcriptomic results may also be limited by the quantity and quality of available gene annotation data, especially for non-model species, as well as the lack of integrative tools required for data interpretation (Morel et al. 2020). The reduced transcriptomics approaches, where a subset of toxicologically relevant genes is measured and which enables the extension of the tested range of chemical concentrations, have helped to reduce the complexity of data analysis and interpretation of transcriptomics analysis in toxicology. However, such an approach limits the potential for the discovery of novel mechanisms of actions of certain emerging pollutants such as nanomaterials (Zhang et al. 2018). Future developments in the field of transcriptomics will likely extend beyond the whole-genome expression on mRNA and will include profiling of regulatory molecules, such as non-coding RNA (ncRNA), microRNA and long non-coding RNA (lncRNA) (Mezencev and Subramaniam 2019).

Proteomics

Although transcriptomics has so far been the most widely applied global approach in (eco)toxicology, proteomics is gaining importance in toxicology as one of the most dynamic and fast-developing fields to analyze changes in the protein expression at the effector level. Proteins are direct mediators of genomic response and therefore directly guide higher-level phenotypic effects. Proteomics and transcriptomics are increasingly recognized as complementary, rather than alternative approaches of expression analysis as it has been demonstrated that there is rarely perfect stoichiometry between transcript and protein levels in terms of abundance due to post-transcriptional and post-translational processes, such as mRNA/protein turnover, alternative splicing and posttranslational modification (Liang et al. 2020).

In proteomic studies typically one of the two approaches is applied: (a) bottom-up approach, where the proteins are first digested by trypsin, and the resulting peptides (~0.8 to 3 kDa) are ionized, separated based on mass/charge ratio (m/z) and detected

by mass spectrometry (MS) or (b) top-down approach (shotgun proteomics), where intact protein ions are introduced in the gas phase and are fragmented (~10 kDa in size) and analyzed by MS to identify the mass of the protein and protein ion fragments, which are then puzzled out to reveal the primary structure of the protein (Fig. 4.3) (Vinh 2019). Most proteome studies employ a bottom-up MS (Fig. 4.3A) where the

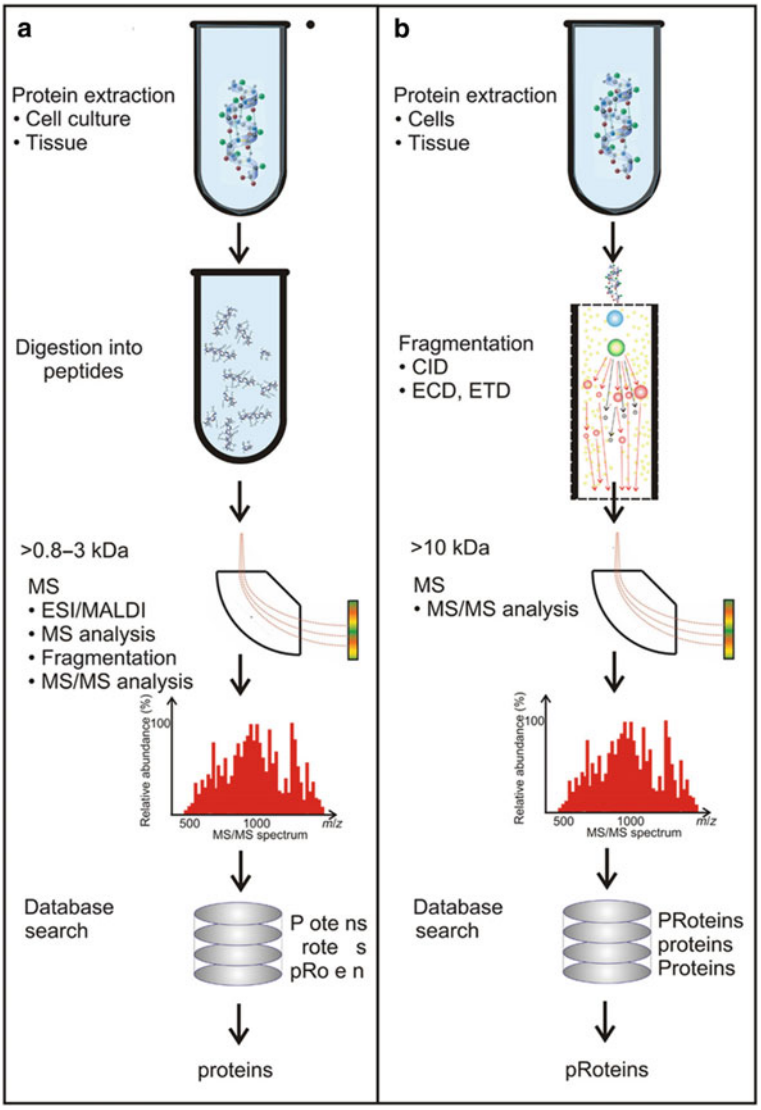

Fig. 4.3 Schematic illustration of two main proteomics strategies: (A) bottom-up approach and (B) top-down approach (shotgun proteomics). CID—collision-induced dissociation, ECD—electron-capture dissociation, ETD—electron-transfer dissociation. Reprinted from (Timp and Timp 2020)

digested peptides (5–10 amino acids long) are usually either ionized in a gas phase directly in an electrospray ionization or matrix-assisted laser desorption/ionization (MALDI) instrument or are first separated by liquid chromatography-MS (LC–MS) and then ionized for MS analysis. The challenges in protein identification using the bottom-up approach include the aspect that due to the proteins being digested into relatively short peptides before identification, the database searches yield only fragments of the complete protein. The assignment of peptides to particular proteins is further complicated by the sequence similarity of some proteins. Another difficulty is associated with the sensitivity of the method which requires a high number of spectra to achieve accurate identification of peptide sequences. It has been reported that about 75% of spectra may be unidentifiable due to signal-to-noise issues, incomplete databases or unknown post-translational modifications of proteins (Griss et al. 2016). Top-down MS on the other hand relies on the analysis of intact proteins (Fig. 4.3B) enabling protein sequencing. Electrospray ionization is used to introduce the intact proteins into the gas phase that are then fragmented either by collision-induced dissociation (CID) or electron-capture dissociation (ECD) or electron-transfer dissociation (ETD) in the MS. The resulting spectra include the masses of proteins and their fragment ions. However, the method can only be used for the identification of proteins < 70 kDa because of the limitation of gas-phase fragmentation and ionization of larger intact proteins. Other shortcomings of the top-down MS include lower sensitivity, proteomic coverage and throughput compared to bottom-up MS (Timp and Timp 2020).

Label-free Proteomics

The most commonly applied separation technique preceding MS has long been two-dimensional electrophoresis (2D-GE), which allows the separation of a large number of proteins based on their isoelectric point and molecular weights. Its main advantages include a visual representation of target proteomes and ease of the detection of post-translational modifications, while its drawbacks include poor reproducibility and the fact that it is time-consuming (López-Pedrouso et al. 2020). Reproducibility, sensitivity, and accuracy of 2D-GE have, however, been significantly improved in 2D differential gel electrophoresis or 2D-DIGE, where different protein samples are labeled with fluorescent tags, allowing to analyze up to three samples in one gel. Still, to overcome the high cost, low sensitivity and limitations of not being able to analyze the entire proteome using 2D gel-based approaches, gel-free proteomics methods have been developed. Consequently, protein identification and quantitation approaches relying on (multidimensional) chromatographic separation of proteins or peptides are increasingly being used. For example, intact proteins can be fractionated using a multidimensional system that consists of chromatofocusing (proteins are fractionated based on isoelectric point) followed by reverse-phase high-performance liquid chromatography (RP-HPLC) (separates proteins based on their surface hydrophobicity). The obtained 2D plots or chromatograms can be used for

comparative analysis of protein expression levels in different samples. Proteins from the collected fractions can be identified by performing the tryptic digestion and tandem MS analysis. Alternatively, a top-down fragmentation of proteins can be applied. Since this approach does not require chemical or metabolic labeling of proteins, the methods are called "label-free" quantitative proteomics. Label-free methods can be used for relative quantification of proteins by either counting the number of peptide-to-spectrum matches obtained for each protein (i.e., spectral counting) or by measuring peptide signal intensities in the extracted ion chromatogram (Lindemann et al. 2017). In spectral counting, the number of MS/MS fragment ion spectra are compared between the samples. In another label-free approach, which involves the extracted ion chromatogram, peptides are quantified by integration of ion intensities over their chromatographic elution profile and differential expression analysis is accomplished by comparing the integrated signals of individual peptides in LC–MS/MS runs of different samples. In label-free approaches, the number of samples to be compared is not limited by the availability of different labels, so unlimited samples can be analyzed and compared, which is one of the advantages of label-free methods (Table 4.1). The use of high-performance MS has enabled the advancement of label-free proteomics by application of two main approaches: data-dependent acquisition (DDA) and data-independent acquisition (DIA). In DDA peptides are identified by searching the fragment spectra in a protein database. DIA data analysis, on the other hand, commonly uses a spectral library constructed from DDA experiments to extract ion chromatograms from DIA data, while the most widely applied software packages include OpenSWATH, Spectronaut, and Skyline (Guan et al. 2020). Since DIA analysis is challenging and protein sequence database searches cannot be applied directly, modified data analysis methods have been recently proposed, such as a data dependent-independent acquisition (DDIA) method (Guan et al. 2020). This approach combines both DDA and DIA in one LC–MS/MS analysis, allowing first to identify peptides in the DDA scans which provides useful information for in-depth interrogation of the DIA scans. The disadvantages of label-free proteomics include the aspect that it does not support multiplexing of samples which means that each sample has to be treated and analyzed individually, resulting in increased variability which can originate from the variations in sample preparation workflow as well as the retention time and m/z values (Table 4.1).

Stable Isotope Labeling-based Proteomics

Multiplexing of samples resulting in higher quantification accuracy and precision is enabled by label-based approaches. Stable isotope labeling, which can be chemical, enzymatic or metabolic, enables improved relative or absolute quantification of up- or downregulation of proteins under toxicant exposure conditions compared to untreated control.

Table 4.1 Advantages and limitations of label-free and stable isotope labeling proteomics methods

Category	Method	Advantages	Limitations
Label-free	2D gel electrophoresis and mass spectrometry	Easy sample preparation Deep proteome coverage Large dynamic range for protein identification	High sample-to-sample variability Quantification is not as efficient as in the labeling-based approaches
	Gel-free mass spectrometry	Comparison of an unlimited number of samples Simple and cost-efficient	Does not allow multiplexing Requires separate sample processing until analysis
Chemical labeling with stable isotope mass tags	ICAT (isotope-coded affinity tag)	Commercially available as a kit Affinity capture of cysteine-containing peptides simplifies a complex tryptic digest for MS	High cost of labeling reagents Not quantitative for low abundance peptides Proteins without cysteine residues are excluded from analysis For comparison of only 2 samples
	ICPL (isotope-coded protein labeling)	Commercially available as a kit Labeling proteins before digestion allows pooling of samples early in the preparation process Nicotinic derivative increases MS signal intensities and fragmentation	Only 60–70% of identified proteins can be quantified Ineffective trypsin digestion of the proteins labeled at lysine residues

(continued)

Table 4.1 (continued)

Category	Method	Advantages	Limitations
	DML (dimethyl labeling)	High reaction yield High accuracy and reproducibility Robust Simple to perform Low cost	Up to 5 samples can be multiplexed Deuterated peptides elute slightly before light ones in reversed-phase chromatography
	iTRAQ (isobaric tags for relative and absolute quantitation)	Commercially available High multiplexing capacity (simultaneous analysis of up to 8 samples) Less complex mass spectra compared to non-isobaric methods	Ratio misrepresentation due to interference of contaminating near isobaric ions High cost Underestimation of fold change in protein expression
	TMT (tandem mass tagging), isobaric labeling	Commercially available High multiplexing capacity (simultaneous analysis of up to 11 samples) Less complex mass spectra compared to non-isobaric methods	Ratio misrepresentation due to interference of contaminating near isobaric ions High cost Underestimation of fold change in protein expression
	DiLeu (N,N-dimethyl leucine) isobaric labeling	Low cost High multiplexing capacity (simultaneous analysis of up to 12 samples) Enhanced collision-induced fragmentation compared to iTRAQ or TMT	In-house synthesis of isobaric tags

(continued)

Table 4.1 (continued)

Category	Method	Advantages	Limitations
Enzyme-catalyzed labeling with stable isotope mass tags	Trypsin-catalyzed $^{18}O/^{16}O$ labeling	Labels peptides non-selectively, without targeting specific amino acids Does not require enrichment of labeled peptides in affinity columns Applicable to clinical samples with limited quantities Can be combined with other stable isotope labeling methods Low cost	Two C-terminal ^{16}O atoms are not always completely exchanged for two ^{18}O atoms which complicates accurate calculation of $^{18}O/^{16}O$ ratios C-terminal peptide of each protein is not labeled and consequently, discrimination is not achievable
Metabolic labeling with stable isotope mass tags	SILAC (stable isotope labeling by amino acids)	Labeling efficiency can be up to 100% Good quantitative repeatability, low protein consumption Suitable for the identification and quantification of membrane proteins Closer to the real state of the sample	Mainly suitable for passagable cells or bacteria, requires 5–6 cell divisions to incorporate the label 'Heavy'-arginine labels can be inserted into proline through arginine catabolism, distorting the ratios of proline-containing light and heavy peptides

Chemical Labeling

Chemical labeling involves adding stable isotope-containing mass tags to proteins or peptides in vitro, after extraction from cells or tissues, i.e., post-harvest labeling. The main chemical labeling methods include isotope-coded affinity tags (ICAT), isotope-coded protein labeling (ICPL), dimethyl labeling, isobaric tags for relative and absolute quantitation (iTRAQ) and tandem mass tagging (TMT) (Table 4.1). ICAT were the first commercially available reagents for isotope labeling of proteins and include biotinylated iodoacetamide or acrylamide derivatives that react with the sulfhydryl groups of cysteine side chains (Gygi et al. 1999). The approach is based on the use of two types of tags, 'heavy' form where the hydrogen or carbon atoms are replaced by deuterium or ^{13}C, respectively, and 'light' form in which H or C atoms have not been modified. One of the forms is used for labeling toxicant-treated sample and the other form for labeling untreated (control) sample. After labeling, 'heavy' and 'light'-tagged samples are pooled, digested to peptides and cysteine-containing peptides are then isolated from the complex sample mixture by biotin-streptavidin affinity chromatography. Quantification of relative abundance of each labeled peptide is afforded by LC–MS analysis where the ratio of peak areas of the 'heavy'/'light' tagged peptide pairs is determined. The advantages of ICAT are that it can be used for large-scale analysis of complex samples and is readily available as a commercial kit, however, the analysis excludes proteins that do not contain cysteines (~10% of the proteome) and does not accurately quantify low-abundance proteins due to recovery bias of the low-abundance biotinylated peptides from the streptavidin column (Chahrour et al. 2015). Similar to ICAT labeling, a technology called ICPL, which incorporates isotope labeling with different numbers of deuterium or carbon atoms to lysine side chains and N-termini of proteins, can also be used for multiplex quantitative analysis (Schmidt et al. 2005). The proteins are commonly labeled prior to digestion, which allows for combining samples early in the sample preparation process. The approach labels lysine residues which means that trypsin digestion, which cleaves selectively at lysine and arginine residues, will not be effective at lysine residues, yielding longer peptides than usual. This effect can either be used as an advantage where longer peptides are desired for identification and quantification or as a disadvantage that can be overcome using double enzymatic digestion. Another disadvantage associated with ICPL labeling is the aspect that a typical LC–MS analysis does not detect all lysine-containing peptides, resulting in the quantification of only 60–70% of identified proteins. Stable-isotope dimethyl labeling by reductive amination, however, achieves labeling of all peptides at the N-terminus and ε-amino group of lysine. The reaction is fast, specific, and low-cost and can be performed by in-solution, online, and on-column. Multiplexing up to five samples has been demonstrated using the dimethyl labeling strategy (Wu et al. 2014). Its disadvantages include lower multiplexing capacity compared to isobaric labeling methods and elution of deuterated peptides earlier than the light forms in reversed-phase chromatography, but due to its versatility and cost-effectiveness, is an increasingly popular method of choice in quantitative proteomics (Tolonen and Haas 2014).

Since non-isobaric labeling methods discussed in the previous paragraphs only allow up to five different tags, isobaric labeling was developed to be able to compare multiple samples in one experiment, i.e., to perform multiplexed quantitative protein analysis. Isobaric tags have identical masses and thus cannot be distinguished chromatographically, but can be quantified based on fragmentation in an MS/MS run when reporter ions with different masses are released. The identical masses of isobaric tags give an advantage compared to other labeling methods to compare the same peptide from each of the samples or conditions as a single peak in the mass spectrum, taking advantage of the reduced density of the data. The most used isobaric labeling reagents are tandem mass tags (TMT) and isobaric tags for absolute and relative quantification (iTRAQ) as these are commercially available (Chahrour et al. 2015). TMT labels consist of a reporter group, an NH_2-reactive group and a linker connecting the two groups. The linker is vulnerable to fragmentation in the second MS run. Because each TMT tag has the same chemical structure, there is no need to modify the labeling protocol or HPLC separation conditions between experiments. In TMT reagent sets that enable multiplexing, the tags of the same set contain equal numbers of ^{13}C and/or ^{15}N atoms which have been incorporated in different locations of the molecular structure, so that the isotopic mass of each label's reporter group is different. Similar to TMT tags, iTRAQ labels consist of an N-hydroxy succinimide ester group that reacts with peptide amine groups, an N-methyl piperazine reporter group and a balancer group which ensures a constant mass increase in the tagged peptides. The tags react with all primary amine groups in peptides, including the N-terminus and ε-amino group of the lysine side-chain, which ensures the labeling of almost all peptides in the sample. For the analyses of iTRAQ and TMT labeled samples, both MALDI and electrospray ionization (ESI) tandem MS have been used, with ESI used several times more frequently than MALDI. Recent literature indicates that the analysis of iTRAQ labeled samples is nowadays commonly made with Orbitrap mass analyzers (Moulder et al. 2018). Both TMT and iTRAQ workflows suffer from ratio misrepresentation due to contaminating near isobaric ions which are co-isolated in MS1 and co-fragmented in MS2. Such interference may cause an underestimation of the differential protein expression in different samples. This problem can be overcome by conducting an additional isolation and fragmentation run of MS3 (Christoforou and Lilley 2012). Another disadvantage of the commercial iTRAQ and TMT tags is their high cost. To overcome this and provide a more affordable isobaric labeling option, in-house synthesized N,N-dimethyl leucine (DiLeu) tags were proposed (Frost et al. 2015). The structure of DiLeu resembles TMT and iTRAQ labels and includes an amine-reactive triazine ester group that binds the N-terminal and primary amino group of the lysine side chain, a balancer group, and a reporter group (Frost et al. 2015). DiLeu labeling allows analysis of up to 12 samples simultaneously and has been reported to provide improved peptide identification and quantification compared to iTRAQ and TMT due to higher reporter ion intensities. Further, co-isolation and co-fragmentation of DiLeu-labeled peptides are reduced by ion mobility (Arul and Robinson 2019). Although isobaric labeling approaches enable high throughput sample analysis, a common problem for reporter ion-based quantification is ratio distortion. To solve this issue, alternative isobaric

tags have been designed, such as the recently proposed isobaric acetyl-isoleucine-proline-glycine (Ac-IPG) tag which enables quantification of peptides based on unique fragments while reducing the complexity of the b-ion series (Tian et al. 2020). The labeling approach relies on a collision-induced dissociation (CID)-cleavable tag and its proteome quantification capability was demonstrated over a tenfold dynamic range when tested by triplex labeling of a yeast proteome.

Enzymatic Labeling

Another group of in vitro labeling methods is enzymatic labeling with stable isotopes. For example, trypsin-catalyzed $^{18}O/^{16}O$ labeling has emerged as a powerful tool for quantitative comparative proteomics. The method is based on trypsin-catalyzed oxygen exchange at the C-terminal carboxyl group of cleaved peptides which generates peptides with a mass difference of 4 Da between the two labeled forms, which can be quantified on an ion trap or MALDI-time-of-flight (TOF) MS in a data-dependent LC–MS/MS acquisition mode (López-Ferrer et al. 2006). One of the disadvantages of the $^{18}O/^{16}O$ labeling method is the incomplete exchange of the two C-terminal ^{16}O atoms for ^{18}O atoms due to the variability in enzyme reaction rates, which causes inaccuracies in the determination of $^{18}O/^{16}O$ ratios. However, methodological and computational solutions have been developed to overcome the quantification issues caused by incomplete ^{18}O incorporation. Thus, considering the relatively low cost and facile procedure of the method, it is considered a preferred method for proteomic profiling of human samples (Chahrour et al. 2015).

Metabolic Labeling

In contrast to chemical labeling, stable isotope labeling by amino acids (SILAC) involves metabolic labeling of proteins during their synthesis in vivo, directly in the cell culture. In SILAC, cell populations are differentially labeled with arginine and lysine with stable isotopes of either ^{2}H, ^{13}C and ^{15}N (heavy culture) and Lys/Arg with no special labeling (light culture) (Lindemann et al. 2017). After the labeling, the two cell cultures subjected to different conditions (i.e., control vs. exposure) are mixed in a 1:1 ratio and processed (proteins are purified, separated and digested) as one sample. Compared to chemical or label-free methods, this technique minimizes variances between different labeled samples, since they are combined in an early step of the workflow after cell growth. This procedure allows for the quantification of differentially expressed proteins with high accuracy, thus allowing for the identification of overexpressed or inhibited proteins between the two studied cellular conditions (Montalvo-Quiros and Luque-Garcia 2019). SILAC also has a very high labeling efficiency (Table 4.1) and allows multiplexing of 2–5 different samples by the means of using distinct isotopic variants of arginine. According to the latest developments in the labeling methodology, sample multiplexing can be increased up to 54 when combining SILAC with chemical labeling methods such as TMT or iTRAQ

(Arul and Robinson 2019). Methods like super-SILAC and spike-SILAC also allow metabolic labeling of tissues or body fluids which extends the applicability of the approach for clinical samples (Lindemann et al. 2017).

Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

In addition to relative quantification methods which are useful in differential expression studies and biomarker discovery, additional methods have been developed which are particularly applied for absolute protein quantification, including AQUA strategy and ICP-MS. The use of ICP-MS for protein quantification is based on either quantification of natural heteroatoms in proteins (such as phosphorus, sulfur, selenium or iodine) if a number of heteroatom-containing amino acids in the peptide is known, or labeling protein functional groups covalently with a metal-containing reagent. In addition to protein quantification, the measurement of phosphorus can be used for the determination of the degree of phosphorylation of selected proteins which makes ICP-MS a useful tool also for the identification of protein modifications. The principles and applications of the absolute protein quantification methods, including the traditional metal detection technique ICP-MS, have been reviewed by (Chahrour et al. 2015).

Application of Proteomics in Toxicology

The prevalent area in toxicology where proteomics is increasingly applied is the elucidation of the toxicity mechanism of emerging pollutants and using the large protein expression datasets to reveal the affected biological pathways. An important class of emerging pollutants is engineered nanomaterials which have been the subject of many toxicity studies, including those which have employed novel omics techniques such as proteomics. Among the omics methods, proteomics appears to be employed at a similar frequency with transcriptomics, both of which have been used in several times higher numbers of studies than metabolomics. For example, among the omics studies of bacterial responses to nanomaterials, proteomics and transcriptomics were each used in 17 and 15 papers while metabolomics was applied only in 3 papers (Mortimer et al. 2021). Several reviews have been recently published about using omics approaches, including proteomics, in nanotoxicology. The reviews have focused either on human health and safety (Costa and Fadeel 2016; Fadeel et al. 2018; Fröhlich 2017), environmental risk assessment (Revel et al. 2017; Mortimer et al. 2021) or agricultural challenges (Majumdar and Keller 2020). Thus, here, only a brief overview of proteomics in nanotoxicology through a few examples, with the focus on methodological approaches, will be given.

Examples of label-free proteomics in nanotoxicity include a recent study where a novel gel- and label-free approach was used to detect proteome changes in model gut bacteria *Escherichia coli*, grown in a biofilm and exposed to Ag nanoparticles

(NPs) at sublethal concentrations (Domingo et al. 2019). A relatively larger number of differentially expressed proteins (212) than in most other studies which have used sub-inhibitory NP concentrations was found when applying the novel method, indicating increased detection sensitivity and dynamic range of the shotgun nanoflow LC–MS/MS. A shotgun nanoflow LC coupled to a Q Exactive Orbitrap MS/MS was also used in another study to investigate the proteomic response of soybeans (*Glycine max*) to the foliar application of copper hydroxide nanowires, for potential application as a pesticide (Majumdar et al. 2021). The study, where proteomics analysis was combined with metabolomics, helped to identify uniquely upregulated biological pathways in the plants sprayed with the nanopesticides compared to the effects of other copper-based agrochemicals. The same shotgun proteomic approach as in the previous study was also used to explore NP-specific responses and the underlying mechanisms in soybeans, using cadmium sulfide-quantum dots (CdS-QD), in comparison with a soluble salt of Cd and micron-sized CdS (Majumdar et al. 2019). Proteomics, combined with targeted analysis of metabolites and gene expression, revealed specific tolerance mechanisms to CdS-QD exposure which differed from these induced by Cd ions. Two complementary proteomics approaches, 2D gel-based and label-free MS were used in parallel to investigate the effects of Ag NPs on the proteome of human intestinal epithelial cells (Caco-2) (Gioria et al. 2018). While the 2D gel-based technique identified less than 100 altered proteins, the label-free MS revealed close to 200–300 regulated proteins upon Ag NP exposure, demonstrating the advantages of high-resolution MS compared to in-gel methods. In addition to relative quantitative proteomics, which is useful for comparing differential protein expression in the exposed and control conditions, absolute protein quantification methods are crucial for nano-bio interaction studies, for example, in the characterization of protein coronas on NPs (Lai et al. 2012).

In addition to label-free approaches, many proteomics studies in toxicology have employed stable isotope labeling techniques. Non-selective isotopic labeling (ICPL) of proteins with ^{12}C (light chains) and ^{13}C (heavy chains) and subsequent peptide identification and quantification using nano HPLC-ESI-Quadrupole TOF (QTOF)-MS/MS were applied to study the mechanisms of mercury toxicity in the nucleus of soil-living amoeba (slime mold) *Dictyostelium discoideum* (Boatti et al. 2017). The ICPL approach was chosen because of the specific characteristics of nuclear proteins (many proteins with low abundance, high isoelectric point and high molecular weight) which did not allow successful separation using 2D gel electrophoresis. Since ICPL labels lysin residues of intact proteins, the subsequent trypsin digestion of lysine-rich proteins, which are abundant in nuclei, was hindered, resulting in longer peptides, considered an advantage in direct analysis with nano HPLC-ESI-QTOF-MS/MS. Quantitative change in core histones and the involvement of pseudouridine synthase in mercury toxicity were identified. Zhang et al. used isobaric labeling (iTRAQ) to study the toxicity mechanisms of one of the most commonly used organophosphorus flame retardants, tris (1,3-dichloro-2-propyl) phosphate, in RAW264.7 macrophage cells (Zhang et al. 2021c). Labeling with an 8-plex kit and analysis on a nanoflow LC coupled to a Q Exactive Orbitrap MS/MS allowed the identification of a total of 180 significantly differentially expressed proteins. Multiplex labeling with iTRAQ has

also been employed in human health risk assessment. To assess the effects of long-term exposure of miners to rare earth elements in soils, Liu et al. (2015) measured the changes in the serum protein levels of the miners. The analysis identified 29 differentially expressed proteins, which were associated with neurovirulence, hepatotoxicity, pathological fibrosis, osteoporosis, and anticoagulation. Several recent studies have employed labeled proteomics approaches to investigate the health effects of atmospheric particles or aerosols. These studies have either used TMT-based isobaric labeling to study $PM_{2.5}$-induced protein profiles in human renal tubular epithelial cells (Li et al. 2021) or lung injury caused by traffic-related particulate matter and gaseous pollutants (Jheng et al. 2021) or stable isotope dimethyl labeling-based proteomics to reveal the effects of naphthalene-derived secondary organic aerosol in BEAS-2B cells (Han et al. 2020).

Proteomics has already been extensively applied as a useful tool for searching protein biomarkers for different pollutants in biomonitoring and human toxicology (Kellie et al. 2019). For example, serum protein levels of rats were measured after exposure to bisphenol A to identify protein biomarkers of susceptibility, using isobaric labeling (TMT) combined with multidimensional protein identification technology (MudPIT) (Betancourt et al. 2014). Identification of protein targets of pollutant toxicity might further enable the development of commercial kits based on the identified protein biomarkers (López-Pedrouso et al. 2020). In addition to expression profiling studies, the identification of protein modifications is another important application of proteomics in human and environmental health research. These include protein modifications associated with cellular stress, such as carbonylation, phosphorylation, and oxidation (Ge et al. 2013; Liang et al. 2020).

Limitations and Future Perspectives of Proteomics in Toxicology

One of the main difficulties limiting the widespread application of proteomics in ecotoxicology is the lack of complete and curated protein databases for many non-model (micro)organisms. To overcome this limitation, new proteomic mass spectrometry technologies are being developed to detect and quantify proteins with increased precision and accuracy. Great efforts are currently being carried out to generate high-quality libraries for data-independent acquisition (DIA) mass spectrometry in non-model organisms to increase the applicability of proteomics in ecotoxicology and biomonitoring (Searle et al. 2020). Additionally, an increasing number of sequenced (meta)genomes is aiding important information in the repertoire of reference databases (Searle et al. 2020; López-Pedrouso et al. 2020).

Improvements in the MS technology and methodological approaches during recent years have been significant, enabling increased coverage and a wider dynamic range in MS-based analysis. Advances have been made in both label-free and isotopic

labeling approaches, however, a comparison of the two methods concluded that, irrespective of the chosen approach, the results were strongly dependent on the experimental design, including MS instruments and settings (Evans et al. 2012). Further, while label-free workflow appeared to be more advantageous for protein identification, there was no difference between isobaric labeling and label-free approach in the protein quantification and discovery potential. Also other authors have highlighted the importance of the workflow, including the sample preparation, protein separation, MS instrument settings and data analysis, in the number of proteins detected (Moulder et al. 2018). While the advantages of isobaric labeling compared to label-free approaches have somewhat diminished owing to the improvements in MS and chromatography, which allow reasonable coverage and high throughput with both DDA and DIA methods, labeling remains the method of choice when complex sample preparation is needed, allowing combining different samples early in the workflow (Moulder et al. 2018).

Isobaric labeling methods can also contribute to the development of single-cell proteomics, an emerging proteomics approach that is complementary to already relatively well-developed single-cell transcriptomics. For the single-cell proteomics to provide comparable information with the transcriptomics approach, the method needs to be improved so that it would be possible to process thousands of cells in a short time, the number of detected proteins would be in a similar order of magnitude with transcripts, and the method would work with a variety of cell systems (Schoof et al. 2021). With these aims in mind, Schoof et al. (2021) recently developed an improved single-celled proteomics workflow that was based on multiplexed TMT labeling and included a specifically tailored software (a python package SCeptre) to provide higher throughput and proteome depth. Single-cell proteomics will enable a step forward in the characterization of the toxicant or pollutant effects and elucidating the underlying modes of action, taking into consideration the heterogeneous response of single cells in a tissue or organism. The efforts in developing and advancing proteomics methods in toxicology will be greatly supported by artificial intelligence-based approaches such as machine learning (Sinitcyn et al. 2018). Overall, computational proteomics will have to keep up with the advances of MS and the increasing datasets generated, with a special focus on enabling the wet lab users to perform the data analysis themselves.

Metabolomics

Metabolomics, an emerging field that studies chemical processes of the metabolome, can be considered as the downstream process of genomics, transcriptomics, and proteomics (Fig. 4.1), and the changes of metabolite levels directly relate to biochemical activity and the phenotype (Johnson et al. 2016). The metabolome represents the complete set of highly diverse small-molecule chemicals (molecular weight $\leq$ 1000 Da) that are found in biological fluids, cells, tissues, organisms or other types of complex biological samples. Environmental metabolomics techniques provide an

understanding of how contaminant stressors lead to changes in metabolites, indicative of changes in biochemical fluxes. These enable the detection of rapid toxic responses earlier than traditional toxicity testing (Labine and Simpson 2020). Metabolomics has been successfully applied to study the effects of various environmental stressors including heavy metals (He et al. 2020), endocrine disruptors (Zhou et al. 2019), particulate matter (Nassan et al. 2021), persistent organic pollutants (Guo et al. 2020), and impacts of environmental factors (e.g., diet and geographical location) (Zhang et al. 2021b).

A common workflow of the environmental metabolomics studies includes the following steps: sample collection, sample preparation, data acquisition using analytical techniques, data analysis, and biological interpretation (Fig. 4.4). Data analysis should be applied to help understand complex chromatograms based on chemometrics. The first step in data analysis is data preprocessing including deconvolution and alignment of data followed by peak annotation using reference libraries. The second step is using univariate and multivariate statistical analysis to determine significantly changed metabolites between perturbed and control states. The final step is the identification of metabolites that have significantly changed (i.e., either up- or down-regulated) in the exposure groups compared to the unexposed control. These results can also lead to the identification of biomarkers, which have been widely applied in toxicology and ecotoxicology. One common method to screen biomarkers is to select highly ranked metabolites based on variable importance in projection scores or other selection methods. Multivariate models are then typically evaluated using cross-validation and permutation testing techniques. Finally, receiver operator characteristic (ROC) curves, which plot one minus specificity (the true negative rate) against sensitivity (the true positive rate), are constructed to evaluate the predictive performance of a biomarker model.

Analytical Methods in Metabolomics

Due to the complexity, dynamics and structural variability of the metabolome, no single analytical protocol exists that can extract, identify and quantify any complete metabolome. Thus, complementary approaches need to be applied. Despite the advances in MS and nuclear magnetic resonance (NMR) spectroscopy that enable the analysis of a broad range of metabolites with high precision and sensitivity, sample preparation and separation are of increasing importance for effective identification of the target set of compounds (Wu et al. 2019). Moreover, as NMR and MS are highly complementary, using both analytical platforms for metabolomic investigations is advantageous, especially in the search for new metabolites (Labine and Simpson 2020).

The main advantages of MS compared to alternative methods is its sensitivity (femto-attomolar range) and high dynamic range and resolution. However, MS detection is limited to metabolites that can be promptly ionized and that yield different ions, which is usually difficult to predict in the case of most samples of unknown

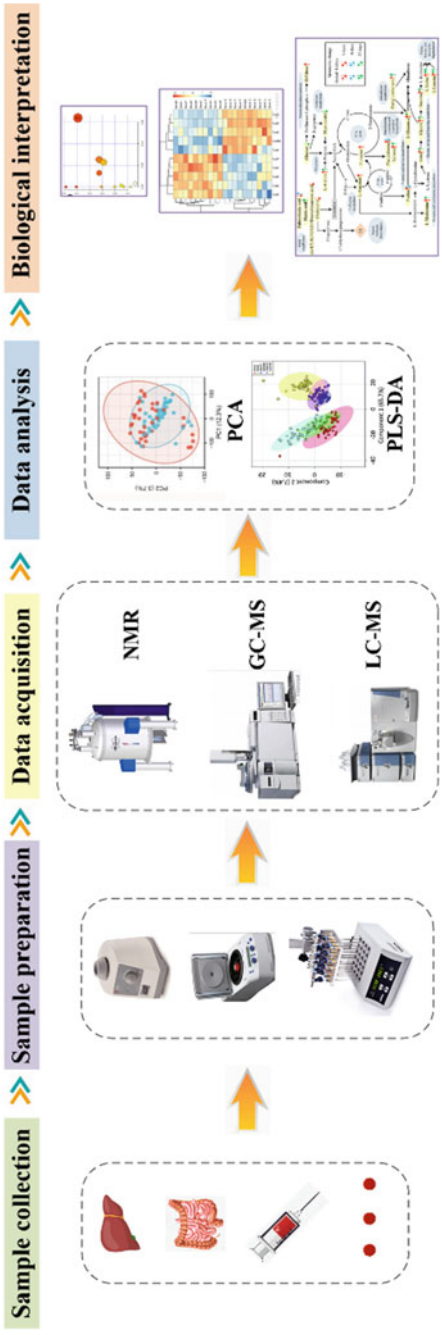

Fig. 4.4 The workflow of metabolomics

composition (Marshall and Powers 2017). MS is usually coupled to liquid chromatography (LC–MS), gas chromatography (GC–MS) or capillary electrophoresis (CE-MS) for analyte separation prior to mass detection by high-resolution and high-accuracy mass analyzers such as Fourier-transform ion cyclotron resonance (FT-ICR) or QTOF. While LC–MS separates soluble analytes based on their polarity, GC–MS can only be applied for volatile and thermally stable compounds. CE-MS involves the separation of highly polar and ionic metabolites in the electric field generated within the capillary (Kovacevic and Simpson 2020). On the other hand, NMR spectroscopy does not require sample separation by chromatography and often requires minimal sample preparation (Takis et al. 2019). It is quantitative and reproducible, can identify unknown metabolites unambiguously, distinguish isomers and even elucidate the structure of unknown compounds. The disadvantage however is its low sensitivity as only the most abundant metabolites (at concentrations greater than 1 μM) are detected (Nagana Gowda and Raftery 2017). Because of the abovementioned advantages, most of the early research applied NMR techniques (particularly ^{1}H NMR, because of the abundance of protons in a variety of nonpolar and polar metabolites). Recently, techniques such as high-resolution magic-angle-spinning NMR were developed allowing for in vivo analysis of intact tissues and cellular specimens without the requirement for metabolite extraction (Labine and Simpson 2020). Moreover, imaging MS (e.g., FT-ICR MS) and live single-cell MS are novel methods for detecting metabolites in situ at the cellular level, enabling detection of the location of the metabolite in the tissues (Wu et al. 2019). Equipped with advanced high-throughput analytical technologies and bioinformatics tools, metabolomics has become an irreplaceable technology to provide insight into global changes in small molecular signatures.

Non-targeted Metabolomics and Targeted Metabolomics

Since the 1990s, metabolomics techniques have developed rapidly. At present, the approach is widely used in various fields of life sciences, providing powerful technical support for interpreting life phenomena, exploring the toxicity of compounds, and discovering biological markers (Nägele 2014; Zhou and Zhao 2021). Based on the methodological approach, metabolomics can be broadly divided into non-targeted and targeted metabolomics. Non-targeted metabolomics is a nonbiased analysis of as many metabolites that can be reliably identified by the means of analytical techniques, data treatment and database matching (Bingol 2018). The non-targeted approach can be used to conduct a comprehensive analysis of all small molecule metabolites to study the dynamic changes of various metabolites and to reveal the involvement of metabolic pathways, without the need of a previous hypothesis, following a "top-down" strategy (Bundy et al. 2008; Hollywood et al. 2006). On the other hand, targeted metabolomics is used for quantitative analysis of a predetermined set of metabolites of interest, commonly driven by a specific hypothesis on specific pathways (Bingol 2018). For example, targeted metabolomics can be focused on bile acid metabolism (Guo et al. 2021), lipid metabolism (lipidomics) (Shanta et al. 2021) or

amino acid metabolism (Zeng et al. 2020). In short, metabolomics has been used in various fields of life science research for in-depth mechanism exploration.

Metabolomics in Ecotoxicology and Human Toxicology

Environmental metabolomics is likely to transform our knowledge of the impacts of environmental stressors, such as organic pollutants, pesticides, endocrine disruptors, pharmaceuticals, heavy metals, and other abiotic stressors in a wide range of model organisms. The application of metabolomics provides novel opportunities to evaluate the risk associated with specific stressors but also can serve as an innovative and informative tool for environmental risk assessment. Metabolomics is superior to current standard toxicity testing due to the opportunity to elucidate contaminant-induced cellular metabolic alternations, even when no significant effects are detected by measuring traditional endpoints such as morphology, mortality, reproduction and weight loss (Tang et al. 2018; Zhu et al. 2020). Metabolomics can also help to identify specific biomarkers and elucidate the mode of action of environmental contaminants as recently demonstrated, for example, in the case of microorganisms (Hu et al. 2021), the crustacean *Daphnia magna* (Jeong and Simpson 2019), other invertebrates (Zhu et al. 2020), aquatic organisms (Zhen et al. 2021) and rodents (Wei et al. 2020).

Humans are exposed to a large variety of environmental contaminants through diet, water, air or direct skin contact. The extent and types of exposure depend on lifestyle, environment, place of residence, occupation, eating habits and air quality, among other factors. Deciphering the potential adverse effects resulting from the actual human exposure to pollutants is a complex exercise and requires collaboration between different disciplines, including epidemiology, toxicology and analytical chemistry. Metabolomics is increasingly used in toxicology, opening new perspectives to study changes associated with chemical exposure at the metabolome level. The origin of the samples used for metabolomic studies in humans is diverse. The most common biological matrices are urine and plasma, but others like saliva, sperm, tissues, or placenta, are also used. Metabolic fingerprints are commonly used to discriminate between sub-populations according to exposure conditions. Beyond that, current developments in metabolomics allow investigation of links between exposures and certain diseases, identify candidate biomarkers of toxicity, and explore adverse outcome pathways (AOP) (Johnson et al. 2016). Since metabolomics can be performed at the individual as well as at the population level, it could have a broad impact on personalized preventive medicine, policy changes, and the understanding of disease mechanisms.

The current metabolomics approaches do not allow to differentiate which component of a chemical mixture produces a specific effect and therefore, for this matter, exposure to single compounds is still needed. However, only exposure experiments to individual compounds do not inform on the potential synergistic or antagonistic effects once these single compounds are integrated into real complex mixtures (Álvarez-Muñoz and Farré 2020). Therefore, metabolomics studies which assess

the effects of single compounds and mixtures are complementary and should be performed in parallel. Methodologically, two kinds of studies can be differentiated working with mixtures: those assessing the impact of complex environmental mixtures (e.g., sewage treatment plant effluents or surface waters) and those evaluating binary mixtures in comparison with the single compounds (Carriquiriborde 2020).

Limitations and Future Perspectives of Metabolomics in Toxicology

One of the main limitations of metabolomics is the lack of standard operating procedures since it is not yet possible to measure all the metabolites using a single extraction procedure. Metabolite identification is the key issue in metabolomics and great progress has been made in this area, but it remains a challenge because in most cases the metabolites detected by NMR and MS have an unknown chemical nature. In many studies, only a small fraction of the features detected are confidently identified as corresponding to a specific molecular structure. A significant amount of work continues to be devoted to the development of metabolite databases and approaches to automate and facilitate metabolite identification (Borts 2019).

Another big challenge in metabolomics has been biomarker validation. Although several studies have demonstrated reproducible patterns of biomarkers, some of these studies have been preliminary and the results have not been validated. For example, many articles which describe metabolomics analysis of similar toxicants, species, and biological specimens have identified largely different sets of putative biomarkers (Borts 2019). Also, there are currently no FDA-approved biomarkers for clinical use, which would have been discovered using metabolomics approaches (Trivedi et al. 2017). This illustrates well the extent of barriers and challenges that exist in establishing validated metabolomics-derived biomarkers of toxicity.

In toxicological studies, the distinction between significant metabolic changes due to the toxicant exposure or damage and inter-individual variability or individual variability under different conditions or over time can be technically complicated. In addition, sometimes too general metabolic pathways have been proposed, where it has not been clear if the effect was a general stress response or specifically related to the toxic mode of action (Carriquiriborde 2020). Overall, to improve the applicability of metabolomics in toxicology, standardized procedures, compatible software, and databases recognized by the scientific community are needed for validation and interpretation of the results. They are of primary importance for the development of future environmental metabolomics (Álvarez-Muñoz and Farré 2020).

The disturbances of metabolic pathways reflect the global profile of biological responses to environmental stressors but are difficult to predict due to highly heterogeneous data from complex biological systems and various pollutant properties. These challenges are being addressed by taking advantage of the machine learning models

which have been recently actively developed. The applicability of machine learning to effectively predict metabolites or metabolic pathways has been demonstrated. For example, multiple machine learning models combined with metabolomics enabled accurate prediction of the disturbance of metabolic pathways by 33 engineered NPs and were further verified by animal experiments (Peng et al. 2020).

Similar to proteomics, the bottleneck of metabolomic analysis is the lack of amplification methods which causes minor metabolites to be undetectable and leads to an incomplete representation of metabolic pathways. Furthermore, compared to gene and protein databases, the metabolite databases, particularly for plants and microorganisms, are relatively poor, which affects the annotation of metabolite functions (Wu et al. 2019; Zhang et al. 2021a). Another limitation of metabolomics is that it provides consequential data without identifying the pathways of cellular mechanisms. Although biomarkers give important information about the potentially deleterious impacts of contaminants on a biochemical level, they are not capable of evaluating the molecular mechanism of action of contaminants (Campos et al. 2012). Integration of metabolomics with transcriptomics or proteomics, therefore, provides a better understanding of the subtle effects of pollutants on living systems (Shin et al. 2018).

Future Perspectives for Omics in Toxicology

While transcriptomics, proteomics and metabolomics are relatively well established and already applied in toxicological research, there are emerging omics methods yet to be extended to toxicology. One of such novel fast-developing omics approaches is translatomics (Fig. 4.1) which investigates all the components in a cell involved in the translation process, including translating mRNAs, ribosomes, tRNAs, regulatory RNAs, ribosome-nascent polypeptide chains and other factors (Zhao et al. 2019). It has been long recognized that translational regulation is one of the most important regulatory functions in the organism but due to technical difficulties, the process has been studied at a global scale for a limited time. The challenges of translatomics are caused by the complexity of the translational machinery which involves various regulatory proteins and macromolecules, and rapid and specific response to environmental signals, which requires new methodological approaches to enable capturing the regulatory responses. Some examples of the multiple methods used in translatomics include polysome profiling, ribosome nascent-chain complex sequencing (RNC-seq), translating ribosome affinity purification (TRAP-seq) and ribosome profiling (Ribo-seq) which can all be used to study translating mRNA (Koppel and Fainzilber 2018). Similarly, there are numerous methodological approaches for studying other components of the translational machinery. Some of the fields closely related to toxicology where translatomics has been applied so far include disease-relevant studies, such as perturbation of global translation in cancer (Wang et al. 2013) or bacterial response to oxidative stress (Zhong et al. 2015). Since translatomics reflects faster and more sensitive biological processes in a cell than transcriptomics or proteomics,

it is expected that, despite the high cost and technical complexity of the current translatomics methods, this new omics approach will be adopted in toxicology studies in the near future.

While omics protocols and analytical capabilities are constantly improving, enabling to better profile global gene and protein expression and metabolites in response to pollutant exposures, it is also becoming more common to combine multiple omics approaches in toxicology studies to gain a more complete overview of the cellular responses at multiple regulatory levels. The integrated omics or multi-omics approach has been recently applied in nanotoxicology, for example, to shed light on the mechanisms of action of soluble metal NPs and the role of metal ions in the exerted toxicity which is an important factor to consider in the case of Ag and ZnO NPs (Dekkers et al. 2018; Maria et al. 2021). The application of multi-omics techniques is expected to become mainstream in toxicology where the need for a systemic understanding of toxicity pathways is deemed crucial for establishing adverse outcome pathways (Canzler et al. 2020). Due to the increasing need to integrate different omics datasets that are usually obtained by using varying data cleaning, normalization and statistical validation approaches, efforts are directed to developing standardized analytical and bioinformatics pipelines for the omics research community (Misra et al. 2019). The complexity of integrating different omics datasets also depends on the type of omics analyses that are being combined and interpreted. For example, the analyses of the proteome and transcriptome usually yield an overall one-to-one correspondence between the obtained sequences which facilitates the comparison and analysis of the data (Sinitcyn et al. 2018). Similar correspondence also exists between genome and proteome data as well as ribosomal profiling and proteomics data. However, when proteomics is combined with metabolomics, no one-to-one correspondence between molecules exists. In these cases, special tools are needed which facilitate metabolic reconstruction and integrate different data types, such as the predictive human metabolic model Recon 2.2. which catalogs all metabolic reactions encoded within the genome and allows integration of information about genes, proteins and reactions (Swainston et al. 2016).

With the importance of microbiota in environmental and human health increasingly recognized, the analysis of changes in microbial communities should become an integral part of toxicity studies. There is growing interest in associating microbiota research in environmental toxicology since studies have shown that a wide range of pollutants induces dysbiosis of gut microbiota in aquatic organisms (Evariste et al. 2019). In addition to the most commonly applied metagenomics analysis which characterizes the changes in the community composition of the microbiota, metatranscriptomics and metaproteomics as more novel techniques are being employed (Shakya et al. 2019; Salvato et al. 2021). Metatranscriptomics and metaproteomics complement the metagenomics data by providing functional information regarding the changes in microbial communities in response to contaminants, thus offering insight into adverse effects of chemicals or pollutants on gut microbiota leading to host physiological alterations. Metatranscriptomics and metaproteomics are rapidly growing fields and are yet to be incorporated into toxicological research of emerging

pollutants. Similar to the multi-omics approach described above, also metagenomics, metatranscriptomics and metaproteomics are being integrated to investigate biological pathways and mechanisms across different molecular scales.

References

Alexander-Dann B, Pruteanu LL, Oerton E et al (2018) Developments in toxicogenomics: understanding and predicting compound-induced toxicity from gene expression data. Mol Omics 14:218–236

Álvarez-Muñoz D, Farré M (2020) Chapter 12 - Future trends in environmental metabolomics analysis. In: Álvarez-Muñoz D, Farré M (eds) Environmental Metabolomics. Elsevier, pp 339–341

Arul AB, Robinson RAS (2019) Sample multiplexing strategies in quantitative proteomics. Anal Chem 91:178–189

Betancourt A, Mobley JA, Wang J et al (2014) Alterations in the rat serum proteome induced by prepubertal exposure to bisphenol A and genistein. J Proteome Res 13:1502–1514

Bingol K (2018) Recent advances in targeted and untargeted metabolomics by NMR and MS/NMR methods. High Throughput 7

Boatti L, Rapallo F, Viarengo A et al (2017) Toxic effects of mercury on the cell nucleus of *Dictyostelium discoideum*. Environ Toxicol 32:417–425

Bodi K, Perera AG, Adams PS et al (2013) Comparison of commercially available target enrichment methods for next-generation sequencing. J Biomol Tech 24:73–86

Borts DJ (2019) Chapter 62 - Biomarkers detection for toxicity testing using metabolomics. In: Gupta RC (ed) Biomarkers in Toxicology (Second Edition). Academic Press, pp 1111–1124

Bumgarner R (2013) Overview of DNA microarrays: types, applications, and their future. Curr Protoc Mol Biol 101:22.1.1–22.1.11

Bundy JG, Davey MP, Viant MR (2008) Environmental metabolomics: a critical review and future perspectives. Metabolomics 5:3

Campos A, Tedesco S, Vasconcelos V et al (2012) Proteomic research in bivalves: towards the identification of molecular markers of aquatic pollution. J Proteomics 75:4346–4359

Canzler S, Schor J, Busch W et al (2020) Prospects and challenges of multi-omics data integration in toxicology. Arch Toxicol 94:371–388

Carriquiriborde P (2020) Chapter 3 - Metabolic profiling of biofluids in fish for identifying biomarkers of exposure and effects for assessing aquatic pollution. In: Álvarez-Muñoz D, Farré M (eds) Environmental Metabolomics. Elsevier, pp 65–89

Chahrour O, Cobice D, Malone J (2015) Stable isotope labelling methods in mass spectrometry-based quantitative proteomics. J Pharm Biomed Anal 113:2–20

Christoforou AL, Lilley KS (2012) Isobaric tagging approaches in quantitative proteomics: the ups and downs. Anal Bioanal Chem 404:1029–1037

Cobb M (2017) 60 years ago, Francis Crick changed the logic of biology. PLoS Biol 15:e2003243

Costa PM, Fadeel B (2016) Emerging systems biology approaches in nanotoxicology: towards a mechanism-based understanding of nanomaterial hazard and risk. Toxicol Appl Pharmacol 299:101–111

Currie RA (2012) Toxicogenomics: the challenges and opportunities to identify biomarkers, signatures and thresholds to support mode-of-action. Mutat Res 746:97–103

Dai J (2018) Reduced transcriptomic approach for screening and prediction of chemical toxicity. Chem Res Toxicol 31:532–533

De Wolf H, De Bondt A, Turner H et al (2016) Transcriptional characterization of compounds: lessons learned from the public LINCS data. Assay Drug Dev Technol 14:252–260

Dekkers S, Williams TD, Zhang J et al (2018) Multi-omics approaches confirm metal ions mediate the main toxicological pathways of metal-bearing nanoparticles in lung epithelial A549 cells. Environ Sci: Nano 5:1506–1517

Domingo G, Villa F, Vannini C et al (2019) Label-free proteomic approach to study the non-lethal effects of silver nanoparticles on a gut bacterium. Front Microbiol 10:2709

Duan Q, Flynn C, Niepel M et al (2014) LINCS Canvas Browser: interactive web app to query, browse and interrogate LINCS L1000 gene expression signatures. Nucleic Acids Res 42:W449–W460

Evans C, Noirel J, Ow SY et al (2012) An insight into iTRAQ: where do we stand now? Anal Bioanal Chem 404:1011–1027

Evariste L, Barret M, Mottier A et al (2019) Gut microbiota of aquatic organisms: a key endpoint for ecotoxicological studies. Environ Pollut 248:989–999

Fadeel B, Farcal L, Hardy B et al (2018) Advanced tools for the safety assessment of nanomaterials. Nat Nanotechnol 13:537–543

Fröhlich E (2017) Role of omics techniques in the toxicity testing of nanoparticles. J Nanobiotechnol 15:84–84

Frost DC, Greer T, Li L (2015) High-resolution enabled 12-plex DiLeu isobaric tags for quantitative proteomics. Anal Chem 87:1646–1654

Ge P, Hao P, Cao M et al (2013) iTRAQ-based quantitative proteomic analysis reveals new metabolic pathways of wheat seedling growth under hydrogen peroxide stress. Proteomics 13:3046–3058

Gioria S, Urbán P, Hajduch M et al (2018) Proteomics study of silver nanoparticles on Caco-2 cells. Toxicol in Vitro 50:347–372

Grimm FA, Iwata Y, Sirenko O et al (2016) A chemical–biological similarity-based grouping of complex substances as a prototype approach for evaluating chemical alternatives. Green Chem 18:4407–4419

Griss J, Perez-Riverol Y, Lewis S et al (2016) Recognizing millions of consistently unidentified spectra across hundreds of shotgun proteomics datasets. Nat Methods 13:651–656

Guan S, Taylor PP, Han Z et al (2020) Data dependent-independent acquisition (DDIA) proteomics. J Proteome Res 19:3230–3237

Guo H, Chen J, Zhang H et al (2021) Exposure to GenX and its novel analogs disrupts hepatic bile acid metabolism in male mice. Environ Sci Technol. https://doi.org/10.1021/acs.est.1c02471

Guo Q, He Z, Liu X et al (2020) High-throughput non-targeted metabolomics study of the effects of perfluorooctane sulfonate (PFOS) on the metabolic characteristics of *A. thaliana* leaves. Sci Total Environ 710:135542

Gygi SP, Rist B, Gerber SA et al (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotechnol 17:994–999

Hamadeh HK, Amin RP, Paules RS et al (2002) An overview of toxicogenomics. Curr Issues Mol Biol 4:45–56

Han J, Wang S, Yeung K et al (2020) Proteome-wide effects of naphthalene-derived secondary organic aerosol in BEAS-2B cells are caused by short-lived unsaturated carbonyls. Proc Natl Acad Sci USA 117:25386–25395

Hayes KR, Bradfield CA (2005) Advances in toxicogenomics. Chem Res Toxicol 18:403–414

He E, Qiu R, Cao X et al (2020) Elucidating toxicodynamic differences at the molecular scale between ZnO nanoparticles and $ZnCl_2$ in *Enchytraeus crypticus* via nontargeted metabolomics. Environ Sci Technol 54:3487–3498

Hollywood K, Brison DR, Goodacre R (2006) Metabolomics: current technologies and future trends. Proteomics 6:4716–4723

House JS, Grimm FA, Jima DD et al (2017) A pipeline for high-throughput concentration response modeling of gene expression for toxicogenomics. Front Genet 8:168–168

Hu C, Liu M, Wan T et al (2021) Disturbances in microbial and metabolic communication across the gut-liver axis induced by a dioxin-like pollutant: an integrated metagenomics and metabolomics analysis. Environ Sci Technol 55:529–537

Jeong T-Y, Simpson MJ (2019) Reproduction stage differentiates the time-course regulation of metabolites in *Daphnia magna*. Environ Sci Technol 53:12764–12773
Jheng Y-T, Putri DU, Chuang H-C et al (2021) Prolonged exposure to traffic-related particulate matter and gaseous pollutants implicate distinct molecular mechanisms of lung injury in rats. Part Fibre Toxicol 18:24
Johnson CH, Ivanisevic J, Siuzdak G (2016) Metabolomics: beyond biomarkers and towards mechanisms. Nat Rev Mol Cell Biol 17:451–459
Judson RS, Mortensen HM, Shah I et al (2012) Using pathway modules as targets for assay development in xenobiotic screening. Mol BioSyst 8:531–542
Kellie JF, Sikorski TW, An B et al (2019) A new era for proteomics. Bioanalysis 11:1731–1735
Koppel I, Fainzilber M (2018) Omics approaches for subcellular translation studies. Molecular Omics 14:380–388
Kovacevic V, Simpson MJ (2020) Chapter 1 - Fundamentals of environmental metabolomics. In: Álvarez-Muñoz D, Farré M (eds) Environmental Metabolomics. Elsevier, pp 1–33
Labine LM, Simpson MJ (2020) The use of nuclear magnetic resonance (NMR) and mass spectrometry (MS)–based metabolomics in environmental exposure assessment. Curr Opin Environ Sci Health 15:7–15
Lai ZW, Yan Y, Caruso F et al (2012) Emerging techniques in proteomics for probing nano-bio interactions. ACS Nano 6:10438–10448
Lamb J, Crawford ED, Peck D et al (2006) The connectivity map: using gene-expression signatures to connect small molecules, genes, and disease. Science 313:1929–1935
Li B, Qin S, Cai Y et al (2021) Proteomic characteristics of PM2.5-induced differentially expressed proteins in human renal tubular epithelial cells. Environ Toxicol Pharmacol 86:103658
Li H-H, Hyduke DR, Chen R et al (2015) Development of a toxicogenomics signature for genotoxicity using a dose-optimization and informatics strategy in human cells. Environ Mol Mutagen 56:505–519
Liang X, Martyniuk CJ, Simmons DBD (2020) Are we forgetting the "proteomics" in multi-omics ecotoxicology? Comp Biochem Physiol Part D Genomics Proteomics 36:100751
Lindemann C, Thomanek N, Hundt F et al (2017) Strategies in relative and absolute quantitative mass spectrometry based proteomics. Biol Chem 398:687–699
Liu H, Wang J, Yang Z et al (2015) Serum proteomic analysis based on iTRAQ in miners exposed to soil containing rare earth elements. Biol Trace Elem Res 167:200–208
Lobenhofer EK, Bushel PR, Afshari CA et al (2001) Progress in the application of DNA microarrays. Environ Health Perspect 109:881–891
López-Ferrer D, Ramos-Fernández A, Martínez-Bartolomé S et al (2006) Quantitative proteomics using 16O/18O labeling and linear ion trap mass spectrometry. Proteomics 6:S4–S11
López-Pedrouso M, Varela Z, Franco D et al (2020) Can proteomics contribute to biomonitoring of aquatic pollution? A critical review. Environ Pollut 267:115473
Lowe R, Shirley N, Bleackley M et al (2017) Transcriptomics technologies. PLoS Comput Biol 13:e1005457
Majumdar S, Keller AA (2020) Omics to address the opportunities and challenges of nanotechnology in agriculture. Crit Rev Environ Sci Technol 51:1–42
Majumdar S, Long RW, Kirkwood JS et al (2021) Unraveling metabolic and proteomic features in soybean plants in response to copper hydroxide nanowires compared to a commercial fertilizer. Environ Sci Technol 55:13477-13489
Majumdar S, Pagano L, Wohlschlegel JA et al (2019) Proteomic, gene and metabolite characterization reveal the uptake and toxicity mechanisms of cadmium sulfide quantum dots in soybean plants. Environ Sci: Nano 6:3010–3026
Maria VL, Licha D, Scott-Fordsmand JJ et al (2021) Multiomics assessment in *Enchytraeus crypticus* exposed to Ag nanomaterials (Ag NM300K) and ions ($AgNO_3$) – Metabolomics, proteomics (& transcriptomics). Environ Pollut 286:117571
Marshall DD, Powers R (2017) Beyond the paradigm: combining mass spectrometry and nuclear magnetic resonance for metabolomics. Prog Nucl Magn Reson Spectrosc 100:1–16

Mav D, Shah RR, Howard BE et al (2018) A hybrid gene selection approach to create the S1500+ targeted gene sets for use in high-throughput transcriptomics. PLoS One 13:e0191105
Mezencev R, Subramaniam R (2019) The use of evidence from high-throughput screening and transcriptomic data in human health risk assessments. Toxicol Appl Pharmacol 380:114706
Minowa Y, Kondo C, Uehara T et al (2012) Toxicogenomic multigene biomarker for predicting the future onset of proximal tubular injury in rats. Toxicology 297:47–56
Misra BB, Langefeld C, Olivier M et al (2019) Integrated omics: tools, advances and future approaches. J Mol Endocrinol 62:R21–R45
Moffat I, Chepelev NL, Labib S et al (2015) Comparison of toxicogenomics and traditional approaches to inform mode of action and points of departure in human health risk assessment of benzo[a]pyrene in drinking water. Crit Rev Toxicol 45:1–43
Montalvo-Quiros S, Luque-Garcia JL (2019) Combination of bioanalytical approaches and quantitative proteomics for the elucidation of the toxicity mechanisms associated to TiO_2 nanoparticles exposure in human keratinocytes. Food Chem Toxicol 127:197–205
Morel E, Jreije I, Tetreault V et al (2020) Biological impacts of Ce nanoparticles with different surface coatings as revealed by RNA-seq in *Chlamydomonas reinhardtii*. NanoImpact 19:100228
Mortimer M, Wang Y, Holden PA (2021) Molecular mechanisms of nanomaterial-bacterial interactions revealed by omics—the role of nanomaterial effect level. Front Bioeng Biotechnol 9:683520
Moulder R, Bhosale SD, Goodlett DR et al (2018) Analysis of the plasma proteome using iTRAQ and TMT-based isobaric labeling. Mass Spectrom Rev 37:583–606
Nagana Gowda GA, Raftery D (2017) Recent advances in NMR-based metabolomics. Anal Chem 89:490–510
Nägele T (2014) Linking metabolomics data to underlying metabolic regulation. Front Mol Biosci 1:22
Nassan FL, Wang C, Kelly RS et al (2021) Ambient PM2.5 species and ultrafine particle exposure and their differential metabolomic signatures. Environ Int 151:106447
Peng T, Wei C, Yu F et al (2020) Predicting nanotoxicity by an integrated machine learning and metabolomics approach. Environ Pollut 267:115434
Revel M, Châtel A, Mouneyrac C (2017) Omics tools: new challenges in aquatic nanotoxicology? Aquat Toxicol 193:72–85
Salvato F, Hettich RL, Kleiner M (2021) Five key aspects of metaproteomics as a tool to understand functional interactions in host-associated microbiomes. PLOS Pathog 17:e1009245
Schmidt A, Kellermann J, Lottspeich F (2005) A novel strategy for quantitative proteomics using isotope-coded protein labels. Proteomics 5:4–15
Schoof EM, Furtwangler B, Uresin N et al (2021) Quantitative single-cell proteomics as a tool to characterize cellular hierarchies. Nat Commun 12:3341
Searle BC, Swearingen KE, Barnes CA et al (2020) Generating high quality libraries for DIA MS with empirically corrected peptide predictions. Nat Commun 11:1548
Shakya M, Lo CC, Chain PSG (2019) Advances and challenges in metatranscriptomic analysis. Front Genet 10:904
Shanta PV, Li B, Stuart DD et al (2021) Lipidomic profiling of algae with microarray MALDI-MS toward ecotoxicological monitoring of herbicide exposure. Environ Sci Technol 55:10558–10568
Shi L, Reid LH, Jones WD et al (2006) The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. Nat Biotechnol 24:1151–1161
Shin TH, Lee DY, Lee HS et al (2018) Integration of metabolomics and transcriptomics in nanotoxicity studies. BMB Rep 51:14–20
Sinitcyn P, Rudolph JD, Cox J (2018) Computational methods for understanding mass spectrometry-based shotgun proteomics data. Annu Rev Biomed Data Sci 1:207–234
Su Z, Łabaj PP, Li S et al (2014) A comprehensive assessment of RNA-seq accuracy, reproducibility and information content by the sequencing quality control consortium. Nat Biotechnol 32:903–914

Swainston N, Smallbone K, Hefzi H et al (2016) Recon 2.2: from reconstruction to model of human metabolism. Metabolomics 12:109–109
Takis PG, Ghini V, Tenori L et al (2019) Uniqueness of the NMR approach to metabolomics. Trends Analyt Chem 120:115300
Tang R, Ding C, Dang F et al (2018) NMR-based metabolic toxicity of low-level Hg exposure to earthworms. Environ Pollut 239:428–437
Tian X, De Vries MP, Permentier HP et al (2020) A collision-induced dissociation cleavable isobaric tag for peptide fragment ion-based quantification in proteomics. J Proteome Res 19:3817–3824
Timp W, Timp G (2020) Beyond mass spectrometry, the next step in proteomics. Sci Ad 6:eaax8978
Tolonen AC, Haas W (2014) Quantitative proteomics using reductive dimethylation for stable isotope labeling. J Vis Exp https://doi.org/10.3791/51416:e51416
Trivedi DK, Hollywood KA, Goodacre R (2017) Metabolomics for the masses: the future of metabolomics in a personalized world. New Horiz Transl Med 3:294–305
Vinh J (2019) CHAPTER 17 Proteomics and proteoforms: bottom-up or top-down, how to use high-resolution mass spectrometry to reach the Grail. In: Kanawati B, Schmitt-Kopplin P (eds) Fundamentals and applications of fourier transform mass spectrometry. Elsevier, pp 529–567
Wang C, Gong B, Bushel PR et al (2014) The concordance between RNA-seq and microarray data depends on chemical treatment and transcript abundance. Nat Biotechnol 32:926–932
Wang P, Xia P, Yang J et al (2018) A reduced transcriptome approach to assess environmental toxicants using zebrafish embryo test. Environ Sci Technol 52:821–830
Wang T, Cui Y, Jin J et al (2013) Translating mRNAs strongly correlate to proteins in a multivariate manner and their translation ratios are phenotype specific. Nucleic Acids Res 41:4743–4754
Wang Z, Gerstein M, Snyder M (2009) RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet 10:57–63
Waters MD, Jackson M, Lea I (2010) Characterizing and predicting carcinogenicity and mode of action using conventional and toxicogenomics methods. Mutat Res 705:184–200
Wei J, Li X, Xiang L et al (2020) Metabolomics and lipidomics study unveils the impact of polybrominated diphenyl ether-47 on breast cancer mice. J Hazard Mater 390:121451
Wu Y, Deng M, Jin Y et al (2019) Toxicokinetics and toxic effects of a Chinese PFOS alternative F-53B in adult zebrafish. Ecotoxicol Environ Saf 171:460–466
Wu Y, Wang F, Liu Z et al (2014) Five-plex isotope dimethyl labeling for quantitative proteomics. Chem Commun 50:1708–1710
Xia P, Zhang X, Zhang H et al (2017) Benchmarking water quality from wastewater to drinking waters using reduced transcriptome of human cells. Environ Sci Technol 51:9318–9326
Zeng S-L, Li S-Z, Xiao P-T et al (2020) Citrus polymethoxyflavones attenuate metabolic syndrome by regulating gut microbiome and amino acid metabolism. Sci Adv 6:eaax6208
Zhang K, Zhao Y (2018) Reduced zebrafish transcriptome atlas toward understanding environmental neurotoxicants. Environ Sci Technol 52:7120–7130
Zhang L-J, Qian L, Ding L-Y et al (2021a) Ecological and toxicological assessments of anthropogenic contaminants based on environmental metabolomics. Environ Sci Ecotechnol 5:100081
Zhang S, Xu M, Shen Z et al (2021b) Green light exposure aggravates high-fat diet feeding-induced hepatic steatosis and pancreatic dysfunction in male mice. Ecotoxicol Environ Saf 225:112802
Zhang W, Wang R, Giesy JP et al (2021c) Proteomic analysis using isobaric tags for relative and absolute quantification technology reveals mechanisms of toxic effects of tris (1,3-dichloro-2-propyl) phosphate on RAW264.7 macrophage cells. J Appl Toxicol 42:190-202
Zhang X, Xia P, Wang P et al (2018) Omics advances in ecotoxicology. Environ Sci Technol 52:3842–3851
Zhao J, Qin B, Nikolay R et al (2019) Translatomics: the global view of translation. Int J Mol Sci 20:212
Zhen H, Teng Q, Mosley JD et al (2021) Untargeted lipidomics for determining cellular and subcellular responses in zebrafish (*Danio rerio*) liver cells following exposure to complex mixtures in U.S. streams. Environ Sci Technol 55:8180–8190

Zhong J, Xiao C, Gu W et al (2015) Transfer RNAs mediate the rapid adaptation of *Escherichia coli* to oxidative stress. PLoS Genet 11:e1005302

Zhou M, Zhao J (2021) A review on the health effects of pesticides based on host gut microbiome and metabolomics. Front Mol Biosci 8:632955–632955

Zhou X, Li Y, Li H et al (2019) Responses in the crucian carp (*Carassius auratus*) exposed to environmentally relevant concentration of 17α-ethinylestradiol based on metabolomics. Ecotoxicol Environ Saf 183:109501

Zhu Y, Wu X, Liu Y et al (2020) Integration of transcriptomics and metabolomics reveals the responses of earthworms to the long-term exposure of TiO_2 nanoparticles in soil. Sci Total Environ 719:137492

Chapter 5
Advancements in a Zebrafish Model for Toxicity Assessment of Nanomaterials

Stephanie Ling Jie Lee and Sijie Lin

Abstract The rise of nanotechnology has led to concerns about its potential risks to the environment and human health. Nanotoxicology, the study on the toxicological effects of nanoparticles, aims to address these concerns through the use of in vitro and in vivo toxicity tests. Although traditional toxicity testing relies primarily on in vivo models such as rodents, there is a shift towards the use of in vitro and alternative models in recent years. While useful for determining tissue-specific toxicity, in vitro cell lines fail to provide information at the whole organismal level. Zebrafish have emerged in the past two decades as a powerful alternative in vivo toxicity model. Their small size, transparency during embryonic and larval stages, rapid development and availability of transgenic zebrafish reporter lines make them highly suitable for high throughput screening (HTS) assays. Also, recent advances in automation, robotic handling and artificial intelligence (AI) assisted data analysis have further enhanced the screening capability. With the establishment of property-toxicity relationships, safer-by-design of nanomaterials have become feasible. This chapter summarizes the key features of the zebrafish toxicity model and HTS which contribute towards its suitability as a nanotoxicity model and highlights applications of zebrafish in nanotoxicity testing.

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Introduction

The Unknown Risks of Nanotechnology—A Necessity for Environmental, Health and Safety Assessment

Nanotechnology has growing applications in industries ranging from manufacturing and consumer products to healthcare. Nanotechnology-based industrial products include paints and coatings as well as catalysts in semiconductor manufacturing. Examples of consumer applications involving nanotechnology encompass smart textiles (Dhineshbabu and Bose 2018), pharmaceutical and personal care products, in particular sunscreens and cosmetics which contain TiO_2 and ZnO (Chiari-Andréo et al. 2019), anti-bacterial silver and iron nanoparticles heavily exploited in biomedical products (Kailasa et al. 2019), and medical applications including anti-bacterial and diagnostic imaging agents, biosensors (Goud et al. 2019), implantable devices (Juanola-Feliu et al. 2014), vaccine adjuvants (Liu and Chen 2016; Mao et al. 2021; Zhu et al. 2020), drug delivery systems (Hossen et al. 2019; Iqbal et al. 2019; Liu et al. 2008; Nava-Arzaluz et al. 2019) for gene therapy (Chen et al. 2016), immunotherapy (Goldberg 2019; Zhang et al. 2019) and photothermal therapy (Robinson et al. 2011) for treatment of cancer (Ma et al. 2019), cardiovascular disease (Chandarana et al. 2018), neurodegenerative diseases (Re et al. 2012), metabolic diseases (Ash et al. 2019) and infectious diseases (Kirtane et al. 2021).

The unique physicochemical properties of nanoscale materials are a double-edged sword. The properties that bring the benefits might potentially contribute towards detrimental effects on organisms and the environment (Colvin 2003; Nel et al. 2006). These concerns about the risks of nanotechnology to the environment and human health gave rise to the discipline of nanotoxicology (Oberdörster et al. 2005). Ideally, before nanotechnology is widely used in the environment or for theragnostic purposes, the toxicity of engineered nanomaterials must be determined using biologically relevant toxicological tests to understand their hazard potential. Knowledge of the interactions at the interface between the biological barriers and nanomaterials referred to as the 'nano-bio interactions' are required for proper risk assessment of engineered nanomaterials (Meng et al. 2018). It is also important to investigate the toxicokinetics (adsorption, distribution, metabolism and excretion), and toxicity mechanisms such as oxidative stress generation, immune and /or inflammatory response activation, endocrine disruption, neurotoxicity, genotoxicity and carcinogenicity exerted by the engineered nanomaterials.

In 2013, Keller et al. (2013) estimated that between 0.4 and 7% of engineered nanomaterials from domestic sewage, water treatment plants, industrial effluents, and surface runoff from landfills (Baalousha et al. 2016; Bäuerlein et al. 2017; Gondikas et al. 2014; Wimmer et al. 2019) are discharged into water bodies. While the proportion of nanomaterials discharged into the environment is estimated to be relatively low, this value is likely to rise with increased reliance on nanotechnology. The nanomaterials found in rivers and lakes pose a serious threat to aquatic ecosystems. Environmental engineered nanoparticles may adversely affect the life cycle of aquatic

organisms (Jiang et al. 2017; Shariati et al. 2020; Zou et al. 2016). Bioaccumulation in aquatic species may also inflict harm on species higher in the food chain (Lekamge et al. 2019; Luo et al. 2016).

Engineered nanoparticles released into aquatic ecosystems may form complex mixtures with other co-occurring aquatic pollutants which may interact in an additive, antagonistic, or synergistic fashion to alter their bioavailability, biodistribution and toxicity (Bundschuh et al. 2018; Li et al. 2020; Naasz et al. 2018). Six main categories were created to describe the different types of interactions depending on their effects on accumulation and toxicity (Naasz et al. 2018). The 'Trojan Horse effect' is one of the mechanisms that explain how environmental toxicants interact with nanoparticles via adsorption onto nanoparticles to hitch a ride into the organism (Naasz et al. 2018). There can be a positive or negative 'Trojan Horse Effect' where toxicity is present (+) or absent (−). The other types of interactions are surface enrichment, retention, inertism, and coalism (Naasz et al. 2018).

Strategies for Assessment of Nanotoxicity

Governmental regulatory agencies recognize the adverse effects unregulated emission of engineered nanomaterials have on the environment and human well-being. In order to keep these emerging contaminants under control, there is an increasing focus on regular water quality monitoring at key water bodies and a shift towards green chemistry. These important issues affecting environmental and human safety (EHS) underscore the importance of nanomaterial hazard identification and risk assessment. Risk assessment would facilitate the design of safer nanoparticles with minimal toxicity suitable for in vivo applications and inflict little to no harm on the environment. A three-tiered system integrating in vitro, in vivo and systems biology has been proposed to provide mechanistic information for human risk assessment (Cote et al. 2012).

Important components of nano EHS risk assessment systems center around (1) development of instruments for environmental detection of engineered nanomaterial exposure, (2) development and validation of toxicological testing methodology for engineered nanomaterials, (3) development of predictive models involving the integration of knowledge on the physicochemical properties of compounds and adverse outcome or phenotypic data from in vitro assays and other in vivo models to establish quantitative structure–activity relationships (QSAR) for prediction of environmental and human health risks leading to the production of safer-by-design nanomaterials, (4) development of effective protocols to understand the risk profile of engineered nanomaterials throughout their life cycle and (5) development of strategic research programs involving interdisciplinary internationally collaborative research which identify risks surrounding the development and use of nanotechnology (Maynard and Aitken 2016; Maynard et al. 2006). Toxicological testing of engineered nanomaterials is broadly based on traditional chemical toxicity testing, for which the gold standard are animal in vivo tests using rodents. However, due to ethical issues, there

has been a shift towards alternative models such as non-mammalian models (e.g., zebrafish and other fish, *Caenorhabditis elegans* and *Daphnia*) in addition to in vitro cell-based assays. The advantages and disadvantages of these alternate models are compared in this chapter.

Advantages of Zebrafish as a Nanotoxicity Model

Zebrafish are a boon for toxicity testing of engineered nanomaterials (Lin et al. 2013a). Benefits of zebrafish embryos and larvae for toxicological testing include: (1) small size that is compatible with the multi-well plates used for high throughput screening (HTS) assays, (2) ease of exposure as test compounds can be easily administered by immersion for zebrafish whereby molecules enter the body via diffusion through skin and gills, (3) use of minute quantities of test compounds per sample, (4) large clutch sizes consisting of hundreds of eggs meaning that toxicological tests can be conducted at large numbers of doses with independent technical and biological replicates in parallel, (5) optical transparency at early developmental stages which facilitates in vivo spatial visualization and imaging of phenotypes using microscopy, (6) rapid embryonic development signifying that acute developmental toxicity testing can be completed within hours or days. These factors enable time, labor and cost savings and reduce problems associated with the disposal of toxic materials. The high levels of biological and technical replication achieved with HTS platforms ensure scientific rigor and statistical power. Furthermore, animal husbandry protocols for zebrafish rearing and breeding have been optimized and standardized. This helps reduce inter-laboratory variation leading to greater reproducibility and reliability of data.

The zebrafish genome sequence has a high level of similarity with the human genome (Howe et al. 2013). Many biological pathways involved in toxicant and environmental stress responses in zebrafish closely resemble those in humans, i.e., responses observed in zebrafish have high predictive value. Numerous complex phenotypes that zebrafish exhibit are highly similar to those observed in higher organisms. High levels of concordance are reported for the toxicity outcomes obtained from zebrafish and mammalian in vivo developmental toxicity studies across many different toxicants (Brannen et al. 2010; Ducharme et al. 2013, 2015). This suggests that zebrafish toxicity assays have a high predictive value for mammalian toxicity studies. The complexity of the zebrafish model enables the studies of the complex interplay between different tissue types and organ systems which in vitro systems are unable to interrogate.

Studies have identified a range of normal behavioral phenotypes of embryonic, larval, and adult zebrafish (Kalueff et al. 2013; Kyzar et al. 2013). Zebrafish embryos are capable of spontaneous muscle contraction at 18 h post fertilization (hpf), touch response at 24 hpf, differentiated touch responses at 48 hpf and free swimming from 96 hpf (Granato et al. 1996). Adult zebrafish display complex behavioral phenotypes relating to aggression, shoaling, courtship and decision making (Kalueff et al. 2013;

Orger and Polavieja 2017). Exposures to neuroactive and neurotoxic agents modify behavioral phenotypes making zebrafish an excellent neurotoxicity model (Kokel et al. 2010; Rihel et al. 2010).

In addition, the zebrafish model is highly amenable to genetic manipulation. Protocols for the generation of transgenic lines and CRISPR-Cas9 genome editing in zebrafish are well established. The generation of zebrafish lines carrying fluorescent reporter genes sensitive to specific stimuli is relatively straightforward. Many transgenic lines have already been developed and characterized. For example, the *casper* transparent zebrafish strain lacks iridophores and pigmented melanocytes and most internal organs can be seen through the skin. Suitable fluorescent lines can be created on the transparent *casper* background to enhance in vivo detection and imaging of fluorescently labeled organs and expand the ability to probe putative toxicity pathways.

Taken together, zebrafish embryotoxicity assays straddle the divide between in vitro assays (high throughput but low predictive value) and mammalian in vivo models (low throughput and high predictive value). They offer great promise for the early stages of hazard identification and prioritization of chemicals for further testing in higher vertebrates.

Zebrafish Toxicity Testing Paradigms

General Methodology for Use of Zebrafish in Toxicity Testing

Zebrafish have been used as an in vivo toxicology model for a few decades. In general, zebrafish toxicity testing at embryonic to larval stages encompasses exposure of zebrafish embryos from early stages of embryonic development (usually from 1 to 6 hpf) to different concentrations of chemicals of interest up to 120 hpf. The chemical is dissolved or dispersed in an embryo medium (containing a carrier solvent if necessary) within a vessel (glass Petri dishes or polystyrene microtiter plate). As a carrier, organic solvents such as dimethyl sulfoxide (DMSO), methanol and acetone are frequently used. The exposure medium is renewed regularly (ideally daily) to maintain high levels of the chemical. At the end of the exposure period, the zebrafish embryos are assessed for the phenotypic endpoints of interest. The most common phenotypes evaluated are mortality, hatching and morphological malformations, i.e., head, spinal and pigment abnormalities and presence of oedemas. Other commonly assessed phenotypes include heart rate and behavior.

Extensive studies have shown that intra- and inter-laboratory variations in protocols and experimental conditions of zebrafish in vivo toxicity assays can influence toxicity outcomes (Hamm et al. 2019). Consortiums representing regulatory, academic and industrial stakeholders, e.g., Systematic Evaluation of the Application of Zebrafish in Toxicology (SEAZIT), have been established to discuss approaches to identify sources causing variability in zebrafish embryo in vivo toxicity assays and

develop experimental guidelines to optimize, harmonize and standardize protocols to improve concordance across laboratories (Ball et al. 2014; Hamm et al. 2019). For this purpose, the Organization for Economic Co-operation and Development (OECD) developed guidelines for zebrafish toxicity testing based on consultation with relevant key laboratories. These guidelines govern chemical testing using zebrafish of different developmental stages (embryonic, larval, juvenile or adult) and assays of different durations (acute or chronic exposure). OECD test guideline 236, also known as the Fish Embryo Acute Toxicity (FET) test, guideline 210 Fish, Early-life Stage Toxicity Test and guideline 212 Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages provide recommendations for toxicity testing with embryonic and larval zebrafish whereas guideline 215 Fish, Juvenile Growth Test deals with exposure in juvenile zebrafish. OECD test guidelines 203 Fish, Acute Toxicity Test, guidelines 230 21-day Fish Assay and 229 Fish Short Term Reproduction Assay are relevant for adult zebrafish. In particular, the FET has gained wide acceptance in the toxicology research community due to its good concordance to adult fish acute toxicity tests (Lammer et al. 2009) and few animal ethical issues. The methods for zebrafish embryotoxicity have been successfully applied in nanotoxicology (Pereira et al. 2019).

State of the Art with Regards to in Vivo Toxicity Testing with Zebrafish

HTS and high content screening (HCS) assays have become popular for toxicity testing in the past one to two decades. Libraries containing hundreds to thousands of chemical compounds can be screened rapidly and reproducibly using HTS and HCS platforms. In the context of toxicity testing, HTS and HCS assays harness sophisticated automated liquid handling systems, robotics, image acquisition, processing and analysis software and increasingly big data processing and artificial intelligence technologies to answer questions regarding the hazardous effects of specific chemical compounds and emerging toxicants on biological systems.

Originally designed to increase the throughput of in vitro toxicological screening, HTS and HCS technology have been adapted for small in vivo models like *C. elegans* and zebrafish embryos. Liquid handling robots and automated microinjection systems (Spaink et al. 2013) have been developed for accurate dispensation of chemicals to zebrafish embryos in HTS assays. The use of robotics-based automated HTS assays accelerates the speed and efficiency at which toxicity assays can be conducted and reduces human error. The key steps in zebrafish HTS are summarized in Fig. 5.1.

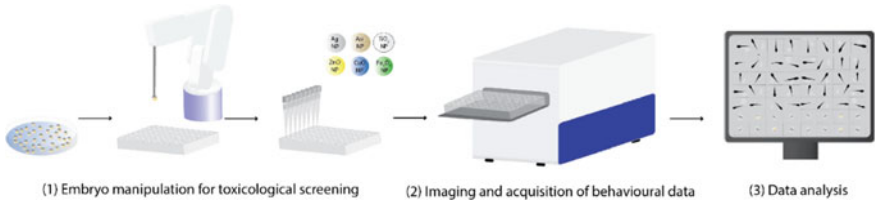

Fig. 5.1 Key steps in zebrafish HTS: (1) embryo manipulation for toxicological screening, (2) imaging and acquisition of behavioral data and (3) data analysis

Embryo Manipulation for Exposure and Screening—Automation and Robotics

Traditionally, zebrafish embryo collection and sorting, chorion removal and transfer into culture plates were performed manually. These processes are laborious and were bottlenecks to zebrafish embryotoxicity testing. Automated systems for zebrafish embryo sorting (Breitwieser et al. 2018; Graf et al. 2011; Pfriem et al. 2012), dechorionation (Mandrell et al. 2012) and arraying of single embryos into microtiter plates (Mandrell et al. 2012) have arisen in the past ten years. The high scalability and efficiency of automated robotic embryo manipulation platforms enabled large swaths of compounds to be tested faster, better and cheaper, contributing greatly to the increased adoption of zebrafish as an in vivo toxicology model.

To satisfy the requirement for large numbers of zebrafish embryos of the same developmental stage to perform HTS assays, dozens to hundreds of adult zebrafish are stimulated to spawn synchronously in specially designed embryo collection systems. High throughput spawning systems take advantage of the natural proclivity for zebrafish to breed in shallow water to induce spawning within a short interval, drastically reducing the time needed to remove breeding partitions and collect embryos from multiple breeding tanks containing small numbers of crosses.

Developing zebrafish embryos are surrounded by a chorion which forms a semi-permeable protective layer around the embryo. The chorion canal pore size of 0.5–0.7 μm (Lee et al. 2007) impedes chemicals larger than the pore from contacting the zebrafish embryo. Due to their small size, single nanoparticles can diffuse through chorion canal pores, however, nanoparticles often agglomerate preventing entry through the chorion (Lee et al. 2007). The physicochemical properties of the compound, including its hydrophobicity or hydrophilicity, polarity, ionic charge, electrostatic properties and the DMSO concentration it is dissolved in, are other factors that affect entry through the chorion (Hamm et al. 2019). Studies have shown that different nanoparticles and their dissolved cations accumulate preferentially at different compartments of the zebrafish embryo (Böhme et al. 2017). Lin et al. (2011) found that Co_3O_4, CuO, NiO, ZnO nanoparticles could not be detected in the chorionic fluid but adsorbed to the chorion instead. Other studies clearly showed that the chorion acts as a barrier to the entry of nanoparticles (Bar-Ilan et al. 2011; Kim and Tanguay 2014; Lin et al. 2011; van Pomeren et al. 2017).

The use of dechorionated embryos and intact embryos (with chorions) for zebrafish embryotoxicity assays varies between laboratories and protocols. This is concerning as the presence or absence of chorions adds variability to comparisons of toxicity outcomes across different assays (Hamm et al. 2019). Dechorionated embryos are reportedly more sensitive to chemical exposure. For many zebrafish embryotoxicity assays, chorions need to be removed before chemical exposure for a more accurate picture of the toxic potential of the chemical (Henn and Braunbeck 2011; Kim and Tanguay 2014; Panzica-Kelly et al. 2015).

Dechorionation enables direct contact of zebrafish embryos with waterborne nanoparticles and other chemicals and improves the sensitivity of in vivo toxicity assays. There are two main methods of zebrafish embryo dechorionation: mechanical and enzymatic removal of chorions. Both approaches have relatively high mortality rates, are time-consuming and require highly trained operators. Dechorionated zebrafish embryos are highly fragile and great care must be taken when transferring them into plates for chemical exposure. Mandrell et al. (2012) modified a shaker platform to create a high throughput zebrafish embryo dechorionation system. It removed chorions from 1600 to 2000 embryos within an hour using a combination of pronase digestion-based dechorionation with mechanical agitation. A literature survey on automated HTS using zebrafish embryo in vivo assays revealed that Mandrell et al. (2012) remains the only automated zebrafish embryo dechorionation system to date.

Several approaches for the automated transfer of zebrafish embryos into microtiter plates have been established. In Mandrell's system, a robotic arm picked transferred single dechorionated embryos from a Petri dish into individual wells of a 96-well plate with low mortality rates (Mandrell et al. 2012). For another approach, 3D printing technology was used to fabricate a 96-well array for high throughput arraying of zebrafish embryos (Yu et al. 2018). Negative pressure was exerted on the array with a vacuum pump to entrap single zebrafish embryos within individual wells. A disadvantage of this array is that the existing design is incompatible with dechorionated zebrafish embryos. Zebrafish embryos were released from a holding plate into a desired receiving 96-well plate with a combination of acceleration and a liquid environment (Zhang et al. 2011). Acceleration of the cell holding device overcame the adhesion forces between the zebrafish embryos and the holding device whereas the liquid environment reduced capillary pressure between the liquid and air. This automated method for the parallel transfer of zebrafish embryos had a high success rate of 94.3%.

Complex Object Parametric Analyzers and Sorters (COPAS) and BioSorter (Union Biometrica) are flow cytometers capable of analyzing and sorting fluorescently labeled transgenic zebrafish embryos (Carvalho et al. 2011). They can also be used to sort dead and live embryos. For the BioSorter system, fluorescent zebrafish embryos can be aspirated from conical tubes, Petri dishes or multiwell plates, are sorted based on fluorescence signals and then dispensed into multiwell plates. The operating principle of COPAS is the same, but the large particle sampler for dispensing zebrafish embryos is sold separately. Both systems have utility for

automated sorting and dispensation of fluorescent transgenic zebrafish embryos into multiwell microtiter plates for HTS assays.

The Vertebrate Automated Screening Technology (VAST) BioImager platform (Union Biometrica) is a streamlined automated system for the manipulation and imaging of zebrafish embryos (Jarque et al. 2018). The basic VAST model is a microscope-mounted platform that integrates an instrument for aspiration of zebrafish embryos from a bulk sample reservoir (a 50-mL conical tube), a capillary where zebrafish embryos are orientated for imaging, image acquisition software for high-resolution image capture and a dispenser which directs the embryos into waste or a storage tube after imaging. An optional large particle sampler with a dispenser adds an additional function of aspirating and dispensing zebrafish embryos from and to multiwell microtiter plates before and after imaging. Although the VAST system is a fully automated system for in vivo screening of zebrafish embryos, its throughput is relatively low since imaging is conducted on individual embryos.

Some applications require direct injection of the engineered nanomaterial into the body of embryos. Successful zebrafish embryo microinjection involves several steps: embryo immobilization, embryo orientation detection and repositioning of embryos into the correct orientation for microinjection, volume calibration and deposition of picolitre volumes of solutions into individual embryos (Wang et al. 2007). Immobilization of zebrafish embryos can be conducted using vacuum-driven adhesion of embryos (Wang et al. 2007; Zhang et al. 2011), with devices involving surface tension or hydrodynamic pressure such as troughs, grooves or specially designed devices (Akagi et al. 2012) or agarose embryo holder grids (Carvalho et al. 2011). A recent study described the use of electrothermally-actuated microgripper for automated manipulation, immobilization and release of zebrafish embryos for microinjections but its current throughput is limited to single embryos (Zhang et al. 2020). Algorithms have been written to detect zebrafish embryo orientation and rotate embryos into the appropriate orientation (Wang et al. 2009). Visual servo-controlled robotic microinjection systems facilitate the injection of thousands of zebrafish embryos at high speed with high accuracy and survival (Wang et al. 2007, 2009; Zhao et al. 2019a).

Letamendia et al. (2012) described the development of a fully automated platform enabling high throughput in vivo screening of cardiotoxic and anti-angiogenic compounds using zebrafish embryos. The system comprised a computer-controlled robotics-enabled automated workflow involving embryo loading, compound dispensation, exposure to compound at constant experimental conditions (e.g., temperature), image acquisition (brightfield and fluorescence image capture as well as video recording) and analysis.

Liquid handling robotics facilitates the preparation and dispensation of nanoparticle suspensions. The robots are attached to multi-channel pipettes where the dispensing volume can be programmed using a computer. They enable automated, accurate pipetting of fixed volumes of nanoparticle suspension into microtiter plates where the exposure of zebrafish embryos will take place. An alternative method for automated dispensing of toxicants utilizes ink-jet technology (Truong et al. 2016). Truong et al. (2016) compared the effectiveness and accuracy of a Hewlett-Packard

D300 digital dispenser ('BioPrinter') with a conventional liquid handling robot at dispensing chemical compounds for zebrafish toxicity assays. The BioPrinter outperformed the liquid handling robot when it came to the precision of chemical delivery—a greater likelihood of adverse phenotypes was observed at the same nominal concentration with the BioPrinter. An existing challenge for the field is to build a fully automated HTS platform that requires minimal to no user intervention.

Imaging and Behavioral Profile Acquisition—High-Content Imaging

Morphological and functional endpoints, i.e., embryo developmental abnormalities, heart rate and behavior, are among the commonly assessed phenotypic readouts of zebrafish in vivo toxicity assays. In the past, the determination of morphological defects involved visual inspection of individual embryos or larvae via microscopy and manual scoring of anatomical structure morphology. Mammalian in vivo embryo-fetal developmental studies were the model for the design of the morphological scoring system in zebrafish embryos (Panzica-Kelly et al. 2010). In this morphological assessment system, the level of severity of a specific abnormality was designated a numerical value on a 5-point score system to grade the morphological defects (Brannen et al. 2010; Panzica-Kelly et al. 2010). The greater the deviation of the morphological abnormality from normal or background levels, the lower score it was assigned. This system focused on identifying morphological defects in 9 main organ systems and structures comprising overall body shape, facial structures, skull structures inclusive of the jaw and pharyngeal arches, brain, heart, somites, notochord, fins and tail (Panzica-Kelly et al. 2010). The body shape was dichotomized as normal or abnormal. Considerable amounts of time and labor had to be expended on operator training and peer review to maintain consistency of morphological score assessment and assignment in this system.

Manual data recording, identification and assignment of phenotypes in zebrafish embryos is often inaccurate, unreliable and subject to operator bias; particularly for the description of subtle phenotypes and embryo morphological phenotypes with a wide range of background variations especially during the early stages of development. Furthermore, manual inspection of morphology, phenotype recognition and morphological scoring of zebrafish embryos for sublethal toxicity assays are inherently time-consuming, labor-intensive and not scalable (Panzica-Kelly et al. 2010). Automation of morphological phenotype feature detection, identification and quantitation would greatly accelerate toxicity screening of compound libraries, dramatically reduce experimental error and further enhance the value and acceptance of zebrafish as an in vivo toxicology model of choice, particularly for high throughput compound screening and hazard identification during early stages of drug development.

HCS approaches couple automated multiparametric microscope-based image acquisition with powerful image processing and analysis algorithms for high throughput automated feature detection (Peravali et al. 2011), quantitation and classification. In this section, we focus on describing the data acquisition instrumentation

(hardware) necessary for conducting HCS assays with the zebrafish model. The algorithms (software) which serve as the lifeblood connecting and automating the various apparatuses responsible for image capture, as well as interpreting and quantifying the visual data collected will be elaborated on in the next section.

HCS systems have been adapted to address different aspects of toxicity. Applications of automated high-resolution imaging platforms for zebrafish toxicity screening encompass visualization and quantification of phenotypic endpoints such as non-invasive detection of heart rate (Gierten et al. 2020; Martin et al. 2019), changes in embryo body length (Lantz-McPeak et al. 2014), numbers of myelinating oligodendrocytes (Early et al. 2018) and behavior. The variety of imaging options offered by automated imaging systems extends from brightfield, fluorescence and confocal microscopy to video recording. At its most basic, high content screening entails concurrent optics-based image recording of multiple samples. An early attempt at developing an economical high content imaging platform was by repurposing flatbed transparency scanners for screening zebrafish (Lessman et al. 2010). Early iterations of automated imaging systems enabled rapid screening of 96 embryos within 31.85 min which is less than 20 s per embryo (Pardo-Martin et al. 2010).

High content imaging systems are commercially available from major biotechnology vendors: ImageXpress Micro systems (Molecular Devices), acumen Cellista (Lin et al. 2011), Cellnsight and ArrayScan suites of HCS platforms (ThermoFisher Scientific), BioSpa™ Live Cell Imaging System (BioTek, Agilent) and the Operetta CLS system (PerkinElmer). Originally designed for imaging of in vitro 2D cell culture-based bioassays, high content imaging systems have been widely adopted for HTS of zebrafish whole organism toxicity assays with both embryos and larvae. They bundle instrumentation enabling acquisition of high-resolution brightfield, fluorescence and/or confocal microscopy images of numerous zebrafish embryos in a high throughput format with image processing and analysis software. Standalone vendor-customized HCS algorithms compatible with existing microscopes are available as well, e.g., Leica HCS A with Leica microscopes.

Automated behavioral screening can be conducted using systems complete with video acquisition instruments and analysis software or standalone specialized automated behavioral tracking and phenotyping software. Some of the software is commercially available whilst others are free to use (Pérez-Escudero et al. 2014). Commercial software tends to have more streamlined workflows and better visualization tools than open-source software. However, the development of open-source automated behavior tracking software is continuously undergoing improvement with new customizable, sophisticated freeware increasingly available. Several open-source automated animal behavior tracking freeware have been tested for use with adult zebrafish (Franco-Restrepo et al. 2019). Newer behavioral tracking tools Bonsai (Lopes et al. 2015), DeepLabCut (Nath et al. 2019), Tracktor (Sridhar et al. 2019) and ezTrack (Pennington et al. 2019) and Ethoflow (Bernardes et al. 2020) are other options worth considering. They reduce the intra-operator and inter-operator variability associated with manual scoring of behavioral phenotypes.

Among commercial software, Noldus has a DanioVision Observation Chamber system which incorporates the Ethovision XT behavioral tracking software with

an observation chamber. Ethovision XT is commonly used for automated behavioral analysis of zebrafish (Duan et al. 2013). Noldus also offers the Ethovision XT software separately and another video tracking software for zebrafish research, called DanioScope. ViewPoint offers a zebrafish-centric automated screening behavioral analysis platform which includes the ZebraBox and ZebraCube apparatuses for behavioral tracking and screening of larval (Javed et al. 2018) and adult zebrafish behavior respectively and proprietary ZebraLab analysis software. The enclosed chambers included with commercial automated zebrafish tracking systems are designed to eliminate disturbance from external factors known to confound behavioral outcomes, e.g., unwanted external light sources, sounds and vibration. Automated behavior observation and tracking systems also come with apparatus enabling programmable control of internal light to monitor photomotor responses or stimulate a startle response. Accessories and software to conduct and analyze other behavioral tests can be purchased separately.

Toxicology Data Analysis—Big Data and Artificial Intelligence

High content imaging systems rapidly and automatically capture thousands of images of individual zebrafish embryos or larvae in high throughput toxicity assays. This creates a need for data storage, management, processing and analysis tools able to store and handle large volumes of optical data efficiently and effectively. Laboratory Information Management Systems (LIMS) are increasingly being used for data management. Zebrafish Acquisition and Analysis Program (ZAAP) is one of the software used (Truong et al. 2016). Big data and artificial intelligence offer solutions to some of the challenges associated with analyzing big datasets. In particular, computer vision and machine learning approaches have widespread applications in toxicity testing, chemical hazard ranking and risk assessment.

Generalized pipelines for computer vision systems include data acquisition, pre-processing, feature extraction, segmentation/detection, image recognition and finally, prediction-making. For zebrafish in vivo toxicity screens, the steps whereby computer vision is employed is as follows: (1) images are captured with an automated image acquisition instrument, usually a microscope or microplate reader, (2) images undergo pre-processing according to pre-defined criteria to reduce noise and identify regions of interest to facilitate downstream processing, (3) feature extraction is performed, (4) the image is converted into quantifiable units during segmentation, (5) the image is compared with images from a training dataset to sort it into categories to construct a decision-making model and (6) the phenotype identified in the image is then classified based on the model constructed (Xu et al. 2010).

Machine learning is a form of artificial intelligence that relies on training a computer to construct classification models to make predictions and decisions on new data based on a training dataset. The effectiveness of machine learning algorithms depends on the quality of a priori knowledge and datasets. Automated image analysis relies heavily on machine learning techniques, predominantly for feature extraction, segmentation, image recognition and classification. For toxicological

applications, machine learning methodologies are used to build rapid, accurate and robust models for phenotype and event classification as well as for the prediction of toxicity outcomes.

Algorithms for high throughput zebrafish toxicity screens perform automated feature detection, recognition and classification of zebrafish eggs (Shang et al. 2019), embryos (Liu et al. 2012; Tharwat et al. 2015) and larval phenotypes (Shang et al. 2020; Teixidó et al. 2018) from images acquired from HCS instrumentation. The key phenotype to be distinguished for zebrafish eggs is whether the eggs are fertilized or unfertilized (Neukum et al. 2019; Shang et al. 2019). A major benefit of being able to conduct high throughput sorting of fertilized and unfertilized eggs at the early stages of fertilization is that it enables the early removal of unfertilized eggs. This prevents unfertilized eggs from being used for zebrafish embryo toxicity tests. Unfertilized eggs may be incorrectly assigned as dead embryos confounding the outcome of the toxicity test (Neukum et al. 2019). At early zebrafish embryonic stages, algorithms focused primarily on automated discrimination of live or dead embryos (Tharwat et al. 2015), hatched, unhatched and dead embryos (Liu et al. 2012), embryos with different pigmentation phenotype patterns (Xu et al. 2010) e.g., wildtype, transparent 1-phenyl 2-thiourea (PTU)-treated embryos, darker transgenic kita-HRAS expressing embryos, homozygous rx3-mutants where eyes are absent (Schutera et al. 2016) and morphological abnormalities (Ishaq et al. 2017; Jeanray et al. 2015; Shang et al. 2020). Leverage of these machine learning algorithms led to the development of commercial automated egg phenotype recognition and sorting platforms and software, e.g., Automated Fish egg sorter (Fraunhofer IPA) and EggSorter (Bionomous).

One of the pioneering machine learning strategies used for automated zebrafish phenotype analysis was Cognition Network Technology, an image analysis approach based on human cognitive processes (Vogt et al. 2009). Traditional machine learning methods such as Support Vector Machine (SVM) (Liu et al. 2012; Schutera et al. 2016) and Random Forest classification (Genest et al. 2019) and newer deep learning algorithms, e.g., Deep Neural Networks (DNN) (Ishaq et al. 2017), convolutional neural networks (CNNs) and Deep Convolution Neural Networks (DCNN) (Shang et al. 2019), are among the machine learning algorithms which have since been used for automated image processing of fish embryos. Major disadvantages of conventional machine learning approaches are the need for an expert to perform manual annotation of image features from training images in the initial training set, small data training sets, the aspect that multiple features are frequently assigned to each image and indistinct inter-class differences (Shang et al. 2020). Shang et al. (2020) utilized two-tier classification with the CNN-derived Xception algorithm to overcome these challenges for automated classification of zebrafish larvae phenotype from brightfield microscope images.

While still in its infancy, the use of machine learning algorithms for video feature extraction and event recognition in behavioral tracking of zebrafish shows promise (Xia et al. 2018; Xu and Cheng 2017). SVM was used successfully for classifying larval zebrafish prey capture behavior (Semmelhack et al. 2014) and looming-evoked escape behavior (Temizer et al. 2015). An unsupervised learning approach has been

used to analyze larval zebrafish locomotor activity (Zhang et al. 2013). CNN architecture was more recently used with the dataset from Semmelhack et al. (2014) to differentiate between larval zebrafish prey and spontaneous swim bouts (Breier and Onken 2020). The accuracy obtained through CNN outperformed Semmelhack's SVM approach (Breier and Onken 2020). Taken together, the benefits deep learning methods provide over conventional machine learning techniques for image and video analysis in zebrafish HCS assays suggest that deep learning algorithms like CNN may be the way of the future for high throughput zebrafish toxicity screens. Machine learning architecture has also been employed for automation of detection and classification of phenotypes associated with cardiovascular health in zebrafish embryos and larvae, i.e., heart beat detection, heart rate quantification and vascular growth (Akerberg et al. 2019; Daetwyler et al. 2019; Gierten et al. 2020; Kang et al. 2018). It is clear that machine learning, especially deep learning approaches are highly beneficial for the detection of neurotoxicity and cardiotoxicity in larval zebrafish toxicity assays.

Artificial intelligence has been instrumental in the field of environmental and human health safety. It has led to breakthroughs in toxicity prediction through the integration of libraries containing physico-chemical properties of thousands of chemical compounds and environmental toxicants with toxicity data from various biological assays using different platforms (Wu and Wang 2018). Computational or in silico toxicological methods such as structure–activity relationship (SAR) and quantitative structure–activity relationship (QSAR) computation modeling have a long history of being used to infer toxicity outcomes from physicochemical properties of chemical compounds. The use of machine learning for computational toxicology is a fast-advancing field. While multiple linear regression used to be the favored technique for QSAR modeling, its popularity was later overtaken by SVM, Random Forest and Ensemble learning and most recently, deep learning methods (Basile et al. 2019; Baskin 2018).

The ToxCast approach of the US EPA integrates toxicity information (e.g., phenotypic endpoints) from multiple HTS assays (in vitro and in vivo) into a computational systems biology to classify and predict toxicity outcomes and hazard potentials for chemicals and toxicants. Machine learning has played a critical role in the construction of toxicity prediction models for human risk assessment. The DeepTox pipeline is a Deep Learning computational model using Deep Neural Network architecture to construct abstract chemical features from HTS assay data to make accurate toxicity predictions (Mayr et al. 2016). In recent years, these approaches have gradually been applied to the field of nanotoxicology with the view of improving the design of engineered nanomaterials through reduction of toxicity (Choi et al. 2018; Furxhi et al. 2020; Peng et al. 2020; Saini and Srivastava 2018; Winkler 2020). The dramatic increase in throughput, accuracy and robustness that artificial intelligence brings to nanotoxicology has the potential to revolutionize the field.

Overview of in Vitro and in Vivo Models in Nanotoxicology and Advantages of Zebrafish as a Toxicology Model

In Vitro Models

Historically, in vitro toxicology involved cell cultures using human and rodent cell lines. In vitro toxicity assays, excellently summarized by Hillegass et al. (2010), provide invaluable knowledge on the harmful effects of nanomaterials in various tissues (Table 5.1). In recent years, fish cell lines have been developed for in vitro assays to substitute in vivo tests (Rehberger et al. 2018). Major zebrafish cell lines include those derived from embryos and adult organs (Table 5.1). Detailed information on the cell lines can be found at the Cellosaurus database (https://web.expasy.org/cellosaurus/) (Bairoch 2018).

Advantages of in vitro toxicology systems include circumventing disadvantages related to the use of whole animal toxicology models i.e., ethical issues and high labor, time and financial costs. However in vitro systems are physiologically and structurally less complex than tissue and organ systems, let alone whole animals. Hence, they are not useful for investigating complex phenotypes. In fish, studies have shown that in vitro cytotoxicity assays are significantly less sensitive than in vivo lethality assays (Rehberger et al. 2018). While emerging advances in in vitro systems such as three-dimensional human organoids may overcome some of these issues (Renner et al. 2020), these technologies are still under development and lack standardized protocols to reduce inter-laboratory variability. Since there is no interaction between tissues and organs for existing 3D human organoid systems, its current utility is limited to exploring tissue-specific or organ-specific effects (Kim et al. 2020).

In Vivo Models

Whole animal toxicity testing is the traditional gold standard for chemical hazard assessment. Rodents have been the preferred toxicology models due to their high levels of biological conservation to humans. However, rodent toxicity testing is hampered by low throughput, ethical issues and costly, time-consuming and labor-intensive protocols. Driven by the 3Rs (Replacement, Reduction and Refinement) principles for humane scientific research, there has been a paradigm shift by international and national regulatory agencies to replace mammalian whole animal toxicity systems. Alternative methods involving the use of in vitro and in silico testing systems as well as non-mammalian animal models are increasingly pursued. Rodent models also fail to account for the adverse effects of environmentally relevant exposure of toxicants on aquatic environments.

Aquatic model organisms such as fathead minnow (*Pimephales promelas*) (Ankley and Villeneuve 2006; Hall et al. 2009; Kurita Oyamada et al. 2020; Laban et al. 2010), medaka (*Oryzias latipes*) (Dong et al. 2016; Kim et al. 2013; Padilla

Table 5.1 Most commonly used cell lines for assessing nanotoxicity

Cell line name	Sampling age	Tissue	Accession number[a]	References
Human cell lines				
HeLa	Adult	Human papillomavirus-related endocervical adenocarcinoma	CVCL_0030	Yehia et al. 2007
A549	Adult	Lung adenocarcinoma	CVCL_0023	Lanone et al. 2009
CaLu3	Adult	Lung adenocarcinoma	CVCL_0609	Kroll et al. 2011
HaCaT	Adult	Skin	CVCL_0038	Kroll et al. 2011
CaCo2	Adult	Colon adenocarcinoma	CVCL_0025	Kroll et al. 2011
Hep-G2	Adult		CVCL_0027	Kroll et al. 2011
THP-1	Child (1 year)	Macrophages from childhood acute monocytic leukemia	CVCL_0006	Lanone et al. 2009
Mouse cell lines				
NIH-3T3	Embryo	Fibroblasts	CVCL_0594	Kroll et al. 2011
RAW264.7	Adult	Macrophages	CVCL_0493	Kroll et al. 2011
Rat cell lines				
RLE-6TN	Adult	Lungs	CVCL_4693	Kroll et al. 2011
NRK-52E	Adult	Kidney	CVCL_0468	Kroll et al. 2011
Rainbow trout cell line				
RT-W1		Gills		George et al. 2012
Zebrafish cell lines				
ZF4	Embryo	Fibroblasts	CVCL_3275	Yan et al. 2021
ZEM2S	Embryo	Fibroblasts	CVCL_3274	Yan et al. 2021
Pac2	Embryo	Fibroblasts	CVCL_5853	Yan et al. 2021
AB.9	Adult	Caudal fin	CVCL_6311	Yan et al. 2021
SJD.1	Adult	Caudal fin	CVCL_5895	Yan et al. 2021
ZG	Adult	Gills	CVCL_R820	Yan et al. 2021
ZFL	Adult	Liver	CVCL_3276	Yan et al. 2021
ZKS	Adult	Renal stroma	CVCL_5905	Yan et al. 2021

(continued)

Table 5.1 (continued)

Cell line name	Sampling age	Tissue	Accession number[a]	References
ZSSJ	Adult	Spleen	CVCL_6E22	Yan et al. 2021

[a] This accession number refers to the Cellosaurus accession number: a unique primary accession number assigned unambiguously to specific cell lines in the Cellosaurus database

et al. 2009) and zebrafish have greatly contributed towards improving understanding of the adverse effects of acute and chronic exposure of engineered nanomaterials on aquatic habitats. While beneficial for aquatic toxicology, toxicity studies using fathead minnow are limited by the relative scarcity of genomic resources available (Saari et al. 2017). Thus, zebrafish has been used as a sentinel species for water quality monitoring of aquatic environments.

Small invertebrate model systems including *Caenorhabditis elegans* (Jung et al. 2015; Kim et al. 2017; Tsai et al. 2021) and *Daphnia* sp. (Fadare et al. 2020; Gao et al. 2018; von Mikecz 2018) have also provided valuable information on the toxicity of nanomaterials. The genome of *C. elegans* has been sequenced, its development is well-studied and it is amenable to genomic manipulation. *C. elegans* is highly amenable to HTS. Long-standing well-optimized protocols exist for *C. elegans* HTS using the COPAS platform (Boyd et al. 2010; Pulak 2006). Major disadvantages of invertebrate systems are low biological complexity and conservation compared to vertebrate systems e.g., different body patterning programs and some aspects of physiology.

Advantages of the Zebrafish Model

Environmental Health and Safety Paradigms

Zebrafish are a key tool for regulatory toxicology, chemical hazard and risk assessment and environmental monitoring especially in the United States of America (USA) and Europe. In addition to the benefits the zebrafish model offers, the strong concordance in toxicity outcomes observed in zebrafish and endemic teleost species contributes towards its popularity as an aquatic toxicology model.

Zebrafish have been used extensively in the national toxicology program of the US Environmental Protection Agency (EPA), called Toxicology in the 21st Century (Tox21), and the European Union (EU) ToxRisk program. The European Chemical Agency (ECHA) frequently uses embryonic zebrafish with the OECD guidelines 210 Fish, Early-Life Stage Toxicity Test, 212 Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages and 236 Fish embryo acute toxicity test (FET) for toxicology testing of chemicals (Busquet et al. 2014). Toxicity testing has also been conducted using adult zebrafish.

Zebrafish embryos younger than 120 hpf are regarded as a 'Replacement' toxicology model. The earliest stages of embryo development, in which they have not gained the abilities of independent feeding and free-living, are not regarded as protected under the European Commission's 2010 directives. It is also generally accepted that zebrafish embryos under 120 hpf are incapable of feeling pain or distress (Strähle et al. 2012). Therefore, from an animal ethical standpoint, scientific research utilizing zebrafish embryos less than 120 hpf of age is not regulated according to animal welfare legislation (Strähle et al. 2012).

Phenotypes Assayed in Zebrafish

Cytotoxicity, teratogenicity, organ and system-specific toxicity encompassing reproductive, gastrointestinal, ocular, oto-, cardio-, hepato-, immuno-, nephro-, neuro-, myo-, and enotoxicity, disruption of the endocrine system and intestinal microbiota are among the other ways nanomaterials exert toxic effects on the body (Chakraborty et al. 2016; Haque and Ward 2018; Pham et al. 2016). While several phenotypic endpoints of toxicity are external and visually apparent, e.g., mortality and hatching interference (Chen et al. 2013; Lin et al. 2013b), engineered nanomaterials also affect internal organ systems where damage assessment is not straightforward. Different phenotypic endpoints are assayed using different batteries of tests (Duan et al. 2013; Mosselhy et al. 2016). Mortality, altered hatching rates, changes in size (head and full body) and gross morphological abnormalities including edema (pericardial, yolk sac and swim bladder), egg development defects, pigmentation disruption (eyes, trunk and fin) and skeletal deformities (skull, operculum, jaw, fin bones and spine) are endpoints frequently assessed for zebrafish embryo and larval toxicity studies (Hamm et al. 2019; Panzica-Kelly et al. 2010). These endpoints can be observed non-invasively in zebrafish larvae using brightfield microscopy (Duan et al. 2013; He et al. 2020). Staining with neutral red, a eurhodin dye, and tissue-specific fluorescent dyes, e.g., the neuromast hair cell-specific dyes 4-Di-1-ASP and DASPEI, enable microscopic visualization of skin and neuromast hair cells, respectively (Peng et al. 2020, 2018). Detection of histopathological abnormalities in internal organs like intestines and gills (Osborne et al. 2015, 2017) are invasive. Fixation, staining with tissue-specific dyes (Alcian blue for mucin staining), tissue sectioning, histological analyses and immunohistochemistry with fixed tissues are required.

Behavioral tests assessing loco- and photomotor activities, as well as touch responses, are used to evaluate neurotoxic effects in live zebrafish (McNeil et al. 2014; Sarasamma et al. 2019; Selderslaghs et al. 2013; Truong et al. 2012; Zhao et al. 2019b). Cardiotoxicity can be evaluated through the monitoring of heart rate. Automated image or video acquisition software is used to record and analyze heart beats per minute (heart rate) in zebrafish embryos. For example, it was determined that exposure to γ-Fe_2O_3 nanoparticles causes embryonic zebrafish to have an abnormally low heart rate (bradycardia) (Pereira et al. 2020).

Molecular pathways modulating functional disruption in zebrafish can be evaluated by measuring established biochemical or genetic biomarkers (Padilla 2014).

Ligands and proteins of interest can be probed using enzyme-linked immunosorbent assays (ELISA) and immunohistochemistry. Reactive oxygen species (ROS) generation due to redox reactions, an indicator of oxidative stress, at the nano-bio interface is associated with skin damage in zebrafish (Peng et al. 2020, 2018). Commercial biochemical kits are used to assess levels of ROS generation and total glutathione depletion. ELISA assays can also be used for the detection and quantification of 8-hydroxy-2′-deoxyguanosine (8-OH-dG) formation (Bar-Ilan et al. 2011). 8-OHdG, an indicator of oxidative stress and oxidative stress induced-genotoxicity, is a type of DNA adduct commonly generated due to toxicity from engineered nanomaterial (Bar-Ilan et al. 2011; Petersen and Nelson 2010). Digestive function is assessed using the EnzChek protease assay kit. Expression levels for genes of interest can be quantified using quantitative real-time polymerase chain reaction (qPCR). Also, in situ hybridization is used to locate the regions of specific genes in tissues and their expression levels. On a larger scale, transcriptomics, proteomics and metabolomics can be interrogated to elucidate the molecular pathways and mechanisms underpinning observed apical effects.

Transgenic Zebrafish Lines Used for Evaluation of Nanotoxicity

Many transgenic zebrafish lines carrying fluorescent reporter genes under the control of regulatory elements specific to inducers such as signaling molecules, chemical compounds and environmental conditions such as high temperature; as well as organs/tissues/cells have been developed. While some of the lines express the fluorescent proteins constitutively, several of these promoters possess inducible response elements that only express the fluorescent marker in response to an inducer (inducible transgenic lines). While in vivo imaging of wild-type zebrafish is usually only possible at embryonic and larval stages, fluorescent transgenic zebrafish lines with the transparent Casper genetic background can be imaged as adult fish. Advantages of transgenic zebrafish lines include high sensitivity and the capability for non-invasive in vivo real-time tracking. In vivo imaging is highly useful for automated imaging-based quantitation of fluorescently labeled cells as well as longitudinal and depuration studies. Several lines have been developed and deployed as biosensors for in situ environmental monitoring and nanotoxicology with great success (Carvan et al. 2001; Lee et al. 2015). The transgenic zebrafish lines fall into two main categories: (1) inducible and (2) constitutive lines (Table 5.2) which can be further dichotomized into lines with ubiquitous or organ/tissue/cell-type-specific expression of fluorescent proteins.

Inducible Transgenic Lines

Expression of fluorescent proteins occurs exclusively in the presence of the inducer for inducible transgenic zebrafish. Depending on the regulatory elements driving transgene expression, the fluorescent proteins can be expressed in most tissues and

Table 5.2 Zebrafish lines used for assessing nanotoxicity

Name	Phenotype	Nanoparticle	Metal Ions	Biological effect	References
Wild-type zebrafish lines					
AB	Pigmented	SiO_2			Liu et al. 2021
WIK	Pigmented	Ag			van Aerle et al. 2013
5D Tropical, 5D	Pigmented	Ag, ZnO, TiO_2, CeO_2 and SnO_2			
Mutant zebrafish line					
Casper (White et al. 2008)	Without pigment	Au			
Inducible transgenic zebrafish lines					
huORFZ	GFP expression in skin and muscle when exposed to Cu^{2+}. GFP expression in skin, olfactory epithelium and pronephric ducts when exposed to Cd^{2+}		Cu^{2+} and Cd^{2+} ions		Lee et al. 2014
Tg(*hsp70:egfp*) (Halloran et al. 2000)	GFP expression in response to oxidative stress (heat shock protein)	CuO, ZnO and NiO		Oxidative stress	Lin et al. 2011
Tg(*ARE:eGFP*)	GFP expression in locations of oxidative stress (antioxidant response element)	TiO_2		Oxidative stress	Bar-Ilan et al. 2011

(continued)

Table 5.2 (continued)

Name	Phenotype	Nanoparticle	Metal Ions	Biological effect	References
Tg(*MT-Ia1:DsRed2*)	Red fluorescent protein expression in dorsal and ventral retina, gills and skin epithelium		Hg^{2+}, Cd^{2+} and Cu^{2+} ions		Pawar et al. 2016
Tg(*mt:egfp*)	GFP expression in the head, eye, pericardium, skin and tail		Zn^{2+} and Cd^{2+} ions		Liu et al. 2016
Organ/tissue/cell type-specific transgenic zebrafish lines					
Tg(*lyz:DsRed2*)	Red fluorescent protein expression in macrophages and neutrophils	Mesoporous silica nanoparticles, TiO_2			He et al. 2020, Sharif et al. 2012, Hall et al. 2007
Tg(*lyz:eGFP*) (Hall et al. 2007)	GFP expression in macrophages and neutrophils	Co_3O_4-based nanoparticles			Peng et al. 2020
Tg(*mpo:GFP*) (Renshaw et al. 2007)	GFP expression in macrophages and neutrophils	SiO_2		Oxidative stress	Duan et al. 2016, Duan et al. 2015
Tg(*mpeg1:mCherry*) (Ellett et al. 2010)	GFP expression in macrophages and neutrophils	Cu, Polystyrene		Oxidative stress	Brun et al. 2018, Evensen et al. 2015

(continued)

Table 5.2 (continued)

Name	Phenotype	Nanoparticle	Metal Ions	Biological effect	References
Tg(*tnfα:eGFP-F*)	GFP expression in macrophages and neutrophils	Cu, Polystyrene		Oxidative stress	Brun et al. 2018
Tg(*il1β:eGFP-F*) (Nguyen-Chi et al. 2014)	GFP expression in keratinocytes at the tip of the caudal fin, in fin buds, retina, neuromasts, gills and thymus, neutrophils and macrophages	Cu, Polystyrene		Inflammatory response	Brun et al. 2018
Tg(*kdrl:egfp*)	GFP expression in heart and blood vasculature	Nano-graphene oxide			Jeong et al. 2015
Tg(*fli1a:egfp*), **Tg(*fli:egfp*)**	GFP expression in heart and blood vasculature	Cadmium selenium (CdSe) quantum dots, Inorganic nanorods, CuO, SiO_2, TiO_2			Duan et al. 2016, Jie et al. 2015
Tg(*cmlc2:eGFP*)	GFP expression in heart and blood vasculature	Ag			Yoo et al. 2016
Tg(*3mnx1:TagBFP*)	GFP expression in motor neurons	CaP-lipid			Chen et al. 2017
Tg(*isl1:GFP*)	GFP expression pan-neuronally throughout the brain	CaP-lipid			Chen et al. 2017

(continued)

Table 5.2 (continued)

Name	Phenotype	Nanoparticle	Metal Ions	Biological effect	References
Tg(*nbt:egfp*)	GFP expression in neurons	SiO_2			Wei et al. 2020
Tg(*gfap:GFP*) (Bernardos and Raymond 2006)	GFP expression in astrocytes of the brain	CaP-lipid			
Tg(HuC:GFP)	GFP expression pan-neuronally throughout the central nervous system	TiO_2			Gu et al. 2021
Tg(*hb9:GFP*)	GFP expression in motor neurons	TiO_2			Gu et al. 2021
Tg(*fabp10a:Dsred*)	Red fluorescent protein expression in liver	SiO_2			Pham et al. 2016

cells (ubiquitous) or specific organs, tissues or cell types (tissue-specific). Inducible zebrafish lines used to assess nanotoxicity include *Tg*(*hsp70:egfp*), *Tg(ARE:egfp),* *Tg*(*mt-Ia1:dsRed2*) and *Tg*(*mt:egfp*) (Table 5.2). In particular, the *Tg*(*hsp70:egfp*) transgenic reporter line where green fluorescent protein (GFP) expression is driven by regulatory elements of the heat shock protein 70 gene (Blechinger et al. 2002; Halloran et al. 2000) has been extensively used for nanotoxicology in zebrafish (Lin et al. 2011; Pan et al. 2013). Interestingly for *Tg*(*hsp70:egfp*) zebrafish, heat treatment-induced ubiquitous GFP expression while CuO nanoparticles induced GFP expression specifically in the head and tail (Lin et al. 2011). Other transgenic lines responsive to environmental stress and toxicants include the transgenic zebrafish reporter for antioxidant response element *Tg*(*ARE:egfp*) (Bar-Ilan et al. 2011), the aryl hydrocarbon receptor transgenic reporter zebrafish line Tg(*cyp1a:gfp*) responsive to dioxin-like compounds and poly aromatic hydrocarbons (Xu et al. 2015), the hypoxia reporter zebrafish line Tg(*phd3::EGFP*) (Santhakumar et al. 2012) and transgenic zebrafish lines responsive to toxic heavy metals, e.g., the transgenic reporters for red fluorescent protein gene under the control of a metallothionein promoter derived from Asian green mussel *Tg*(*mt-Ia1:dsRed2*) (Pawar et al. 2016) and enhanced GFP gene driven by zebrafish metallothionein promoter *Tg*(*mt:egfp*) (Liu et al. 2016). These transgenic lines are good indicators of chemical compounds that induce oxidative stress and oxidative toxicity in embryonic zebrafish.

Constitutive Transgenic Lines

In constitutive transgenic zebrafish, fluorescent proteins are expressed continually. Constitutive tissue-specific reporter lines have been used for the assessment of organ, tissue or cell-type specific nanotoxicity (Poon et al. 2017) (Table 5.2). Blood vessel-specific transgenic lines include Tg(*flia:egfp*) (Duan et al. 2018) with the GFP gene under the control of zebrafish *fli* promoter variants and the transgenic reporter line under the cardiac myosin light chain 2 promoter Tg(*cmcl2:egfp*) (Huang et al. 2003). Co-exposure of silica nanoparticles with methylmercury in *Tg*(*fli-1:EGFP*) transgenic zebrafish embryos reduced the numbers of vascular endothelial cells below that with exposure of silica nanoparticles and methylmercury alone, suggesting that it caused damage of vascular endothelial cells (Duan et al. 2016). *Tg*(*lfabp:DsRed*) which tags liver cells with the red fluorescent protein under the control of the regulatory elements of the liver-type fatty acid-binding protein (lfabp) gene has utility as a biosensor for hepatotoxicity (Pham et al. 2016). Pham et al., 2016 showed that silica nanoparticles do not elicit hepatotoxicity in zebrafish embryos using the *Tg*(*lfabp:DsRed*) zebrafish line.

The physicochemical properties of some engineered nanomaterials can modulate immune responses and stimulate inflammatory responses. In some cases, redox reactions at the nano-bio interface may trigger injury of the skin epithelium leading to an immune response. Transgenic zebrafish lines with fluorescently tagged macrophages and neutrophils (Torraca et al. 2014) are good reporters of immunotoxicity since environmental toxicants frequently affect immune responses

adversely (Xu et al. 2018). These transgenic reporter lines include *Tg*(*lyz:DsRed2*), *g*(*coro1a:eGFP*) and *Tg*(*mpeg1:EGFP*). Macrophages and neutrophils express GFP in the *Tg*(*coro1a:eGFP*) reporter line (Li et al. 2012). Macrophages specifically express GFP in the *Tg*(*mpeg1:EGFP*) zebrafish line (Ellett et al. 2010). For the *Tg*(*lyz:DsRed2)* transgenic zebrafish line, only neutrophils are labeled with a fluorescent red protein (He et al. 2020). Exposure of *Tg*(*mpo:GFP*) transgenic zebrafish embryos to a mixture of silica nanoparticles and methylmercury produced more neutrophils in caudal vein than with exposure to silica nanoparticles and methylmercury alone (Duan et al. 2016). This suggests an inflammatory response is mounted in response to co-exposure with silica nanoparticles and methylmercury (Duan et al. 2016).

Muscles and neurons co-operatively mediate several zebrafish behavioral phenotypes. Therefore, transgenic zebrafish lines with fluorescent reporter genes under the control of the regulatory elements of muscle and neuron-specific genes can be used for in vivo monitoring of zebrafish behavior. The *TgBAC*(*hspb11:GFP*) zebrafish line expresses GFP under the control of the small heat shock protein *hspb11* gene regulatory elements. *hspb11* is involved in slow muscle myofibril organization and/or maintenance and its expression was loosely correlated with activation of nicotinic acetylcholine receptor (nAChR) in muscles (Klüver et al. 2011). This reporter line acts as a sensitive biosensor of chemical compounds that impair muscle integrity leading to motor dysfunction (Shahid et al. 2016). It serves well as a tool for visualization of locomotor activity for behavioral assays.

Transgenic zebrafish where important modulators of neurological activity are fluorescently labeled have potential as tools for elucidating mechanisms of action underpinning neurotoxic effects of nanomaterials. Promising targets include neural subtype-specific proteins and receptors essential for neuromodulation such as neurotransmitters (Feng et al. 2019; Jing et al. 2018; Sun et al. 2018; Wan et al. 2021). *Tg*(*olig2:egfp*) reporter line expresses GFP in motor neurons, oligodendrocytes and Purkinje neurons (Shin et al. 2003). The vesicular monoamine transporter 2 (vmat2) promoter for the transgenic line *ETvmat2:GFP* tags most monoaminergic neurons with fluorescent green protein facilitating their visualization in zebrafish (Wen et al. 2008). In the *Tg*(*dat:EGFP*) transgenic line, GFP labels dopaminergic neurons of the ventral diencephalon (vDC) clusters, amacrine cells in the retina, olfactory bulb, pretectum, and caudal hypothalamus (Xi et al. 2011). The transgenic line Tg(*gfap:GFP*) expresses GFP in glial cells driven by the zebrafish glial fibrillary acidic protein (GFAP) regulatory elements (Bernardos and Raymond 2006). *Tg*(*nkx2.2a:mEGFP*) transgenic zebrafish express GFP in ventral axons, enabling visualization and length measurements of ventral axons of oligodendrocyte-lineage cells (Kirby et al. 2006; Zhang and Gong 2013). Exposure of the reporter line to low concentrations of five neurotoxins (acetaminophen, atenolol, atrazine, ethanol and lindane) dramatically reduced the lengths of ventral axons, demonstrating its usefulness as a biosensor of neurotoxicity.

Neuromast hair cells located at the lateral line of zebrafish are mechanosensory cells structurally and functionally orthologous to inner ear cells in humans (Ton and Parng 2005). Several transgenic reporter lines express GFP and GCaMP3.0

in the hair cells of the lateral line under control of hair cell-specific promoters pou4f3 (brn3c) and myosin6 respectively: *Tg*(*pou4f3:gap43-GFP*) (Xiao et al. 2005) and *Tg*(*myo6b:GCaMP3*)*w78* (Esterberg et al. 2013). The *Tg*(*pou4f3:gap43-GFP*) reporter line demonstrated that zebrafish embryos suffered from ototoxicity upon silver nanoparticle exposure (Yoo et al. 2016).

In addition, triple transgenic zebrafish lines in which different organs or different cell lineages within an organ are labeled with different fluorescent proteins have been created for toxicology testing (Cornet et al. 2017; Koiwa et al. 2019). These lines allow monitoring of multiple phenotypic endpoints involving different organ systems or cell types in single organisms reflecting different types of toxicity. This is highly valuable for elucidation of mechanisms of action of chemical compounds and environmental pollutants which cause systemic toxicity. For example, Koiwa et al. (2019) developed a triple transgenic zebrafish line in which different neural cell lineages were labeled with different fluorescent proteins: neurons with a Cerulean cyan fluorescent protein, astrocytes with an mCherry red fluorescent protein and oligodendrocytes with an mCitrine yellow fluorescent protein (Koiwa et al. 2019). It facilitated interrogation into how chemical compounds elicited neurotoxicity by affecting neuronal differentiation into the three neural cell subtypes. Toxicant responsive genes with potential utility as biomarkers can be identified from toxicogenomic experiments. Biomarkers can be used downstream for the evidence-based design of transgenic zebrafish lines suitable for toxicological monitoring (Padilla 2014).

Studies Using Zebrafish to Evaluate Engineered Nanomaterials Toxicity

Extensive studies have shown the potential of engineered nanomaterials to inflict toxicity on embryonic and larval zebrafish (Lin et al. 2011) (Table 5.3). Zinc oxide nanoparticles have deleterious effects on hatching (Chen et al. 2013; Xia et al. 2011; Zhao et al. 2016). It targets the zebrafish hatching enzyme, ZHE1 to inhibit hatching (Lin et al. 2013b). Lin et al. (2013b) showed that CuO, Cr_2O_3 and NiO nanoparticles can also disrupt ZHE1. High concentrations of Congo red (CR) functionalized iron oxide nanoparticles (CR@Fe_3O_4) increased mortality and reduced hatching rates of zebrafish embryos (Jurewicz et al. 2020). Several studies point towards the neurotoxic effect of silver and gold nanoparticles on zebrafish embryos and larvae (Ašmonaitė et al. 2016; McNeil et al. 2014; Powers et al. 2011; Truong et al. 2012; Zhao et al. 2019b). Alterations in zebrafish larval and adult behavior have been reported for CuO (McNeil et al. 2014; Thit et al. 2017) and silica nanoparticles (Duan et al. 2013; Li et al. 2019, 2014).

Zebrafish in vivo toxicity studies with nanoparticles have also helped elucidate the physicochemical properties which contribute to various types of toxicity and aided hazard ranking for risk assessment. Zebrafish embryo in vivo assays demonstrated that the toxicity of silver nanoplates could be attributed to the presence of surface

Table 5.3 Representative studies using zebrafish for assessing nanotoxicity

Nanoparticle	Modification	Stage	Duration	Phenotypes				References
				Mortality	Hatching	Histopathology	Behavior	
Ag	Citrate-coated and PVP-coated	Embryos, larvae	0 to 5 days	✓	✓	✓	✓	Powers et al. 2011
Ag	None	Embryos, larvae					✓	McNeil et al. 2014
Ag	None	Larvae	4 h				✓	McNeil et al. 2014
Ag	None	Adult	4 h, 4 days, 4 days with 7-day depuration			✓	✓	Osborne et al. 2015
Ag	Citrate-coated	Embryos, larvae	4 hpf to 24, 48, 72 and 96 hpf				✓	Zhao et al. 2019b
Au	Negatively, neutral and positively charged	Embryos, larvae	6 to 120 hpf				✓	Truong et al. 2012
C_{60} and C_{70}	None	Embryos, larvae	24 to 96 hpf	✓		✓	✓	Usenko et al. 2007
C_{60}	None	Adult	12 days				✓	Sarasamma et al. 2018
C_{70}	None	Adult	7, 14, 21 days				✓	Sarasamma et al. 2019
Co_3O_4	None	Embryos, larvae	24 h					Peng et al. 2018

(continued)

Table 5.3 (continued)

Nanoparticle	Modification	Stage	Duration	Phenotypes				References
				Mortality	Hatching	Histopathology	Behavior	
Co_3O_4	Cu-doped, Cr-doped, Fe-doped, Mn-doped, PdO conjugated	Embryos, larvae	24, 48 and 72 hpf	✓		✓		Peng et al. 2020
Cr_2O_3	None	Embryos, larvae	24 h		✓	✓		Peng et al. 2018
CuO	None	Embryos, larvae	1 to 5 days	✓	✓		✓	Thit et al. 2017
CuO	None	Larvae	4 h	✓	✓		✓	McNeil et al. 2014
Fe_3O_4	None	Embryos, larvae	24 h			✓		Peng et al. 2018
Fe_3O_4	Congo Red conjugated	Embryos, larvae		✓	✓	✓		Jurewicz et al. 2020
GaAs	None	Embryos, larvae, adult	4 h pulse exposure (3 times)	✓	✓	✓		Osborne et al. 2017
GaP	None	Embryos, larvae	4 h pulse exposure (3 times)	✓	✓	✓		Osborne et al. 2017
InAs	None	Embryos, larvae, adult	4 h pulse exposure (3 times)					Osborne et al. 2017
InP	None	Embryos, larvae	4 h pulse exposure (3 times)	✓	✓	✓	✓	Osborne et al. 2017

(continued)

Table 5.3 (continued)

Nanoparticle	Modification	Stage	Duration	Phenotypes				References
				Mortality	Hatching	Histopathology	Behavior	
NiO	None	Embryos, larvae	4 h pulse exposure (3 times)		✓			Peng et al. 2018
Silica	None	Embryos	4 to 96 hpf	✓	✓	✓	✓	Duan et al. 2013
SiO_2	None	Larvae	3 days	✓	✓	✓	✓	Xue et al. 2013
SiO_2	None	Adult		✓	✓	✓	✓	Li et al. 2014
Supramagnetic FeO	Dextran, chitosan, polyethylene glycol, carboxy-silane and silica	Larvae		✓	✓	✓	✓	Oliveira et al. 2020
TiO_2	None	Embryos, larvae	5 days				✓	Chen et al. 2011
TiO_2	None	Larvae	4 h					McNeil et al. 2014
TiO_2	Light treated	Embryos, larvae	6 h			✓		He et al. 2020
ZnO	None	Embryos, larvae	to 144 hpf		✓		✓	Chen et al. 2013
ZnO	None	Larvae		✓	✓	✓		Wehmas et al. 2015
ZnO	Fe-doped	Embryos, larvae		✓	✓			Xia et al. 2011

defects (George et al. 2012), size of nanoparticles (Osborne et al. 2017), and aspect ratio (Ji et al. 2012; Lin et al. 2014). Zebrafish in vivo toxicity assays also uncovered the mechanism of action underlying toxicity for several nanoparticles. As(III) and As(V) ion shedding from nano-sized particulates (nm-InAs and nm-GaAs) disrupted Na^+/K^+ ion channels (Osborne et al. 2017) and shed metal ions from CuO, ZnO, Cr_2O_3, and NiO nanoparticles interfered with zebrafish hatching (Lin et al. 2013b). The induction of oxidative stress is also a key mechanism for nanotoxicity (He et al. 2020; Osborne et al. 2017; Peng et al. 2020). ROS formation (He et al. 2020; Peng et al. 2020), up-regulation of the oxidative stress biomarkers heme oxygenase-1 (HO-1), nuclear factor-erythroid-2-related factor (Nrf2a) and interleukin-1β (IL-1 β) accompanied with induction of lipid peroxidation and DNA damage (He et al. 2020) were clear indicators that oxidative stress was a major culprit underpinning the acute toxicity of NiO, Cr_2O_3, Co_3O_4 and TiO_2 nanoparticles on zebrafish embryos and larvae (He et al. 2020; Peng et al. 2020).

Zebrafish toxicity studies have provided ample evidence of the Trojan Horse effect of complex mixtures of nanomaterials and environmental toxicants: benzo(α)pyrene interacting with carbon nanopowders altered uptake of benzo(a)pyrene and gene expression profiles in embryonic zebrafish (Binelli et al. 2017), administration of TiO_2 nanoparticles with a BDE-209; a polybrominated diphenyl ether congener used as a brominated flame retardant, augmented BDE-209 bioaccumulation, affected thyroid function and induced neurotoxicity (Wang et al. 2014), co-exposure of zebrafish larvae to TiO_2 nanoparticles and cypermethrin, an insecticide, increased bioaccumulation of cypermethrin and cypermethrin-induced neurotoxicity (Li et al. 2018). Chen et al., 2017 showed that co-exposure to TiO_2 nanoparticles and bisphenol A can disrupt gut microbiota in adult zebrafish (Chen et al. 2018). Similarly, a recent in vivo study showed that zebrafish embryos exposed to gold nanoparticles mixed individually with the common surfactants Polysorbate 20, Polysorbate 80 or sodium dodecyl sulfate exhibited synergistic toxicity (Ginzburg et al. 2018). Co-exposure of zebrafish embryos to ZnO nanoparticles and dissolved organic matter attenuated the toxic effects on hatching and improved survival compared to ZnO nanoparticle exposure alone (Kteeba et al. 2017). In some cases, the toxic effects of co-exposure of nanoparticles and environmental pollutants can span across generations (Guo et al. 2019). Co-administration of TiO_2 nanoparticles with BPA elevated thyroid endocrine disruption in female zebrafish beyond that observed with BPA exposure alone. The maternal transfer caused significant neurotoxicity in offspring (Guo et al. 2019).

Although the adsorption of benzo(a)pryene onto fullerene reduced the bioaccumulation and uptake of fullerene, the combined exposure induced greater cytotoxicity and genotoxicity in zebrafish embryos than with fullerene alone (Della Torre et al. 2018). Adsorption of polycyclic aromatic hydrocarbons (PAH) to nanoplastics appeared to reduce bioaccumulation and toxicity of PAH (Trevisan et al. 2020, 2019). Interestingly, Trevisan et al. (2019) and (2020) found that exposure to nanoplastics-adsorbed PAH protected zebrafish embryos from PAH toxicity but disrupted mitochondrial function, a phenomenon seen with exposure to nanoplastics alone.

Potential of Zebrafish for Nanomedicine Exploration

Nanomaterials have been harnessed for the development of imaging, diagnostics and therapies against major diseases including cancer and neurodegenerative diseases (Pelaz et al. 2017). In the body, nanocarriers improve drug solubility, enable drugs to pass through the blood–brain barrier and tumor endothelium, prevent opsonization of drugs, protect biologics based on genes, RNA, peptides or proteins from premature degradation, enable controlled drug release and targeted delivery of drugs to specific tissue and cell types and therefore enhance treatment efficacy and reducing undesired toxicity (de Lázaro and Mooney 2021; Ventola 2012). Different cancer cell eradicating approaches such as thermotherapy (Quintanilla et al. 2019), immunotherapy (Irvine and Dane 2020) and radiation therapy (Kotb et al. 2016) can be achieved and improved with nanomaterials. For imaging applications, direct monitoring is possible for inorganic nanoparticles whereas organic particles can be used to encapsulate contrast agents (Cui et al. 2017; Fan et al. 2016; Pratt et al. 2016). Furthermore, it is possible to combine imaging, diagnostic and therapeutic function through formulation (de Lázaro and Mooney 2021; Feng et al. 2020).

Zebrafish play an important role in the development of nanomedicines as a proof-of-concept model for evaluation of drug efficacy and as a toxicity model, with the use of rapid HTS assays (Gutiérrez-Lovera et al. 2017; Sieber et al. 2019). Zebrafish lack adaptive immunity early in development (up to 3–6 weeks post fertilization) (Lam et al. 2004) therefore human and mouse cancer cell xenografts are not rejected in embryos and during early larval stages. This favors their use as cancer models. Zebrafish models of amyloidogenesis and Alzheimer's disease were also created via human islet amyloid polypeptide (IAPP) and/or amyloid beta (Aβ) injection into zebrafish embryos. Developing zebrafish are used for visualization of the cancer cell targeting ability of nanomedicines (Qin et al. 2020; Evensen et al. 2015; Kocere et al. 2020). Protocols have been developed using transgenic zebrafish embryos and larvae xenografted with human and mouse cancer cells (Qin et al. 2020). These zebrafish models improved understanding of the toxicity, circulation, distribution and accumulation of nanomedicines in vivo as well as their effects on cancer cells (Evensen et al. 2015; Kocere et al. 2020).

Zebrafish have been used to evaluate the uptake of poly(ethylene glycol)-co-poly(ε-caprolactone) (PEG-PCL) nanoparticle-encapsulated ginkgolide B, a promising drug against Parkinson's disease, and its ability to cross various biological barriers (Zhao et al. 2020). Another study assessed the distribution, toxicity and efficacy of ginkgolide B nanocrystals against Parkinson's disease (Liu et al. 2020). Other studies have used zebrafish to determine whether carbon dots which block β-secretase activity (BACE) to lower Aβ toxicity can be transported across the zebrafish embryonic blood–brain barrier (Han et al. 2017), to investigate the effectiveness of carbon quantum dots at mitigating amyloid peptide (IAPP and Aβ) aggregation (Koppel et al. 2020), to assess the ability of biomimetic nanomaterials to inhibit amyloidogenesis in vivo (Javed et al. 2019, 2018) and to characterize the effectiveness of chitosan nanoparticles conjugated with the naturally derived compound chrysin to improve

the symptoms of Alzheimer's disease (Saleem 2020). These studies demonstrate the scope of zebrafish as a tool for toxicity testing and evaluation of the efficacy of nanotechnology-based therapies against cancer and neurodegenerative diseases.

Implications and Future Prospects of Using Zebrafish for Risk Assessment of Nanomaterials and Emerging Contaminants

It is clear that nanotechnology and its applications show substantial utility and will increasingly pervade more aspects of our lives. The widespread use of nanotechnology is not without its risks. Zebrafish assays play an important part in demystifying the unknown effects of nanotechnology on the environment and human health. Integration of HTS assays and AI-assisted analysis tools on zebrafish is expected to generate large quantities of information which facilitate rapid hazard identification and enable hazard ranking for prioritization of toxicity testing for existing engineered nanomaterials and emerging contaminants (Ginsberg et al. 2019). From a regulatory context, the wealth of information gleaned would be used to guide regulation of the design, use and disposal of new engineered nanomaterials and emerging contaminants to mitigate the EHS risks.

References

Akagi J, Khoshmanesh K, Evans B et al (2012) Miniaturized embryo array for automated trapping, immobilization and microperfusion of zebrafish embryos. PLoS ONE 7(5):e36630. https://doi.org/10.1371/journal.pone.0036630

Akerberg AA, Burns CE, Burns CG et al (2019) Deep learning enables automated volumetric assessments of cardiac function in zebrafish. Dis Model Mech 12(10):dmm.040188. https://doi.org/10.1242/dmm.040188

Ankley GT, Villeneuve DL (2006) The fathead minnow in aquatic toxicology: Past, present and future. Aquat Toxicol 78(1):91–102. https://doi.org/10.1016/j.aquatox.2006.01.018

Ash GI, Kim D, Choudhury M (2019) Promises of nanotherapeutics in obesity. Trends Endocrinol Metab 30(6):369–383. https://doi.org/10.1016/j.tem.2019.04.004

Ašmonaitė G, Boyer S, de Souza KB et al (2016) Behavioural toxicity assessment of silver ions and nanoparticles on zebrafish using a locomotion profiling approach. Aquat Toxicol 173:143–153. https://doi.org/10.1016/j.aquatox.2016.01.013

Baalousha M, Cornelis G, Kuhlbusch TAJ et al (2016) Modeling nanomaterial fate and uptake in the environment: current knowledge and future trends. Environ Sci Nano 3(2):323–345. https://doi.org/10.1039/C5EN00207A

Bairoch A (2018) The cellosaurus, a cell-line knowledge resource. J Biomol Tech 29(2):25–38. https://doi.org/10.7171/jbt.18-2902-002

Ball JS, Stedman DB, Hillegass JM et al (2014) Fishing for teratogens: a consortium effort for a harmonized zebrafish developmental toxicology assay. Toxicol Sci 139(1):210–219. https://doi.org/10.1093/toxsci/kfu017

Bar-Ilan O, Louis K, Yang S et al (2011) Titanium dioxide nanoparticles produce phototoxicity in the developing zebrafish. Nanotoxicology 6:670–679. https://doi.org/10.3109/17435390.2011.604438

Basile AO, Yahi A, Tatonetti NP (2019) Artificial intelligence for drug toxicity and safety. Trends Pharmacol Sci 40(9):624–635. https://doi.org/10.1016/j.tips.2019.07.005

Baskin I (2018) Machine learning methods in computational toxicology. Methods Mol Biol 1800:119–139. https://doi.org/10.1007/978-1-4939-7899-1_5

Bäuerlein PS, Emke E, Tromp P et al (2017) Is there evidence for man-made nanoparticles in the Dutch environment? Sci Total Environ 576:273–283. https://doi.org/10.1016/j.scitotenv.2016.09.206

Bernardes RC, Lima MAP, Guedes RNC et al (2020) Ethoflow: computer vision and artificial intelligence-based software for automatic behavior analysis. bioRxiv:2020.2007.2023.218255. https://doi.org/10.1101/2020.07.23.218255

Bernardos RL, Raymond PA (2006) GFAP transgenic zebrafish. Gene Expr Patterns 6(8):1007–1013. https://doi.org/10.1016/j.modgep.2006.04.006

Binelli A, Del Giacco L, Santo N et al (2017) Carbon nanopowder acts as a Trojan-horse for benzo(α)pyrene in Danio rerio embryos. Nanotoxicology 11(3):371–381. https://doi.org/10.1080/17435390.2017.1306130

Blechinger S, Warren J, Kuwada J et al (2002) Developmental toxicology of cadmium in living embryos of a stable transgenic zebrafish line. Environ Health Perspect 110:1041–1046. https://doi.org/10.1289/ehp.021101041

Böhme S, Baccaro M, Schmidt M et al (2017) Metal uptake and distribution in the zebrafish (Danio rerio) embryo: differences between nanoparticles and metal ions. Environ Sci Nano 4(5):1005–1015. https://doi.org/10.1039/C6EN00440G

Boyd W, McBride S, Rice J et al (2010) A high-throughput method for assessing chemical toxicity using a Caenorhabditis elegans reproduction assay. Toxicol Appl Pharmacol 245:153–159. https://doi.org/10.1016/j.taap.2010.02.014

Brannen KC, Panzica-Kelly JM, Danberry TL et al (2010) Development of a zebrafish embryo teratogenicity assay and quantitative prediction model. Birth Defects Res B Dev Reprod Toxicol 89(1):66–77. https://doi.org/10.1002/bdrb.20223

Breier B, Onken A (2020) Analysis of video feature learning in two-stream CNNs on the example of zebrafish swim bout classification. arXiv:1912.09857

Breitwieser H, Dickmeis T, Vogt M et al (2018) Fully automated pipetting sorting system for different morphological phenotypes of zebrafish embryos. SLAS Technol 23(2):128–133. https://doi.org/10.1177/2472630317745780

Brun NR, Koch BEV, Varela M et al (2018) Nanoparticles induce dermal and intestinal innate immune system responses in zebrafish embryos. Environ Sci Nano 5(4):904–916. https://doi.org/10.1039/C8EN00002F

Bundschuh M, Filser J, Lüderwald S et al (2018) Nanoparticles in the environment: where do we come from, where do we go to? Environ Sci Eur 30(1):6–6. https://doi.org/10.1186/s12302-018-0132-6

Busquet F, Strecker R, Rawlings J et al (2014) OECD validation study to assess intra- and inter-laboratory reproducibility of the zebrafish embryo toxicity test for acute aquatic toxicity testing. Regul Toxicol Pharmacol 69(3):496–511. https://doi.org/10.1016/j.yrtph.2014.05.018

Carvalho R, de Sonneville J, Stockhammer OW et al (2011) A high-throughput screen for Tuberculosis progression. PLoS ONE 6(2):e16779. https://doi.org/10.1371/journal.pone.0016779

Carvan M, Sonntag D, Cmar C et al (2001) Oxidative stress in zebrafish cells: potential utility of transgenic zebrafish as a deployable sentinel for site hazard ranking. Sci Total Environ 274:183–196. https://doi.org/10.1016/S0048-9697(01)00742-2

Chakraborty C, Sharma AR, Sharma G et al (2016) Zebrafish: a complete animal model to enumerate the nanoparticle toxicity. J Nanobiotechnol 14(1):65. https://doi.org/10.1186/s12951-016-0217-6

Chandarana M, Curtis A, Hoskins C (2018) The use of nanotechnology in cardiovascular disease. Appl Nanosci 8(7):1607–1619. https://doi.org/10.1007/s13204-018-0856-z
Chen J, Guo Z, Tian H et al (2016) Production and clinical development of nanoparticles for gene delivery. Mol Ther Methods Clin Dev 3:16023. https://doi.org/10.1038/mtm.2016.23
Chen L, Guo Y, Hu C et al (2018) Dysbiosis of gut microbiota by chronic coexposure to titanium dioxide nanoparticles and bisphenol A: Implications for host health in zebrafish. Environ Pollut 234:307–317. https://doi.org/10.1016/j.envpol.2017.11.074
Chen L, Watson C, Morsch M et al (2017) Improving the delivery of SOD1 antisense oligonucleotides to motor neurons using calcium phosphate-lipid nanoparticles. Front Neurosci 11:476. https://doi.org/10.3389/fnins.2017.00476
Chen T-H, Lin C-C, Meng P-J (2013) Zinc oxide nanoparticles alter hatching and larval locomotor activity in zebrafish (Danio rerio). J Hazard Mater 277:134–140. https://doi.org/10.1016/j.jhazmat.2013.12.030
Chen T-H, Lin C-Y, Tseng M-C (2011) Behavioral effects of titanium dioxide nanoparticles on larval zebrafish (Danio rerio). Mar Pollut Bull 63(5):303–308. https://doi.org/10.1016/j.marpolbul.2011.04.017
Chiari-Andréo BG, Almeida-Cincotto MGJd, Oshiro JA et al (2019) Nanoparticles for cosmetic use and its application. In: Grumezescu AM (ed) Nanoparticles in pharmacotherapy. William Andrew Publishing, Chapter 5, p 113–146. https://doi.org/10.1016/B978-0-12-816504-1.00013-2
Choi J-S, Ha MK, Trinh TX et al (2018) Towards a generalized toxicity prediction model for oxide nanomaterials using integrated data from different sources. Sci Rep 8(1):6110. https://doi.org/10.1038/s41598-018-24483-z
Colvin VL (2003) The potential environmental impact of engineered nanomaterials. Nat Biotechnol 21(10):1166–1170. https://doi.org/10.1038/nbt875
Cornet C, Calzolari S, Miñana-Prieto R et al (2017) ZeGlobalTox: an innovative approach to address organ drug toxicity using zebrafish. Int J Mol Sci 18(4):864
Cote I, Anastas PT, Birnbaum LS et al (2012) Advancing the next generation of health risk assessment. Environ Health Perspect 120(11):1499–1502. https://doi.org/10.1289/ehp.1104870
Cui MY, Dong Z, Cai H et al (2017) Folate-targeted polymeric micelles loaded with superparamagnetic iron oxide as a contrast agent for magnetic resonance imaging of a human tongue cancer cell line. Mol Med Rep 16(5):7597–7602. https://doi.org/10.3892/mmr.2017.7565
Daetwyler S, Günther U, Modes CD et al (2019) Multi-sample SPIM image acquisition, processing and analysis of vascular growth in zebrafish. Development 146(6):dev173757. https://doi.org/10.1242/dev.173757
de Lázaro I, Mooney DJ (2021) Obstacles and opportunities in a forward vision for cancer nanomedicine. Nat Mater. https://doi.org/10.1038/s41563-021-01047-7
Della Torre C, Maggioni D, Ghilardi A et al (2018) The interactions of fullerene C_{60} and Benzo(α)pyrene influence their bioavailability and toxicity to zebrafish embryos. Environ Pollut 241:999–1008. https://doi.org/10.1016/j.envpol.2018.06.042
Dhineshbabu NR, Bose S (2018) Smart textiles coated with eco-friendly UV-blocking nanoparticles derived from natural resources. ACS Omega 3(7):7454–7465. https://doi.org/10.1021/acsomega.8b00822
Dong W, Wei L, Jingfeng Y et al (2016) Developmental toxicity from exposure to various forms of mercury compounds in medaka fish (Oryzias latipes) embryos. PeerJ 4:e2282. https://doi.org/10.7717/peerj.2282
Duan J, Hu H, Li Q et al (2016) Combined toxicity of silica nanoparticles and methylmercury on cardiovascular system in zebrafish (Danio rerio) embryos. Environ Toxicol Pharmacol 44:120–127. https://doi.org/10.1016/j.etap.2016.05.004
Duan J, Liang S, Yu Y et al (2018) Inflammation–coagulation response and thrombotic effects induced by silica nanoparticles in zebrafish embryos. Nanotoxicology 12:1–15. https://doi.org/10.1080/17435390.2018.1461267

Duan J, Yu Y, Li Y et al (2015) Low-dose exposure of silica nanoparticles induces cardiac dysfunction via neutrophil-mediated inflammation and cardiac contraction in zebrafish embryos. Nanotoxicology 10:1–11. https://doi.org/10.3109/17435390.2015.1102981

Duan J, Yu Y, Shi H et al (2013) Toxic Effects of Silica Nanoparticles on Zebrafish Embryos and Larvae. PLoS ONE 8(9):e74606. https://doi.org/10.1371/journal.pone.0074606

Ducharme NA, Peterson LE, Benfenati E et al (2013) Meta-analysis of toxicity and teratogenicity of 133 chemicals from zebrafish developmental toxicity studies. Reprod Toxicol 41:98–108. https://doi.org/10.1016/j.reprotox.2013.06.070

Ducharme NA, Reif DM, Gustafsson J-A et al (2015) Comparison of toxicity values across zebrafish early life stages and mammalian studies: Implications for chemical testing. Reprod Toxicol 55:3–10. https://doi.org/10.1016/j.reprotox.2014.09.005

Early JJ, Marshall-Phelps KLH, Williamson JM et al (2018) An automated high-resolution in vivo screen in zebrafish to identify chemical regulators of myelination. eLife 7:e35136. https://doi.org/10.7554/eLife.35136

Ellett F, Pase L, Hayman J et al (2010) Mpeg1 promoter transgenes direct macrophage-lineage expression in zebrafish. Blood 117:e49-56. https://doi.org/10.1182/blood-2010-10-314120

Esterberg R, Hailey DW, Coffin AB et al (2013) Disruption of intracellular calcium regulation is integral to aminoglycoside-induced hair cell death. J Neurosci 33(17):7513–7525. https://doi.org/10.1523/jneurosci.4559-12.2013

Evensen L, Johansen P, Koster G et al (2015) Zebrafish as a model system for characterization of nanoparticles against cancer. Nanoscale 8(2):862–877. https://doi.org/10.1039/c5nr07289a

Fadare OO, Wan B, Liu K et al (2020) Eco-Corona vs Protein Corona: effects of humic substances on corona formation and nanoplastic particle toxicity in Daphnia magna. Environ Sci Technol 54(13):8001–8009. https://doi.org/10.1021/acs.est.0c00615

Fan Y, Guo R, Shi X et al (2016) Modified nanoemulsions with iron oxide for magnetic resonance imaging. Nanomaterials 6(12):223

Feng J, Zhang C, Lischinsky JE et al (2019) A genetically encoded fluorescent sensor for rapid and specific in vivo detection of norepinephrine. Neuron 102(4):745–761. https://doi.org/10.1016/j.neuron.2019.02.037

Feng L, Gai S, He F et al (2020) Multifunctional Bismuth Ferrite Nanocatalysts with optical and magnetic functions for Ultrasound-Enhanced Tumor Theranostics. ACS Nano 14(6):7245–7258. https://doi.org/10.1021/acsnano.0c02458

Franco-Restrepo JE, Forero DA, Vargas RA (2019) A review of freely available, open-source software for the automated analysis of the behavior of adult zebrafish. Zebrafish 16(3):223–232. https://doi.org/10.1089/zeb.2018.1662

Furxhi I, Murphy F, Mullins M et al (2020) Practices and trends of machine learning application in nanotoxicology. Nanomaterials 10:116. https://doi.org/10.3390/nano10010116

Gao M, Zhang Z, Lv M et al (2018) Toxic effects of nanomaterial-adsorbed cadmium on Daphnia magna. Ecotoxicol Environ Saf 148:261–268. https://doi.org/10.1016/j.ecoenv.2017.10.038

Genest D, Puybareau E, Léonard M et al (2019) High throughput automated detection of axial malformations in medaka embryo. Comput Biol Med 105:157–168. https://doi.org/10.1016/j.compbiomed.2018.12.016

George S, Lin S, Ji Z et al (2012) Surface defects on plate-shaped silver nanoparticles contribute to its hazard potential in a fish gill cell line and zebrafish embryos. ACS Nano 6(5):3745–3759. https://doi.org/10.1021/nn204671v

Gierten J, Pylatiuk C, Hammouda OT et al (2020) Automated high-throughput heartbeat quantification in medaka and zebrafish embryos under physiological conditions. Sci Rep 10(1):2046. https://doi.org/10.1038/s41598-020-58563-w

Ginsberg GL, Fedinick KP, Solomon GM et al (2019) New toxicology tools and the emerging paradigm shift in environmental health decision-making. Environ Health Perspect 127(12):125002. https://doi.org/10.1289/EHP4745

Ginzburg AL, Truong L, Tanguay RL et al (2018) Synergistic toxicity produced by mixtures of biocompatible gold nanoparticles and widely used surfactants. ACS Nano 12(6):5312–5322. https://doi.org/10.1021/acsnano.8b00036

Goldberg MS (2019) Improving cancer immunotherapy through nanotechnology. Nat Rev Cancer 19(10):587–602. https://doi.org/10.1038/s41568-019-0186-9

Gondikas AP, Fvd K, Reed RB et al (2014) Release of TiO_2 nanoparticles from sunscreens into surface waters: a one-year survey at the old danube recreational lake. Environ Sci Technol 48(10):5415–5422. https://doi.org/10.1021/es405596y

Goud KY, Satyanarayana M, Hayat A et al (2019). Nanomaterial-based electrochemical sensors in pharmaceutical applications. In: Grumezescu AM (ed) Nanoparticles in pharmacotherapy. William Andrew Publishing, Chapter 7, p 195–216. https://doi.org/10.1016/B978-0-12-816504-1.00015-6

Graf SF, Hötzel S, Liebel U et al (2011) Image-based fluidic sorting system for automated zebrafish egg sorting into multiwell plates. J Lab Autom 16(2):105–111. https://doi.org/10.1016/j.jala.2010.11.002

Granato M, van Eeden FJ, Schach U et al (1996) Genes controlling and mediating locomotion behavior of the zebrafish embryo and larva. Development 123(1):399–413

Gu J, Guo M, Huang C et al (2021) Titanium dioxide nanoparticle affects motor behavior, neurodevelopment and axonal growth in zebrafish (Danio rerio) larvae. Sci Total Environ 754:142315. https://doi.org/10.1016/j.scitotenv.2020.142315

Guo Y, Chen L, Wu J et al (2019) Parental co-exposure to bisphenol A and nano-TiO_2 causes thyroid endocrine disruption and developmental neurotoxicity in zebrafish offspring. Sci Total Environ 650:557–565. https://doi.org/10.1016/j.scitotenv.2018.09.007

Gutiérrez-Lovera C, Vázquez-Ríos A, Guerra-Varela J et al (2017) The potential of zebrafish as a model organism for improving the translation of genetic anticancer nanomedicines. Genes 8(12):349

Hall C, Flores MV, Storm T et al (2007) The zebrafish lysozyme C promoter drives myeloid-specific expression in transgenic fish. BMC Dev Biol 7(1):42. https://doi.org/10.1186/1471-213X-7-42

Hall S, Bradley T, Moore JT et al (2009) Acute and chronic toxicity of nano-scale TiO_2 particles to freshwater fish, cladocerans, and green algae, and effects of organic and inorganic substrate on TiO_2 toxicity. Nanotoxicology 3(2):91–97. https://doi.org/10.1080/17435390902788078

Halloran MC, Sato-Maeda M, Warren JT et al (2000) Laser-induced gene expression in specific cells of transgenic zebrafish. Development 127(9):1953–1960. https://doi.org/10.1242/dev.127.9.1953

Hamm JT, Ceger P, Allen D et al (2019) Characterizing sources of variability in zebrafish embryo screening protocols. Altex 36(1):103–120. https://doi.org/10.14573/altex.1804162

Han X, Jing Z, Wu W et al (2017) Biocompatible and blood–brain barrier permeable carbon dots for inhibition of Aβ fibrillation and toxicity, and BACE1 activity. Nanoscale 9(35):12862–12866. https://doi.org/10.1039/C7NR04352J

Haque E, Ward A (2018) Zebrafish as a model to evaluate nanoparticle toxicity. Nanomaterials 8:561. https://doi.org/10.3390/nano8070561

He Y, Peng G, Jiang Y et al (2020) Environmental hazard potential of nano-photocatalysts determined by nano-bio interactions and exposure conditions. Small 16(21):1907690. https://doi.org/10.1002/smll.201907690

Henn K, Braunbeck T (2011) Dechorionation as a tool to improve the fish embryo toxicity test (FET) with the zebrafish (Danio rerio). Comp Biochem Physiol C Toxicol Pharmacol 153:91–98. https://doi.org/10.1016/j.cbpc.2010.09.003

Hillegass JM, Shukla A, Lathrop SA et al (2010) Assessing nanotoxicity in cells in vitro. Wiley Interdiscip Rev Nanomed Nanobiotechnol 2(3):219–231. https://doi.org/10.1002/wnan.54

Hossen S, Hossain MK, Basher MK et al (2019) Smart nanocarrier-based drug delivery systems for cancer therapy and toxicity studies: A review. J Adv Res 15:1–18. https://doi.org/10.1016/j.jare.2018.06.005

Howe K, Clark MD, Torroja CF et al (2013) The zebrafish reference genome sequence and its relationship to the human genome. Nature 496(7446):498–503. https://doi.org/10.1038/nature12111

Huang C-J, Tu C-T, Hsiao C-D et al (2003) Germ-line transmission of a myocardium-specific GFP transgene reveals critical regulatory elements in the cardiac myosin light chain 2 promoter of zebrafish. Dev Dyn 228(1):30–40. https://doi.org/10.1002/dvdy.10356

Iqbal J, Behl G, Walker G et al (2019) Nanoparticulate drug delivery systems for the oral administration of macromolecular drugs. In: Grumezescu AM (ed) Nanoparticles in pharmacotherapy. William Andrew Publishing, Chapter 6, p 147–193. https://doi.org/10.1016/B978-0-12-816504-1.00017-X

Irvine DJ, Dane EL (2020) Enhancing cancer immunotherapy with nanomedicine. Nat Rev Immunol 20(5):321–334. https://doi.org/10.1038/s41577-019-0269-6

Ishaq O, Sadanandan SK, Wählby C (2017) Deep fish: deep learning-based classification of zebrafish deformation for high-throughput screening. SLAS Discov 22(1):102–107. https://doi.org/10.1177/1087057116667894

Jarque S, Fetter E, Veneman WJ et al (2018) An automated screening method for detecting compounds with goitrogenic activity using transgenic zebrafish embryos. PLoS ONE 13(8):e0203087. https://doi.org/10.1371/journal.pone.0203087

Javed I, Peng G, Xing Y et al (2019) Inhibition of amyloid beta toxicity in zebrafish with a chaperone-gold nanoparticle dual strategy. Nat Comm 10(1):3780. https://doi.org/10.1038/s41467-019-11762-0

Javed I, Yu T, Peng G et al (2018) In vivo mitigation of amyloidogenesis through functional-pathogenic double-protein Coronae. Nano Lett 18(9):5797–5804. https://doi.org/10.1021/acs.nanolett.8b02446

Jeanray N, Marée R, Pruvot B et al (2015) Phenotype classification of zebrafish embryos by supervised learning. PLoS ONE 10(1):e0116989. https://doi.org/10.1371/journal.pone.0116989

Jeong J, Cho H-J, Choi M et al (2015) In vivo toxicity assessment of angiogenesis and the live distribution of nano-graphene oxide and its PEGylated derivatives using the developing zebrafish embryo. Carbon 93:431–440. https://doi.org/10.1016/j.carbon.2015.05.024

Ji Z, Wang X, Zhang H et al (2012) Designed synthesis of CeO_2 nanorods and nanowires for studying toxicological effects of high aspect ratio nanomaterials. ACS Nano 6(6):5366–5380. https://doi.org/10.1021/nn3012114

Jiang C, Castellon BT, Matson CW et al (2017) Relative contributions of copper oxide nanoparticles and dissolved copper to Cu uptake kinetics of gulf killifish (Fundulus grandis) embryos. Environ Sci Technol 51(3):1395–1404. https://doi.org/10.1021/acs.est.6b04672

Jie C, Ichihara G, Shimada Y et al (2015). Copper oxide nanoparticles reduce vasculogenesis in transgenic zebrafish through down-regulation of vascular endothelial growth factor expression and induction of apoptosis. J Nanosci Nanotechnol 15. https://doi.org/10.1166/jnn.2015.9762

Jing M, Zhang P, Wang G et al (2018) A genetically encoded fluorescent acetylcholine indicator for in vitro and in vivo studies. Nat Biotechnol 36(8):726–737. https://doi.org/10.1038/nbt.4184

Juanola-Feliu E, Miribel-Català PL, Páez Avilés C et al (2014) Design of a customized multipurpose nano-enabled implantable system for in-vivo theranostics. Sensors (basel) 14(10):19275–19306. https://doi.org/10.3390/s141019275

Jung S-K, Qu X, Aleman-Meza B et al (2015) Multi-endpoint, high-throughput study of nanomaterial toxicity in Caenorhabditis elegans. Environ Sci Technol 49(4):2477–2485. https://doi.org/10.1021/es5056462

Jurewicz A, Ilyas S, Uppal JK et al (2020) Evaluation of magnetite nanoparticle-based toxicity on embryo-larvae stages of zebrafish (Danio rerio). ACS Appl Nano Mater 3(2):1621–1629. https://doi.org/10.1021/acsanm.9b02330

Kailasa SK, Park T-J, Rohit JV et al (2019) Antimicrobial activity of silver nanoparticles. In: Grumezescu AM (ed) Nanoparticles in pharmacotherapy. William Andrew Publishing, Chapter 14, p 461–484. https://doi.org/10.1016/B978-0-12-816504-1.00009-0

Kalueff AV, Gebhardt M, Stewart AM et al (2013). Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond. Zebrafish 10(1):70–86. https://doi.org/10.1089/zeb.2012.0861

Kang C-P, Tu H-C, Fu T-F et al (2018) An automatic method to calculate heart rate from zebrafish larval cardiac videos. BMC Bioinformatics 19(1):169. https://doi.org/10.1186/s12859-018-2166-6

Keller AA, McFerran S, Lazareva A et al (2013) Global life cycle releases of engineered nanomaterials. J Nanopart Res 15(6):1692. https://doi.org/10.1007/s11051-013-1692-4

Kim J, Kim K-T, Lee B-G et al (2013) Developmental toxicity of Japanese medaka embryos by silver nanoparticles and released ions in the presence of humic acid. Ecotoxicol Environ Saf 92:57–63. https://doi.org/10.1016/j.ecoenv.2013.02.004

Kim J, Koo B-K, Knoblich JA (2020) Human organoids: model systems for human biology and medicine. Nat Rev Mol Cell Biol 21(10):571–584. https://doi.org/10.1038/s41580-020-0259-3

Kim JH, Lee SH, Cha YJ et al (2017). C. elegans-on-a-chip for in situ and in vivo Ag nanoparticles' uptake and toxicity assay. Sci Rep 7(1):40225. https://doi.org/10.1038/srep40225

Kim K-T, Tanguay RL (2014) The role of chorion on toxicity of silver nanoparticles in the embryonic zebrafish assay. Environ Anal Health Toxicol 29:e2014021–e2014020. https://doi.org/10.5620/eht.e2014021

Kirby BB, Takada N, Latimer AJ et al (2006) In vivo time-lapse imaging shows dynamic oligodendrocyte progenitor behavior during zebrafish development. Nat Neurosci 9(12):1506–1511. https://doi.org/10.1038/nn1803

Kirtane AR, Verma M, Karandikar P et al (2021) Nanotechnology approaches for global infectious diseases. Nat Nanotechnol 16(4):369–384. https://doi.org/10.1038/s41565-021-00866-8

Klüver N, Yang L, Busch W et al (2011) Transcriptional response of zebrafish embryos exposed to neurotoxic compounds reveals a muscle activity dependent hspb11 expression. PLoS ONE 6(12):e29063. https://doi.org/10.1371/journal.pone.0029063

Kocere A, Resseguier J, Wohlmann J et al (2020) Real-time imaging of polymersome nanoparticles in zebrafish embryos engrafted with melanoma cancer cells: localization, toxicity and treatment analysis. EBioMedicine 58:102902. https://doi.org/10.1016/j.ebiom.2020.102902

Koiwa J, Shiromizu T, Adachi Y et al (2019) Generation of a triple-transgenic zebrafish line for assessment of developmental neurotoxicity during neuronal differentiation. Pharmaceuticals 12:145. https://doi.org/10.3390/ph12040145

Kokel D, Bryan J, Laggner C et al (2010) Rapid behavior-based identification of neuroactive small molecules in the zebrafish. Nat Chem Biol 6(3):231–237. https://doi.org/10.1038/nchembio.307

Koppel K, Tang H, Javed I et al (2020) Elevated amyloidoses of human IAPP and amyloid beta by lipopolysaccharide and their mitigation by carbon quantum dots. Nanoscale 12(23):12317–12328. https://doi.org/10.1039/D0NR02710C

Kotb S, Detappe A, Lux F et al (2016) Gadolinium-based nanoparticles and radiation therapy for multiple brain melanoma metastases: proof of concept before phase I trial. Theranostics 6(3):418–427. https://doi.org/10.7150/thno.14018

Kroll A, Dierker C, Rommel C et al (2011) Cytotoxicity screening of 23 engineered nanomaterials using a test matrix of ten cell lines and three different assays. Part Fibre Toxicol 8(1):9. https://doi.org/10.1186/1743-8977-8-9

Kteeba S, Eladawi H, El-Rayis O et al (2017) Zinc oxide nanoparticle toxicity in embryonic zebrafish: mitigation with different natural organic matter. Environ Pollut 230:1125–1140. https://doi.org/10.1016/j.envpol.2017.07.042

Kurita Oyamada H, Brown C, Kroll K et al (2020) Toxicity assessment of a novel oil dispersant based on silica nanoparticles using Fathead minnow. Aquat Toxicol 229:105653. https://doi.org/10.1016/j.aquatox.2020.105653

Kyzar E, Stewart AM, Landsman S et al (2013) Behavioral effects of bidirectional modulators of brain monoamines reserpine and d-amphetamine in zebrafish. Brain Res 1527:108–116. https://doi.org/10.1016/j.brainres.2013.06.033

Laban G, Nies LF, Turco RF et al (2010) The effects of silver nanoparticles on fathead minnow (Pimephales promelas) embryos. Ecotoxicology 19(1):185–195. https://doi.org/10.1007/s10646-009-0404-4

Lam S, Chua HL, Gong Z et al (2004) Development and maturation of the immune system in zebrafish, Danio rerio: a gene expression profiling, in situ hybridization and immunological study. Dev Comp Immunol 28:9–28. https://doi.org/10.1016/S0145-305X(03)00103-4

Lammer E, Carr GJ, Wendler K et al (2009) Is the fish embryo toxicity test (FET) with the zebrafish (Danio rerio) a potential alternative for the fish acute toxicity test? Comp Biochem Physiol C Toxicol Pharmacol 149(2):196–209. https://doi.org/10.1016/j.cbpc.2008.11.006

Lanone S, Rogerieux F, Geys J et al (2009) Comparative toxicity of 24 manufactured nanoparticles in human alveolar epithelial and macrophage cell lines. Part Fibre Toxicol 6(1):14. https://doi.org/10.1186/1743-8977-6-14

Lantz-McPeak S, Guo X, Cuevas E et al (2014) Developmental toxicity assay using high content screening of zebrafish embryos. J Appl Toxicol 35(3):261–272. https://doi.org/10.1002/jat.3029

Lee H-C, Lu P-N, Huang H-L et al (2014) Zebrafish transgenic line huORFZ is an effective living bioindicator for detecting environmental toxicants. PLoS ONE 9(3):e90160. https://doi.org/10.1371/journal.pone.0090160

Lee KJ, Nallathamby PD, Browning LM et al (2007) In vivo imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos. ACS Nano 1(2):133–143. https://doi.org/10.1021/nn700048y

Lee O, Green JM, Tyler CR (2015) Transgenic fish systems and their application in ecotoxicology. Crit Rev Toxicol 45(2):124–141. https://doi.org/10.3109/10408444.2014.965805

Lekamge S, Miranda AF, Ball AS et al (2019) The toxicity of coated silver nanoparticles to Daphnia carinata and trophic transfer from alga Raphidocelis subcapitata. PLoS ONE 14(4):e0214398. https://doi.org/10.1371/journal.pone.0214398

Lessman CA, Taylor MR, Orisme W et al (2010) Use of flatbed transparency scanners in zebrafish research: versatile and economical adjuncts to traditional imaging tools for the danio rerio laboratory. In: Detrich HW, Westerfield M, Zon LI (eds) Methods in cell biology, vol 100. Academic Press, Chapter 12, p 295–322. https://doi.org/10.1016/B978-0-12-384892-5.00012-8

Letamendia A, Quevedo C, Ibarbia I et al (2012) Development and validation of an automated high-throughput system for zebrafish in vivo screenings. PLoS ONE 7(5):e36690. https://doi.org/10.1371/journal.pone.0036690

Li L, Yan B, Shi Y-Q et al (2012) Live imaging reveals differing roles of macrophages and neutrophils during zebrafish tail fin regeneration. J Biol Chem 287(30):25353–25360. https://doi.org/10.1074/jbc.M112.349126

Li M, Liu W, Slaveykova VI (2020) Effects of mixtures of engineered nanoparticles and metallic pollutants on aquatic organisms. Environments 7(4):27

Li M, Wu Q, Wang Q et al (2018) Effect of titanium dioxide nanoparticles on the bioavailability and neurotoxicity of cypermethrin in zebrafish larvae. Aquat Toxicol 199:212–219. https://doi.org/10.1016/j.aquatox.2018.03.022

Li X, Ji X, Wang R et al (2019) Zebrafish behavioral phenomics employed for characterizing behavioral neurotoxicity caused by silica nanoparticles. Chemosphere 240:124937. https://doi.org/10.1016/j.chemosphere.2019.124937

Li X, Liu B, Li X-L et al (2014) SiO_2 nanoparticles change colour preference and cause Parkinson's-like behaviour in zebrafish. Sci Rep 4(1):3810. https://doi.org/10.1038/srep03810

Lin S, Wang X, Ji Z et al (2014) Aspect ratio plays a role in the hazard potential of CeO_2 nanoparticles in mouse lung and zebrafish gastrointestinal tract. ACS Nano 8(5):4450–4464. https://doi.org/10.1021/nn5012754

Lin S, Zhao Y, Ji Z et al (2013b) Zebrafish high-throughput screening to study the impact of dissolvable metal oxide nanoparticles on the hatching Enzyme, ZHE1. Small 9(9–10):1776–1785. https://doi.org/10.1002/smll.201202128

Lin S, Zhao Y, Nel AE et al (2013a) Zebrafish: an in vivo model for nano EHS studies. Small 9(9–10):1608–1618. https://doi.org/10.1002/smll.201202115

Lin S, Zhao Y, Xia T et al (2011) High content screening in zebrafish speeds up hazard ranking of transition metal oxide nanoparticles. ACS Nano 5(9):7284–7295. https://doi.org/10.1021/nn202116p

Liu L, Yan Y, Wang J et al (2016) Generation of mt:egfp transgenic zebrafish biosensor for the detection of aquatic zinc and cadmium. Environ Toxicol Chem 35(8):2066–2073. https://doi.org/10.1002/etc.3362

Liu P, Wang S, Chang Z et al (2021) Combined toxicity of silica nanoparticles and cadmium chloride on the cardiovascular system of zebrafish (Danio rerio) larvae. Comp Biochem Physiol C Toxicol Pharmacol 239:108895. https://doi.org/10.1016/j.cbpc.2020.108895

Liu R, Lin S, Rallo R et al (2012) Automated phenotype recognition for zebrafish embryo based in vivo high throughput toxicity screening of engineered nano-materials. PLoS ONE 7(4):e35014. https://doi.org/10.1371/journal.pone.0035014

Liu Y, Chen C (2016) Role of nanotechnology in HIV/AIDS vaccine development. Adv Drug Deliv Rev 103:76–89. https://doi.org/10.1016/j.addr.2016.02.010

Liu Y, Liu W, Xiong S et al (2020) Highly stabilized nanocrystals delivering Ginkgolide B in protecting against the Parkinson's disease. Int J Pharm 577:119053. https://doi.org/10.1016/j.ijpharm.2020.119053

Liu Z, Chen K, Davis C et al (2008) Drug delivery with carbon nanotubes for in vivo cancer treatment. Cancer Res 68(16):6652. https://doi.org/10.1158/0008-5472.CAN-08-1468

Lopes G, Bonacchi N, Frazão J et al (2015) Bonsai: an event-based framework for processing and controlling data streams. Front Neuroinform 9:7. https://doi.org/10.3389/fninf.2015.00007

Luo X, Xu S, Yang Y et al (2016) Insights into the ecotoxicity of silver nanoparticles transferred from Escherichia coli to Caenorhabditis elegans. Sci Rep 6(1):36465. https://doi.org/10.1038/srep36465

Ma Z, Wan H, Wang W et al (2019) A theranostic agent for cancer therapy and imaging in the second near-infrared window. Nano Res 12(2):273–279. https://doi.org/10.1007/s12274-018-2210-x

Mandrell D, Truong L, Jephson C et al (2012) Automated zebrafish chorion removal and single embryo placement: optimizing throughput of zebrafish developmental toxicity screens. J Lab Autom 17(1):66–74. https://doi.org/10.1177/2211068211432197

Mao L, Chen Z, Wang Y et al (2021) Design and application of nanoparticles as vaccine adjuvants against human corona virus infection. J Inorg Biochem 219:111454. https://doi.org/10.1016/j.jinorgbio.2021.111454

Martin WK, Tennant AH, Conolly RB et al (2019) High-throughput video processing of heart rate responses in multiple wild-type embryonic zebrafish per imaging field. Sci Rep 9(1):145. https://doi.org/10.1038/s41598-018-35949-5

Maynard AD, Aitken RJ (2016) "Safe handling of nanotechnology" ten years on. Nat Nanotechnol 11(12):998–1000. https://doi.org/10.1038/nnano.2016.270

Maynard AD, Aitken RJ, Butz T et al (2006) Safe handling of nanotechnology. Nature 444(7117):267–269. https://doi.org/10.1038/444267a

Mayr A, Klambauer G, Unterthiner T et al (2016) DeepTox: toxicity prediction using deep learning. Front Environ Sci 3:80. https://doi.org/10.3389/fenvs.2015.00080

McNeil PL, Boyle D, Henry TB et al (2014) Effects of metal nanoparticles on the lateral line system and behaviour in early life stages of zebrafish (Danio rerio). Aquat Toxicol 152:318–323. https://doi.org/10.1016/j.aquatox.2014.04.022

Meng H, Leong W, Leong KW et al (2018) Walking the line: The fate of nanomaterials at biological barriers. Biomaterials 174:41–53. https://doi.org/10.1016/j.biomaterials.2018.04.056

Mosselhy DA, He W, Li D et al (2016) Silver nanoparticles: in vivo toxicity in zebrafish embryos and a comparison to silver nitrate. J Nanopart Res 18(8):222. https://doi.org/10.1007/s11051-016-3514-y

Naasz S, Altenburger R, Kühnel D (2018) Environmental mixtures of nanomaterials and chemicals: The Trojan-horse phenomenon and its relevance for ecotoxicity. Sci Total Environ 635:1170–1181. https://doi.org/10.1016/j.scitotenv.2018.04.180

Nath T, Mathis A, Chen AC et al (2019) Using DeepLabCut for 3D markerless pose estimation across species and behaviors. Nat Protoc 14(7):2152–2176. https://doi.org/10.1038/s41596-019-0176-0

Nava-Arzaluz MG, Piñón-Segundo E, Ganem-Rondero A (2019) Lipid nanocarriers as skin drug delivery systems. In: Grumezescu AM (ed) Nanoparticles in pharmacotherapy. William Andrew Publishing, Chapter 11, p 311–390. https://doi.org/10.1016/B978-0-12-816504-1.00007-7

Nel A, Xia T, Mädler L et al (2006) Toxic potential of materials at the nanolevel. Science 311:622–627. https://doi.org/10.1126/science.1114397

Neukum A, Bartschat A, Breitwieser H et al (2019) Automated classification of fertilized zebrafish embryos. Zebrafish 16(3):326–328. https://doi.org/10.1089/zeb.2019.1728

Nguyen-Chi M, Phan QT, Gonzalez C et al (2014) Transient infection of the zebrafish notochord with E. coli induces chronic inflammation. Dis Model Mech 7(7):871–882. https://doi.org/10.1242/dmm.014498

Oberdörster G, Oberdörster E, Oberdörster J (2005) Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ Health Perspect 113:823–839. https://doi.org/10.1289/ehp.7339

Oliveira EMN, Selli GI, von Schmude A et al (2020) Developmental toxicity of iron oxide nanoparticles with different coatings in zebrafish larvae. J Nanopart Res 22(4):87. https://doi.org/10.1007/s11051-020-04800-2

Orger MB, Polavieja GGd (2017) Zebrafish behavior: opportunities and challenges. Annu Rev Neurosci 40(1):125–147. https://doi.org/10.1146/annurev-neuro-071714-033857

Osborne OJ, Lin S, Chang CH et al (2015) Organ-specific and size-dependent Ag nanoparticle toxicity in gills and intestines of adult zebrafish. ACS Nano 9(10):9573–9584. https://doi.org/10.1021/acsnano.5b04583

Osborne OJ, Lin S, Jiang W et al (2017) Differential effect of micron- versus nanoscale III–V particulates and ionic species on the zebrafish gut. Environ Sci Nano 4(6):1350–1364. https://doi.org/10.1039/C6EN00675B

Padilla S (2014) Biomarkers of toxicity in zebrafish. In: Gupta RC (ed) Biomarkers in toxicology, 1st edn. Academic Press, USA, p 103–112. doi:https://doi.org/10.1016/B978-0-12-404630-6.00005-1

Padilla S, Cowden J, Hinton D et al (2009). Use of medaka in toxicity testing. Curr Protoc Toxicol, Chapter 1:Unit1.10. https://doi.org/10.1002/0471140856.tx0110s39

Pan Y, Leifert A, Graf M et al (2013) High-sensitivity real-time analysis of nanoparticle toxicity in green fluorescent protein-expressing zebrafish. Small 9(6):863–869. https://doi.org/10.1002/smll.201201173

Panzica-Kelly JM, Zhang CX, Augustine-Rauch KA (2015) Optimization and performance assessment of the chorion-off [dechorinated] zebrafish developmental toxicity assay. Toxicol Sci 146(1):127–134. https://doi.org/10.1093/toxsci/kfv076

Panzica-Kelly JM, Zhang CX, Danberry TL et al (2010) Morphological score assignment guidelines for the dechorionated zebrafish teratogenicity assay. Birth Defects Res B Dev Reprod Toxicol 89(5):382–395. https://doi.org/10.1002/bdrb.20260

Pardo-Martin C, Chang T-Y, Koo BK et al (2010) High-throughput in vivo vertebrate screening. Nat Methods 7(8):634–636. https://doi.org/10.1038/nmeth.1481

Pawar N, Gireesh-Babu P, Sabnis S et al (2016) Development of a fluorescent transgenic zebrafish biosensor for sensing aquatic heavy metal pollution. Transgenic Res 25(5):617–627. https://doi.org/10.1007/s11248-016-9959-z

Pelaz B, Alexiou C, Alvarez-Puebla RA et al (2017) Diverse applications of nanomedicine. ACS Nano 11(3):2313–2381. https://doi.org/10.1021/acsnano.6b06040

Peng G, He Y, Wang X et al (2020) Redox activity and nano-bio interactions determine the skin injury potential of Co_3O_4-based metal oxide nanoparticles toward zebrafish. ACS Nano 14(4):4166–4177. https://doi.org/10.1021/acsnano.9b08938

Peng G, He Y, Zhao M et al (2018) Differential effects of metal oxide nanoparticles on zebrafish embryos and developing larvae. Environ Sci Nano 5(5):1200–1207. https://doi.org/10.1039/C8EN00190A

Pennington ZT, Dong Z, Feng Y et al (2019) ezTrack: an open-source video analysis pipeline for the investigation of animal behavior. Sci Rep 9(1):19979. https://doi.org/10.1038/s41598-019-56408-9

Peravali R, Gehrig J, Giselbrecht S et al (2011) Automated feature detection and imaging for high-resolution screening of zebrafish embryos. Biotechniques 50(5):319–324. https://doi.org/10.2144/000113669

Pereira AC, Gomes T, Ferreira Machado MR et al (2019) The zebrafish embryotoxicity test (ZET) for nanotoxicity assessment: from morphological to molecular approach. Environ Pollut 252:1841–1853. https://doi.org/10.1016/j.envpol.2019.06.100

Pereira AC, Gonçalves BB, Brito RdS et al (2020) Comparative developmental toxicity of iron oxide nanoparticles and ferric chloride to zebrafish (Danio rerio) after static and semi-static exposure. Chemosphere 254:126792. https://doi.org/10.1016/j.chemosphere.2020.126792

Pérez-Escudero A, Vicente-Page J, Hinz R et al (2014) IdTracker: Tracking individuals in a group by automatic identification of unmarked animals. Nat Methods 11:743–748. https://doi.org/10.1038/nmeth.2994

Petersen E, Nelson B (2010) Mechanisms and measurements of nanomaterial-induced oxidative damage to DNA. Anal Bioanal Chem 398:613–650. https://doi.org/10.1007/s00216-010-3881-7

Pfriem A, Pylatiuk C, Alshut R et al (2012) A modular, low-cost robot for zebrafish handling. Annu Int Conf IEEE Eng Med Biol Soc 2012:980–983. https://doi.org/10.1109/embc.2012.6346097

Pham D-H, De Roo B, Nguyen X-B et al (2016) Use of zebrafish larvae as a multi-endpoint platform to characterize the toxicity profile of silica nanoparticles. Sci Rep 6(1):37145. https://doi.org/10.1038/srep37145

Poon KL, Wang X, Lee SGP et al (2017) Editor's highlight: transgenic zebrafish reporter lines as alternative in vivo organ toxicity models. Toxicol Sci 156(1):133–148. https://doi.org/10.1093/toxsci/kfw250

Powers C, Slotkin T, Seidler F et al (2011) Silver nanoparticles alter zebrafish development and larval behavior: distinct roles for particle size, coating and composition. Neurotoxicol Teratol 33:708–714. https://doi.org/10.1016/j.ntt.2011.02.002

Pratt EC, Shaffer TM, Grimm J (2016) Nanoparticles and radiotracers: advances toward radionanomedicine. Wiley Interdiscip Rev Nanomed Nanobiotechnol 8(6):872–890. https://doi.org/10.1002/wnan.1402

Pulak R (2006) Techniques for analysis, sorting, and dispensing of C. elegans on the COPAS™ flow-sorting system. In: Strange K (ed) C. elegans: Methods and applications. Humana Press, Totowa, NJ, USA, p 275–286. https://doi.org/10.1385/1-59745-151-7:275

Qin X, Laroche FFJ, Peerzade SAMA et al (2020) In vivo targeting of xenografted human cancer cells with functionalized fluorescent silica nanoparticles in zebrafish. J vis Exp 159:e61187. https://doi.org/10.3791/61187

Quintanilla M, García I, de Lázaro I et al (2019) Thermal monitoring during photothermia: hybrid probes for simultaneous plasmonic heating and near-infrared optical nanothermometry. Theranostics 9(24):7298–7312. https://doi.org/10.7150/thno.38091

Re F, Gregori M, Masserini M (2012) Nanotechnology for neurodegenerative disorders. Maturitas 73(1):45–51. https://doi.org/10.1016/j.maturitas.2011.12.015

Rehberger K, Kropf C, Segner H (2018) In vitro or not in vitro: a short journey through a long history. Environ Sci Eur 30(1):23. https://doi.org/10.1186/s12302-018-0151-3

Renner H, Grabos M, Becker KJ et al (2020). A fully automated high-throughput workflow for 3D-based chemical screening in human midbrain organoids. eLife 9:e52904. https://doi.org/10.7554/eLife.52904

Renshaw S, Loynes C, Trushell D et al (2007) A transgenic zebrafish model of neutrophilic inflammation. Blood 108:3976–3978. https://doi.org/10.1182/blood-2006-05-024075

Rihel J, Prober DA, Arvanites A et al (2010) Zebrafish behavioral profiling links drugs to biological targets and rest/wake regulation. Science 327(5963):348–351. https://doi.org/10.1126/science.1183090

Robinson JT, Tabakman SM, Liang Y et al (2011) Ultrasmall reduced graphene oxide with high near-infrared absorbance for photothermal therapy. J Am Chem Soc 133(17):6825–6831. https://doi.org/10.1021/ja2010175

Saari TW, Schroeder AL, Ankley GT et al (2017) First-generation annotations for the fathead minnow (Pimephales promelas) genome. Environ Toxicol Chem 36(12):3436–3442. https://doi.org/10.1002/etc.3929

Saini B, Srivastava S (2018) Nanotoxicity prediction using computational modelling—review and future directions. IOP Conference Series: Materials Science and Engineering 348:012005. https://doi.org/10.1088/1757-899X/348/1/012005

Saleem S (2020) Chitosan nanoparticles alleviate Alzheimer's disease in zebrafish by adopting a dual strategy. Alzheimers Dement 16(S9):e037238. https://doi.org/10.1002/alz.037238

Santhakumar K, Judson EC, Elks PM et al (2012) A zebrafish model to study and therapeutically manipulate hypoxia signaling in tumorigenesis. Cancer Res 72(16):4017–4027. https://doi.org/10.1158/0008-5472.Can-11-3148

Sarasamma S, Audira G, Juniardi S et al (2018) Evaluation of the effects of Carbon 60 nanoparticle exposure to adult zebrafish: a behavioral and biochemical approach to elucidate the mechanism of toxicity. Int J Mol Sci 19(12):3853. https://doi.org/10.3390/ijms19123853

Sarasamma S, Audira G, Samikannu P et al (2019) Behavioral impairments and oxidative stress in the brain, muscle, and gill caused by chronic exposure of C_{70} nanoparticles on adult zebrafish. Int J Mol Sci 20:5795. https://doi.org/10.3390/ijms20225795

Schutera M, Dickmeis T, Mione M et al (2016) Automated phenotype pattern recognition of zebrafish for high-throughput screening. Bioengineered 7(4):261–265. https://doi.org/10.1080/21655979.2016.1197710

Selderslaghs I, Hooyberghs J, Blust R et al (2013) Assessment of the developmental neurotoxicity of compounds by measuring locomotor activity in zebrafish embryos and larvae. Neurotoxicol Teratol 37:44–56. https://doi.org/10.1016/j.ntt.2013.01.003

Semmelhack JL, Donovan JC, Thiele TR et al (2014). A dedicated visual pathway for prey detection in larval zebrafish. eLife 3:e04878. https://doi.org/10.7554/eLife.04878

Shahid M, Takamiya M, Stegmaier J et al (2016) Zebrafish biosensor for toxicant induced muscle hyperactivity. Sci Rep 6(1):23768. https://doi.org/10.1038/srep23768

Shang S, Lin S, Cong F (2020) Zebrafish larvae phenotype classification from bright-field microscopic images using a two-tier deep-learning pipeline. Appl Sci 10(4):1247

Shang S, Long L, Lin S et al (2019) Automatic zebrafish egg phenotype recognition from bright-field microscopic images using deep convolutional neural network. Appl Sci 9(16):3362

Shariati F, Poordeljoo T, Zanjanchi P (2020) The acute toxicity of SiO_2 and Fe_3O_4 nano-particles on Daphnia magna. SILICON 12(12):2941–2946. https://doi.org/10.1007/s12633-020-00393-6

Sharif F, Porta F, Meijer AH et al (2012) Mesoporous silica nanoparticles as a compound delivery system in zebrafish embryos. Int J Nanomedicine 7:1875–1890. https://doi.org/10.2147/ijn.S26547

Shin J, Park H-C, Topczewska JM et al (2003) Neural cell fate analysis in zebrafish using olig2 BAC transgenics. Methods Cell Biol 25(1):7–14. https://doi.org/10.1023/B:MICS.0000006847.09037.3a

Sieber S, Grossen P, Bussmann J et al (2019) Zebrafish as a preclinical in vivo screening model for nanomedicines. Adv Drug Deliv Rev 151–152:152–168. https://doi.org/10.1016/j.addr.2019.01.001

Spaink HP, Cui C, Wiweger MI et al (2013) Robotic injection of zebrafish embryos for high-throughput screening in disease models. Methods 62(3):246–254. https://doi.org/10.1016/j.ymeth.2013.06.002

Sridhar VH, Roche DG, Gingins S (2019) Tracktor: image-based automated tracking of animal movement and behaviour. Methods Ecol Evol 10(6):815–820. https://doi.org/10.1111/2041-210X.13166

Strähle U, Scholz S, Geisler R et al (2012) Zebrafish embryos as an alternative to animal experiments—a commentary on the definition of the onset of protected life stages in animal welfare regulations. Reprod Toxicol 33(2):128–132. https://doi.org/10.1016/j.reprotox.2011.06.121

Sun F, Zeng J, Jing M et al (2018) A genetically encoded fluorescent sensor enables rapid and specific detection of dopamine in flies, fish, and mice. Cell 174(2):481–496. https://doi.org/10.1016/j.cell.2018.06.042

Teixidó E, Kießling TR, Krupp E et al (2018) Automated morphological feature assessment for zebrafish embryo developmental toxicity screens. Toxicol Sci 167(2):438–449. https://doi.org/10.1093/toxsci/kfy250

Temizer I, Donovan JC, Baier H et al (2015) A visual pathway for looming-evoked escape in larval zebrafish. Curr Biol 25(14):1823–1834. https://doi.org/10.1016/j.cub.2015.06.002

Tharwat A, Gaber T, Fouad MM et al (2015) Towards an automated zebrafish-based toxicity test model using machine learning. Procedia Comput Sci 65:643–651. https://doi.org/10.1016/j.procs.2015.09.008

Thit A, Skjolding LM, Selck H et al (2017) Effects of copper oxide nanoparticles and copper ions to zebrafish (Danio rerio) cells, embryos and fry. Toxicol in Vitro 45(Pt 1):89–100. https://doi.org/10.1016/j.tiv.2017.08.010

Ton C, Parng C (2005) The use of zebrafish for assessing ototoxic and otoprotective agents. Hear Res 208(1):79–88. https://doi.org/10.1016/j.heares.2005.05.005

Torraca V, Masud S, Spaink HP et al (2014) Macrophage-pathogen interactions in infectious diseases: new therapeutic insights from the zebrafish host model. Dis Model Mech 7(7):785–797. https://doi.org/10.1242/dmm.015594

Trevisan R, Uzochukwu D, Di Giulio RT (2020) PAH sorption to nanoplastics and the Trojan Horse effect as drivers of mitochondrial toxicity and PAH localization in zebrafish. Front Environ Sci 8:78. https://doi.org/10.3389/fenvs.2020.00078

Trevisan R, Voy C, Chen S et al (2019) Nanoplastics decrease the toxicity of a complex PAH mixture but impair mitochondrial energy production in developing zebrafish. Environ Sci Technol 53(14):8405–8415. https://doi.org/10.1021/acs.est.9b02003

Truong L, Bugel SM, Chlebowski A et al (2016) Optimizing multi-dimensional high throughput screening using zebrafish. Reprod Toxicol 65:139–147. https://doi.org/10.1016/j.reprotox.2016.05.015

Truong L, Saili KS, Miller JM et al (2012) Persistent adult zebrafish behavioral deficits results from acute embryonic exposure to gold nanoparticles. Comp Biochem Physiol C Toxicol 155(2):269–274. https://doi.org/10.1016/j.cbpc.2011.09.006

Tsai M-H, Chao H-R, Jiang J-J et al (2021) Toxicity of low-dose graphene oxide nanoparticles in an in-vivo wild type of Caenorhabditis elegans model. Aerosol Air Qual Res 21(5):200559. https://doi.org/10.4209/aaqr.200559

Usenko C, Harper S, Tanguay R (2007) In vivo evaluation of carbon fullerene toxicity using embryonic zebrafish. Carbon 45:1891–1898. https://doi.org/10.1016/j.carbon.2007.04.021

van Aerle R, Lange A, Moorhouse A et al (2013) Molecular mechanisms of toxicity of silver nanoparticles in zebrafish embryos. Environ Sci Technol 47(14):8005–8014. https://doi.org/10.1021/es401758d

van Pomeren M, Brun NR, Peijnenburg WJGM et al (2017) Exploring uptake and biodistribution of polystyrene (nano)particles in zebrafish embryos at different developmental stages. Aquat Toxicol 190:40–45. https://doi.org/10.1016/j.aquatox.2017.06.017

Ventola CL (2012) The nanomedicine revolution: part 2: current and future clinical applications. P T 37(10):582–591

Vogt A, Cholewinski A, Shen X et al (2009) Automated image-based phenotypic analysis in zebrafish embryos. Dev Dyn 238(3):656–663. https://doi.org/10.1002/dvdy.21892

von Mikecz A (2018) Lifetime eco-nanotoxicology in an adult organism: where and when is the invertebrate C. elegans vulnerable? Environ Sci Nano 5(3):616–622. https://doi.org/10.1039/C7EN01061C

Wan J, Peng W, Li X et al (2021) A genetically encoded sensor for measuring serotonin dynamics. Nat Neurosci 24:746–752. https://doi.org/10.1038/s41593-021-00823-7

Wang Q, Chen Q, Zhou P et al (2014) Bioconcentration and metabolism of BDE-209 in the presence of titanium dioxide nanoparticles and impact on the thyroid endocrine system and neuronal development in zebrafish larvae. Nanotoxicology 8(sup1):196–207. https://doi.org/10.3109/17435390.2013.875232

Wang W, Liu X, Gelinas D et al (2007) A fully automated robotic system for microinjection of zebrafish embryos. PLoS ONE 2(9):e862. https://doi.org/10.1371/journal.pone.0000862

Wang WH, Liu XY, Sun Y (2009) High-throughput automated injection of individual biological cells. IEEE Trans Autom Sci Eng 6(2):209–219. https://doi.org/10.1109/TASE.2008.917136

Wehmas LC, Anders C, Chess J et al (2015) Comparative metal oxide nanoparticle toxicity using embryonic zebrafish. Toxicol Rep 2:702–715. https://doi.org/10.1016/j.toxrep.2015.03.015

Wei J, Liu J, Liang S et al (2020) Low-dose exposure of silica nanoparticles induces neurotoxicity via neuroactive ligand-receptor interaction signaling pathway in zebrafish embryos. Int J Nanomed 15:4407–4415. https://doi.org/10.2147/IJN.S254480

Wen L, Wei W, Gu W et al (2008) Visualization of monoaminergic neurons and neurotoxicity of MPTP in live transgenic zebrafish. Dev Biol 314:84–92. https://doi.org/10.1016/j.ydbio.2007.11.012

White R, Sessa A, Burke C et al (2008) Transparent adult zebrafish as a tool for in vivo transplantation analysis. Cell Stem Cell 2:183–189. https://doi.org/10.1016/j.stem.2007.11.002

Wimmer A, Markus AA, Schuster M (2019) Silver nanoparticle levels in river water: real environmental measurements and modeling approaches—a comparative study. Environ Sci Technol Lett 6(6):353–358. https://doi.org/10.1021/acs.estlett.9b00211

Winkler DA (2020) Role of artificial intelligence and machine learning in nanosafety. Small 16(36):2001883. https://doi.org/10.1002/smll.202001883

Wu Y, Wang G (2018) Machine learning based toxicity prediction: from chemical structural description to transcriptome analysis. Int J Mol Sci 19:2358. https://doi.org/10.3390/ijms19082358

Xi Y, Yu M, Godoy R et al (2011) Transgenic zebrafish expressing green fluorescent protein in dopaminergic neurons of the ventral diencephalon. Dev Dyn 240(11):2539–2547. https://doi.org/10.1002/dvdy.22742

Xia C, Fu L, Liu Z et al (2018) Aquatic toxic analysis by monitoring fish behavior using computer vision: a recent progress. J Toxicol 2018:2591924. https://doi.org/10.1155/2018/2591924

Xia T, Zhao Y, Sager T et al (2011) Decreased dissolution of ZnO by iron doping yields nanoparticles with reduced toxicity in the rodent lung and zebrafish embryos. ACS Nano 5(2):1223–1235. https://doi.org/10.1021/nn1028482

Xiao T, Roeser T, Staub W et al (2005) A GFP-based genetic screen reveals mutations that disrupt the architecture of the zebrafish retinotectal projection. Development 132(13):2955–2967. https://doi.org/10.1242/dev.01861

Xu H, Li C, Li Y et al (2015) Generation of Tg(cyp1a:gfp) transgenic zebrafish for development of a convenient and sensitive in vivo assay for aryl hydrocarbon receptor activity. Mar Biotechnol 17(6):831–840. https://doi.org/10.1007/s10126-015-9669-1

Xu H, Zhang X, Li H et al (2018) Immune response induced by major environmental pollutants through altering neutrophils in zebrafish larvae. Aquat Toxicol 201:99–108. https://doi.org/10.1016/j.aquatox.2018.06.002

Xu X, Xu X, Huang X et al (2010) A high-throughput analysis method to detect regions of interest and quantify zebrafish embryo images. J Biomol Screen 15(9):1152–1159. https://doi.org/10.1177/1087057110379155

Xu Z, Cheng XE (2017) Zebrafish tracking using convolutional neural networks. Sci Rep 7(1):42815. https://doi.org/10.1038/srep42815

Xue J-Y, Li X, Sun M-Z et al (2013) An assessment of the impact of SiO_2 nanoparticles of different sizes on the rest/wake behavior and the developmental profile of zebrafish larvae. Small 9(18):3161–3168. https://doi.org/10.1002/smll.201300430

Yan N, Tang BZ, Wang W-X (2021) Intracellular trafficking of silver nanoparticles and silver ions determined their specific mitotoxicity to the zebrafish cell line. Environ Sci Nano 8(5):1364–1375. https://doi.org/10.1039/D1EN00021G

Yehia HN, Draper RK, Mikoryak C et al (2007) Single-walled carbon nanotube interactions with HeLa cells. J Nanobiotechnology 5(1):8. https://doi.org/10.1186/1477-3155-5-8

Yoo MH, Rah YC, Choi J et al (2016) Embryotoxicity and hair cell toxicity of silver nanoparticles in zebrafish embryos. Int J Pediatr Otorhinolaryngol 83:168–174. https://doi.org/10.1016/j.ijporl.2016.02.013

Yu T, Jiang Y, Lin S (2018) A 3-dimensional (3D)-printed template for high throughput zebrafish embryo arraying. J vis Exp 136:e57892. https://doi.org/10.3791/57892

Zhang H, Lenaghan S, Connolly M et al (2013) Zebrafish larva locomotor activity analysis using machine learning techniques, 12th International Conference on Machine Learning and Applications, Miami, FL, USA, p 161–166, https://doi.org/10.1109/ICMLA.2013.35

Zhang P, Zhai Y, Cai Y et al (2019) Nanomedicine-based immunotherapy for the treatment of cancer metastasis. Adv Mater 31(49):1904156. https://doi.org/10.1002/adma.201904156

Zhang X, Gong Z (2013) Fluorescent transgenic zebrafish Tg(nkx2.2a:mEGFP) provides a highly sensitive monitoring tool for neurotoxins. PloS ONE 8:e55474. https://doi.org/10.1371/journal.pone.0055474

Zhang X, Lu Z, Gelinas D et al (2011) Batch transfer of zebrafish embryos into multiwell plates. IEEE Trans Autom Sci Eng 8:625–632. https://doi.org/10.1109/TASE.2011.2121903

Zhang Z, Yu Y, Song P et al (2020) Automated manipulation of zebrafish embryos using an electrothermal microgripper. Microsyst Technol 26(6):1823–1834. https://doi.org/10.1007/s00542-019-04728-1

Zhao G, Wang Z, Xu L et al (2019b) Silver nanoparticles induce abnormal touch responses by damaging neural circuits in zebrafish embryos. Chemosphere 229:169–180. https://doi.org/10.1016/j.chemosphere.2019.04.223

Zhao X, Ren X, Zhu R et al (2016) Zinc oxide nanoparticles induce oxidative DNA damage and ROS-triggered mitochondria-mediated apoptosis in zebrafish embryos. Aquat Toxicol 180:56–70. https://doi.org/10.1016/j.aquatox.2016.09.013

Zhao Y, Sun H, Sha X et al (2019a) A review of automated microinjection of zebrafish embryos. Micromachines 10(1):7

Zhao Y, Xiong S, Liu P et al (2020) Polymeric nanoparticles-based brain delivery with improved therapeutic efficacy of ginkgolide B in parkinson's disease. Int J Nanomedicine 15:10453–10467. https://doi.org/10.2147/IJN.S272831

Zhu M, Du L, Zhao R et al (2020) Cell-penetrating nanoparticles activate the inflammasome to enhance antibody production by targeting microtubule-associated protein 1-light chain 3 for degradation. ACS Nano 14(3):3703–3717. https://doi.org/10.1021/acsnano.0c00962

Zou X, Li P, Huang Q, Zhang H (2016) The different response mechanisms of Wolffia globosa: Light-induced silver nanoparticle toxicity. Aquat Toxicol 176:97–105. https://doi.org/10.1016/j.aquatox.2016.04.019

Part II
Toxicology of Nanomaterials and Emerging Contaminants—State of the Art

Chapter 6
Ecotoxicity of Nanomaterials to Freshwater Microalgae and Fish

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Abstract Currently, engineered nanomaterials are used in a wide range of applications and enter the aquatic environment directly via consumer applications and industrial waste or by unintended discharge. In the coming years, the production rate of nanomaterials is bound to grow, and so are their predicted environmental levels. The toxicological approaches are of significant importance and require noteworthy attention for a sustainable ecosystem. The risk assessment of nanomaterials is, however, a very intricate process. Thus, in this chapter, we aim to provide basic information on the toxic aspects of engineered nanomaterials to freshwater microalgae and fish. The initial section deals with the release of nanomaterials and the principles of their toxicity. The later part of the chapter discusses the toxic impacts of metallic, carbon-based, and metal oxide nanoparticles.

Introduction

Nanotechnology in recent years has diversified its applications in various fields of medicine, consumer products, and also the environment. Nanomaterials are defined as materials with the external dimensions in the nanoscale or having an internal or surface structure in the nanoscale (1–100 nm range). They occur in various forms that include nanoparticles (NPs), nanotubes, nanocomposites, nanofibers, and nanowires. The behavior of most nanomaterials in the environment depends on their size, shape, surface reactivity, and degree of agglomeration (Sengul and Asmatulu 2020). The unique physicochemical properties such as extremely small size, large surface area to volume ratio, and size-dependent optical properties are the reasons that make the NPs versatile (Sajid et al. 2015). For instance, titanium dioxide and zinc oxide NPs have been widely used in cosmetic and beauty products as sun-guard to shield the skin against the penetration of harmful ultraviolet rays (Stark et al. 2015). Gold NPs are widely explored in the biomedical industry owing to their easy modification,

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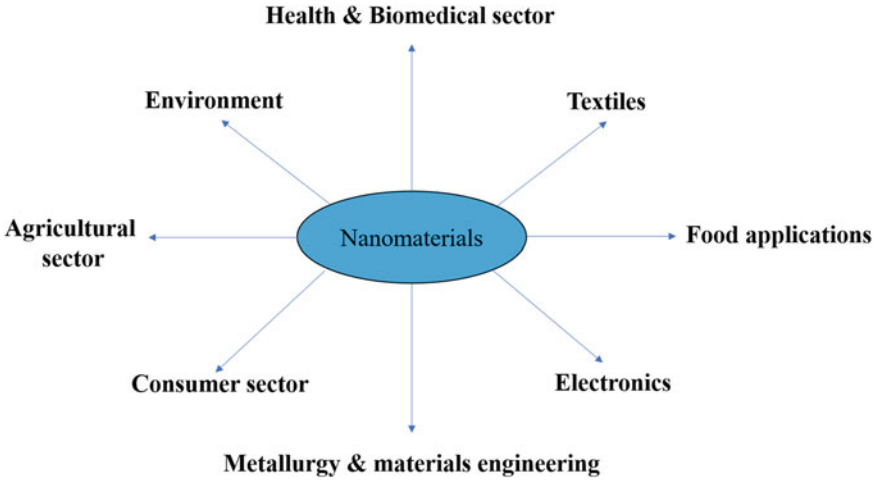

Fig. 6.1 Applications of nanomaterials in different sectors

tunable size, and strong optical properties (Jia et al. 2017). Silver NPs are vastly utilized as antimicrobial agents due to their antibacterial, antifungal, antifilarial, and antiviral properties (Cameron et al. 2018).

Despite their application across various fields (Fig. 6.1), NPs pose a threat of exposure and unfavorable effects on the environment and organisms. With a surge in their production, it is quite certain that the nanomaterials will end up in the aquatic systems (Moore 2006). This is concerning because most of the industrial wastes are washed off into the water bodies (lakes, drainage ditches, rivers, and oceans) despite safety measures. The accidental spillage or the permitted release of the NPs in the form of industrial effluent can result in direct exposure to humans via skin contact, inhalation of aerosols, and direct ingestion of contaminated water or food and vegetables coated with NPs (Liu et al. 2014). Besides, indirect exposure could also result from the ingestion of fish and mollusks contaminated with NPs expelled into the water bodies.

Even though the use of NPs has contributed significantly to the improvement of various fields over recent years, their application raises serious concerns regarding the exposure and adverse effects on the environment and organisms. Recent studies in this area have investigated the toxicological impacts and the various hazards of the exposure of NPs towards the environment but there is still a notable gap of knowledge regarding the toxicities of different NPs and their effects on freshwater organisms.

Release of NPs in the Environment

The discharge of nanomaterials in the aquatic ecosystem can either be accidental or deliberate. They are released into the environment during the different phases of their life cycle, from production to release (Nowack and Bucheli 2007). The run-offs from the nanotechnology-based industries are one of the major sources of nanomaterials in the aquatic environment (Daughton 2004). Once entered the aquatic environment, the fate of the nanomaterials depends upon several factors such as natural organic matter content, pH, and ionic strength. The nanomaterials can easily enter the aquatic organisms via endocytosis and phagocytosis and are passed onto the higher trophic levels via ingestion of those lower-level organisms. Hence, it is of utmost importance to study nanomaterials' potential toxic effects and health hazards.

Principles of NP Toxicity

Several factors may alter the toxicity of NPs such as (1) physicochemical properties of NP, (2) functional behavior of NPs, and (3) interaction with other pollutants in the aquatic environment (Turan et al. 2019). NPs tend to show unique and greater toxicity as compared to their bulk counterparts. This can be attributed to their small size and relatively high surface area. The toxicity of NPs generally depends on their size. Particle size affects the cellular uptake of NPs in organisms. A study conducted by Chithrani and Chan (2007) showed that there could be an optimal size for NP uptake. Similarly, the surface charge of the particle plays a crucial role in determining the toxicity of the NPs. Overall, the positively charged NPs are quickly adsorbed to cells as compared to the negatively charged ones due to the net negative charge of the cell surfaces. Therefore, the higher toxicity of the positively charged NPs could be attributed to their higher cellular uptake (Oh et al. 2012). In addition to particle size and surface charge, the shape can also influence the toxicity of the NPs. It determines whether NPs are phagocytosed by the cells. In addition to phagocytosis, NPs can enter the cells by physically piercing or rupturing the cell membranes. Carbon nanotubes (CNTs), for example, have been suggested by several researchers to employ their toxicity by the virtue of their needle-like structure that provides a high aspect ratio to rupture the cell membranes. This can apply to some of the multiwall carbon nanotubes (MWCNTs) with a relatively high diameter and rigidity (Nagai et al. 2011). Since the toxicity of NPs is affected by the material properties, the toxic effects of NPs will be discussed in the next sections by the main classes of nanomaterials based on the composition (Fig. 6.2.).

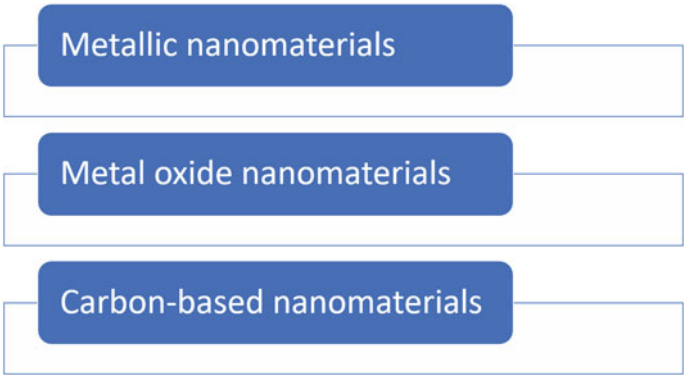

Fig. 6.2 Classification of nanomaterials based on material composition

Toxic Effects of Metallic NPs

Metallic NPs are usually composed of a metal core of an inorganic metal. This is generally covered with a shell consisting of organic or inorganic materials or metal oxide (Khan 2019). Metallic NPs have various applications in day-to-day life. Since the new techniques of NP production have become economically feasible, there has been a surge in the use of metallic NPs in various consumer products like shampoos, creams, footwear, clothing, and also plastic containers (Diegoli et al. 2008).

Even though NPs have proved to be essential in a broad aspect, the fact that they pose various health risks to organisms, as well as the environment, cannot be avoided. In recent years, studies conducted by several researchers using gold and silver NPs have proved this notion. But still, there is a gap of knowledge when it comes to determining the toxic effects of specific metal NPs in freshwater organisms. This is discussed in the next sections.

Gold

Gold NPs (Au-NPs) have been extensively studied for potential use in the biomedical field especially for diagnostics, drug delivery, therapeutics, and cancer treatment (Jia et al. 2017). This is primarily because of the unique characteristics of the Au-NPs. The increased use of Au-NPs has led to its increased diffusion into the environment that comes with an unavoidable risk towards the aquatic organisms. The cytotoxic effects of Au-NPs have been reported in mammalian cells, and also their shape, size, and external coating play a major role in enhancing their toxic effects (Chueh et al. 2014). Although studies on the toxic effects of Au-NPs have been done on mammalian cell lines, research on their toxicity on aquatic organisms is still scarce.

Hence it is crucial to investigate the harmful effects of Au-NPs on aquatic organisms to provide a background for ecotoxicological hazard review.

The ecotoxic effects of two polymer-coated Au-NPs were observed by Hoecke et al. on the freshwater microalgae *Pseudokirchneriella subcapitata* (Van Hoecke et al. 2013). In aquatic environments, agglomeration/aggregation of Au-NPs is common and does not necessarily decrease NP toxicity but could facilitate ingestion. Gilroy et al. (2014) established the potential transfer of Au-NPs in the food webs and stated that most NPs that remained in the digestive tract did not affect reproduction and were eliminated with ejection. Iswarya et al. (2017) explored the impact of Zn^{2+} present in the freshwater environment at an average concentration of <0.05 mg/L, on the toxicity of Au-NPs with different sizes and surface capping (citrate and PVP) to the green alga *Scenedesmus obliquus*. They found that as the concentration of Au-NPs increased, the relative toxicity of all the types of Au-NPs tested increased. Citrate-capped Au-NPs were found to be more toxic than PVP-capped Au-NPs, and the toxicity depended also on NP size. It was confirmed that Zn ions showed an antagonistic ability to interfere with the toxicity caused by Au-NPs on green algae *Scenedemus* sp. independently from the surface capping.

Zebrafish (*Danio rerio*) are increasingly being employed as an in vivo model to assess Au-NP toxicity. The impact of Au-NP (12 and 50 nm) exposure in the food of zebrafish showed that exposure even in the low doses can result in various cellular dysfunctions and cause genomic alterations (Geffroy et al. 2012). Smaller malpigmented eyes were seen after zebrafish embryos were exposed to 1.3 nm Au-NPs functionalized with a cationic ligand, N,N,N-trimethylammoniumethanethiol (TMAT-Au-NPs) (Kim et al. 2013). This was related to the increase in cell death in the eyes caused by the overexpression of genes p53 and bax. The effects of contaminated sediment containing Au-NPs were investigated during a 20-day exposure study of *D. rerio*. The chronic exposure resulted in a series of detrimental effects on the tissues of the organism including increased expression of genes involved in oxidative stress, mitochondrial metabolism, and modifications in the genome (Dedeh et al. 2015). A study comparing the different terminal modifications of Au-NPs on zebrafish using peptide-capped Au-NPs revealed that the terminal alteration was essential with terminal histidines causing higher toxicity than terminal tryptophans, and methionine causing the least toxicity (Harper et al. 2014). The biodistribution of differently shaped Au-NPs (nanospheres, nanorods, nano-urchins, and nanobipyramids) on the toxicity of zebrafish revealed shape-dependent biodistribution patterns after exposure to different-shaped gold particles. The differently shaped particles were found to be distributed in different ratios in the digestive organs such as the gall bladder, liver, and pancreas. The biodistribution patterns suggested that long-term exposure could cause shape-dependent sublethal consequences (van Pomeren et al. 2019).

Silver

Studies exposing cultures of algae and zooplankton to engineered Ag-NPs have revealed several factors that may alter the toxicity of Ag-NPs (Zhao and Wang 2010; Das et al. 2013). The trophic transfer of Ag-NPs may be altered by the nature of the exposure (waterborne or diet borne) (Zhao and Wang 2011). Significant effort has been made to study the contribution of silver ions on Ag-NP toxicity to algae and zooplankton (Navarro et al. 2008b; Das et al. 2013). It has been shown that the presence of algae may trigger the release of silver ions from Ag-NPs and consequently alter their toxicity (Navarro et al. 2008b). At the organism level, physiological and functional characteristics may also alter the toxicity of Ag-NPs (Oukarroum et al. 2012; Pokhrel et al. 2013). For instance, *Chlorella vulgaris* could efficiently detoxify Ag-NP induced ROS species via the induction of antioxidant enzymes, allowing photosynthesis to continue even at high Ag-NPs concentrations (Qian et al. 2016). The toxicity of Ag-NPs was higher in cultures at the early phases of growth. Finally, Ag-NP toxicity was different depending on the examined species according to species sensitivity distributions (SSDs) (Coll et al. 2016). This was also observed in the present literature review where vulnerability to Ag-NP toxicity was higher for *D. magna* compared to other daphnids (Völker et al. 2013), for *D. galeata* compared to *D. magna* and *Bosmina longirostris* (Sakamoto et al. 2015), for *Dunaliella tertiolecta* compared to *C. vulgaris* (Oukarroum et al. 2012), and for *Microcystis aeruginosa* (prokaryotic) compared to *C. vulgaris* (eukaryotic) (Qian et al. 2016).

In a size-dependent (30–72 nm) in vivo study conducted on zebrafish, it was observed that Ag-NPs were able to diffuse into the embryos via Brownian motion through chorionic pores, thereby generating toxicity (Lee et al. 2012). Bar-Ilan et al. (2009), on the other hand, synthesized Ag-NPs of various sizes (3, 10, 50, and 200 nm) and applied them to zebrafish embryos in a rearing container. They discovered size-independent mortality rates after 120 h post-fertilization (Bar-Ilan et al. 2009). In another investigation, Ag-NPs were discovered to have a size-dependent effect on the neural development of zebrafish embryos. Four-nm Ag-NPs were taken up more efficiently than 10-nm Ag-NPs in this circumstance, with the exposed zebrafish embryos' heads accumulating more Ag-NPs than the trunks (Xin et al. 2015). As a result, the size-dependent toxicity profile of Ag-NPs remains a point of contention. In both fish cell lines and zebrafish embryos, George et al. (2012) confirmed the surface defect-driven toxicity of Ag-NPs. Surface reactivity caused by crystal defects increased the toxicity of Ag nanoplates compared to other Ag-NPs. According to another study, Ag-peptide NPs were substantially more biocompatible than citrate-coated Ag-NPs (Lee et al. 2013). The study showed that the combination of numerous physicochemical properties of the NPs defined their harmful effects on embryonic development, emphasizing the significance of investigating their effects one factor at a time. Another investigation revealed that exposing Ag-NPs to simulated sunlight increased their embryonic toxicity (George et al. 2014). Zebrafish exposed to Ag-NPs during their early development experienced a variety of side effects, including a decrease in heart rate, damage to neuromast hair cells, and smaller but statistically

significant increases in mortality and teratogenicity (Yoo et al. 2016). After chronic exposure to Ag-NPs, a recent study looked at the reproductive toxicity and associated probable adverse outcome pathway (AOP) in zebrafish. Adult zebrafish (three months old) were treated with varying concentrations (0, 10, 33, and 100 μg/L) of Ag-NPs for five weeks and the results were observed. Female zebrafish fertility was dramatically reduced after exposure to 33 and 100 μg/L Ag-NPs, which was accompanied by an increase in apoptotic cells in the ovarian and testicular tissue (Ma et al. 2018). The size-related effects of chronic Ag-NPs exposure on intestinal Na/K-ATPase and SOD activities in adult zebrafish were suggested in a recent study. The study also revealed Ag-NPs had higher toxicity in the intestine than in the liver, showing that Ag-NPs have organ-specific toxicity. It was also demonstrated in this study that the response of zebrafish to Ag-NPs was sex-dependent since the males showed more susceptibility as compared to the females (Bao et al. 2020).

Toxic Effects of Carbon-based Nanomaterials

Carbon nanomaterials (CNMs) can be described as the allotropes of carbon that have at least one of their dimensions in the range of 1- 100 nm. The major classes of CNMs are fullerenes, CNTs, graphene, and carbon black. These materials can originate in diverse ways, some of these could be liberated into the environment naturally (in consequence of forest fires or volcanic eruptions), whereas others could be produced by anthropogenic combustions or could also be manufactured in industries (Freixa et al. 2018). Due to their unique physicochemical, mechanical, and electrical properties, they have been used in various fields such as engineering, sports equipment, optics, automotive industry, cosmetic and medical applications (Navarro et al. 2008a). The increased usage of CNMs has increased the exposure risk of the aquatic environment to CNMs. Hence it is evident that methodologies are devised to study the effects of CNMs on the organisms to get cumulative knowledge on their toxic effects and potential bioaccumulation.

Algae are one of the most sensitive organisms to CNMs. It is reported that the toxicity of CNMs to algal cells can be both directly related to their exposure as well as to the indirect effects such as shading effects by the nanomaterials (resulting in reduced light absorption and photosynthesis) and to the nutrient depletion caused by the absorption of nutrients on CNMs (Schwab et al. 2011; Long et al. 2012; Zhao et al. 2017). For instance, Zhao et al. (2017) studied the toxicity of graphene nanomaterials to freshwater algae (*Chlorella pyrenoidosa*) showing that graphene significantly decreased the membrane integrity of algal cells. Sensitivity to CNM exposure differs between species. For instance, the growth rate of *C. vulgaris* was more strongly affected at lower CNT concentrations (EC_{50} = 1.8 mg/L) than that the growth rate of *P. subcapitata* (EC_{50} = 20 mg/L) (Schwab et al. 2011). Similar toxicity of CNTs for *P. subcapitata* (EC_{50} = 17.95 mg/L) was also reported in another study (Lukhele et al. 2015). Moreover, differences in toxicity to CNMs observed between planktonic and biofilm communities have been attributed to the

presence of extracellular polymeric substances (EPS) matrix (Luongo and Zhang 2010; Rodrigues and Elimelech 2010), where planktonic communities were more severely affected by CNM exposure. The protective role of EPS to pollutants has been widely studied (Flemming and Wingender 2010). Other studies have observed fast overproduction of EPS in algae after exposure to high concentrations of CNMs, which was interpreted as a natural defensive mechanism against double-walled CNTs (Verneuil et al. 2015b), MWCNT (Verneuil et al. 2015a), or graphene (Garacci et al. 2017).

The bioaccumulation and distribution of multiwalled CNTs were recently investigated using a zebrafish model, which revealed a bioaccumulation factor of 16 L/kg fish wet weight (Maes et al. 2014). Li et al. (2015) discovered CNT-induced biochemical changes in zebrafish. They showed that CNT exposure can activate the brain and cause gonadal changes. In another study, the toxicity of functionalized CNTs of various lengths was assessed in zebrafish embryos, with the conclusion that the length of CNTs has a significant impact on their toxicity profile in vivo (Cheng and Cheng 2012). Another study found that single-wall (SW)CNTs functionalized with polyethylene glycol increased mortality, delayed hatching, and decreased overall larval length only at the highest dosage examined (1 mg/L), with no evidence of genotoxicity or nanotube uptake by tissues (Girardi et al. 2017). A study using oxidized-MWCNT along with Cd showed that oxidized-MWCNT promoted apoptosis and necrosis in ZFL (zebrafish liver cell lines) cells and increased Cd toxicity at low concentrations, most likely through a "Trojan horse" and/or synergistic action (Morozesk et al. 2018). In another study, Ren et al. (2021) explored the effect of MWCNTs on the enantioselectivity of bioaccumulation of a chiral insecticide indoxacarb in zebrafish and found that MWCNTs did not affect the preferential bioaccumulation pattern of R-(-)-indoxacarb. However, the amount of R-(-)-indoxacarb that accumulated in zebrafish was 65% higher when co-exposed with MWCNTs compared to single exposure (Ren et al. 2021).

Toxic Effects of Metal Oxide NPs

Metal oxide NPs (MOx NPs) include both synthesized and naturally found particles that are in the nanoscale range. MOx NPs, especially the engineered ones, have gained popularity in recent years owing to their diversity in the crystal structure, intriguing magnetic and electronic properties, and the existence of metal–oxygen bonding (Amde et al. 2017). They are used in almost all fields, which include medicine, biomedical applications, material chemistry, agriculture, environmental remediation, and catalysis (Chavali and Nikolova 2019). The endless applications have paved the way for their deliberate and accidental release into the aquatic environment. However, in the aquatic matrix, MOx NPs undergo various physicochemical transformations that alter their pristine nature (Garner et al. 2017) and subsequently their toxic impact on aquatic species. Thus, the following sections address the toxic effects of different MOx NPs and their mechanisms of toxic action on aquatic species.

Titanium Dioxide NPs

TiO_2 NPs are the most frequently used metal oxide NPs in multiple commercial sections such as topical sunscreens, light-emitting diodes, surface coatings, and disinfectant sprays (Saxena and Harish 2018). Such large-scale use of TiO_2 NPs has prompted several researchers to study their impacts on the aquatic environment. Toxic effects associated with TiO_2 NPs on freshwater microalgae include the shading effect (Zhang et al. 2020b), oxidative stress generation (Gao et al. 2020), cellular membrane damage (Roy et al. 2020), and a decrease in photosynthetic efficiency (Middepogu et al. 2018). These toxic effects vary with differences in particle size, crystalline form (Chen et al. 2019b), and illumination conditions (Iswarya et al. 2018). Smaller-sized particles have a larger specific surface area to volume ratio that increases the likelihood of interaction with the algal surface. Moreover, the different crystalline forms of TiO_2 NPs display dissimilar toxic effects due to the differences in semiconductor bandgap and surface chemistry. Since TiO_2 NPs are photocatalysts, light source plays a vital role in imparting toxic effects. The activation of TiO_2 NPs in the presence of UV illuminations generated reactive oxygen species (ROS) in the medium that augmented the toxic effects (Sendra et al. 2017; Roy et al. 2020). Until now, ROS generation has been described as the early stress response and the basic mechanism of TiO_2 NPs toxicity in freshwater microalgae. However, Middepogu et al. (2018) supported a paradigm shift in the toxic mechanism of TiO_2 NPs from oxidative stress to metabolic disruptions involved in photosynthesis. Besides all these particle-associated and experimental factors, various environmental parameters such as pH and temperature also alter the properties and the toxicological effects of TiO_2 NPs on microalgae (Zhang et al. 2020b).

In freshwater fishes such as *D. rerio* and *Carassius gibelio*, TiO_2 NPs stimulated the immune system with ROS generation, lysosomal membrane destruction, lipid peroxidation, protein carbonylation, DNA damage, and lastly apoptosis (Bobori et al. 2020). Dietary uptake of TiO_2 NPs caused morphological alterations in the kidney, intestine, and liver and biochemical changes in the liver of *D. rerio* (Cunha and de Brito-Gitirana 2020). Besides, TiO_2 NPs altered the gene expression associated with the development of the dorsoventral axis and neural network of *D. rerio* embryos (Kansara et al. 2020). Mechanistic investigation using shotgun proteomics revealed that the chronic exposure of TiO_2 NPs altered the insulin-responsive compartment of *D. rerio* offspring (Chen et al. 2019a). Overall, the evaluation of the toxic effects of TiO_2 NPs on microalgae and fishes has taken a shift from using conventional toxicity endpoints (such as mortality and oxidative stress determination) to a more specific mechanistic approach.

Zinc Oxide NPs

ZnO NPs have a wurtzite structure and are used in paints, pigments, lubricants, ceramic glass, fire retardants, and batteries because of their optoelectronic, catalytic, and antimicrobial properties (Saxena and Harish 2018). Exposure to a very low or environmentally relevant concentration of ZnO NPs is known to affect the growth and lipid content, induce plasmolysis, destruct the membrane, and disrupt thylakoids in the chloroplast of *Scenedesmus* sp. (Meng et al. 2018; Aravantinou et al. 2020). As discussed earlier, NP transformation in the water matrix can alter their toxic effects. The presence of bovine serum albumin reduced the toxic effects of ZnO NPs on *C. pyrenoidosa* by forming a protein corona on the surface of ZnO NPs (Janani et al. 2020) whereas the presence of phosphate in water transformed ZnO NPs into zinc phosphate and hopeite resulting in their reduced toxic effects to *Chlorella sorokiniana* (Zhang et al. 2020a). Unlike TiO_2 NPs, ZnO NPs can undergo dissociation and the released ions are internalized by the algal cells that impart toxic effects (Ye et al. 2018). Moreover, it is essential to infer the toxic effects through feedback between microalgae and the aquatic ecosystem. Tang et al. (2018) investigated such environmental feedback caused by the release of algal organic matter to the aquatic environment using standard analytical parameters, such as excitation-emission matrices, molecular weight distribution, hydrophilic and hydrophobic properties, and microcystin-LR.

The toxicity of ZnO NPs in fish has been assessed based on behavior (Campos et al. 2019), physiological markers (Chupani et al. 2018), and molecular biological approaches (Hou et al. 2019). With the gastrointestinal route being the most important exposure pathway in aquatic species, toxicity studies have been conducted incorporating ZnO NPs in the feed. Chronic exposure of *Cyprinus carpio* to dietary ZnO NPs (50 and 500 mg/kg of feed) did not affect the blood biochemistry, hematology, lipid peroxidation, and Zn accumulation levels but affected liver and kidney function (Chupani et al. 2018). Likewise, Dekani et al. (2019) revealed that the dietary exposure of *C. carpio* to ZnO NPs resulted in greater accumulation in the target organs and caused higher toxicity than the dietary exposure to organic and inorganic forms of Zn. Using the observational assessment of fish behavior, Campos et al. (2019) found that ZnO NPs can induce food demotivation and alter the anti-predatory defensive behavior of *Oreochromis niloticus*, which suggests possible neurotoxicity. Besides, ZnO NPs were toxic to developing vascular and nervous systems of *D. rerio* and its succeeding generations (Kteeba et al. 2018). However, these toxic effects were reversed in the presence of dissolved organic matter. At the molecular level, ZnO NPs inhibited the growth and development of *D. rerio* by affecting the cell cycle processes (Hou et al. 2019).

Cerium Oxide NPs

CeO_2 NPs are extremely versatile owing to their unique surface area and redox activity, and high stability that makes them a potential candidate in the manufacture of biosensors, catalysis, corrosion-resistant coatings, therapeutic agents, drug delivery vectors, and anti-parasitic ointments (Nadeem et al. 2020). Very few studies have reported the toxic effects of CeO_2 NPs on microalgae. Pulido-Reyes et al. (2019) demonstrated that the surface coating of CeO_2 NPs completely modifies the interaction of NPs with algal cells and also influences the mechanism of toxic effects. Pristine CeO_2 NPs damaged the cell membrane and reduced the metabolic activity while the PVP-coated CeO_2 NPs induced toxicity (ROS generation) without damage to the cell membrane. These conflicting effects of CeO_2 NPs will be very useful during the manufacturing of safer-by-design NPs. CeO_2 NPs are insoluble under environmental pH > 7.5. However, the small fraction of dissolved Ce^{3+} can produce harmful effects (Röhder et al. 2014). In contrast, Kosak née Röhder et al. (2018) reported a very high median effective concentration of CeO_2 NPs and Ce^{3+} probably due to the negligible uptake of CeO_2 NPs in the wild type *Chlamydomonas reinhardtii* and relatively slow uptake of Ce^{3+} in both the wild type and cell wall free mutant of *C. reinhardtii*. Similarly, Xiong et al. (2020) also reported a negligible effect of CeO_2 NPs on the growth and pigments of *Scenedesmus obliquus*. Recently, Hund-Rinke et al. (2020) studied the attachment behavior of three sub-types of CeO_2 NPs to algae and found a correlation between growth inhibition (*Raphidocelis subcapitata*) and attachment efficiency of CeO_2 NPs. To sum up, the toxic effects of CeO_2 NPs majorly depend on the dissociation of Ce ions in the experimental matrix and subsides in the presence of a surface coating.

Copper Oxide NPs

Similar to TiO_2 NPs, CuO also has a strong light absorption reaction that makes them an essential photocatalyst. They are used in products such as catalysts, sensors, surfactants, and antimicrobials (Wu et al. 2020). Most studies described the toxic effects related to CuO NPs by the release of Cu^{2+} from the NPs as the ionic form is highly toxic (Joonas et al. 2019; Wu et al. 2020). To decipher biological processes, it is important to consider the metabolomes such as lipids, glycans, and the array of small molecules along with the genome, the transcriptome, and the proteome, as the metabolomes function as a substrate and product of biochemical reactions and aid in biological regulation (Doerr 2016). Wang et al. (2020) used such an approach (global metabolomics) and found similar metabolic responses (lipid bilayer remodeling, perturbation of glutathione metabolism, and accumulation of osmoregulants and chlorophyll intermediates) in *C. vulgaris* after treatment with CuO NPs, CuO microparticles (1 and 10 mg/L), and Cu ions (0.08 and 0.8 mg/L), and also confirmed dissolution as a major driving factor resulting in the metabolic reprogramming of

algae. Alho et al. (2020) also proposed the shedding of Cu^{2+} from CuO NPs, which was the cause of toxic effects, as both NPs and ions had similar toxicity targets and responses in *R. subcapitata.*

NP toxicity assessments in the matrix representing the natural environment are understated. The correlation of study settings with real exposure conditions is bound to enhance the ecotoxicological results of NPs. In support of this, Joonas et al. (2019) studied the behavior and toxic effects of CuO NPs and their ions in nutrient-adjusted natural water. A decrease in the toxicity of CuO NPs and Cu ions was observed due to the differences in bioavailability arising from the binding of Cu ions to natural organic matter. Similarly, Yin et al. (2020) reported that the presence of *C. reinhardtii* significantly affected the fate (reduced colloidal stability, adsorption, and assimilation) and toxic effects of CuO NPs.

In contrast to the shedding of Cu ions from CuO NPs that induced toxic effects in microalgae, CuO NPs as a whole were responsible for inducing ROS in gills and increasing the number of cells in the early apoptotic and necrotic phases of *Hyphessobrycon eques* (Mansano et al. 2018). Although the presence of clay particles and humic acid-induced heteroagglomeration with CuO NPs and decreased the bioavailability of CuO NPs, altered levels of developmental gene expression and abnormalities were observed in the embryos of *D. rerio* (Kansara et al. 2019). Canli et al. (2018) reported that the exposure to CuO NPs (0, 1, 5, and 25 mg/L) altered the serum biomarkers levels in freshwater fish *Oreochromis niloticus.* Boyle et al. (2020) investigated the effects of pH and intermittent pulse on the toxicity of CuO NPs to *D. rerio* and found that CuO NPs were more toxic in pulse exposure and acidic conditions. In the study by Braz-Mota et al. (2018), species-specific metabolic stress responses to CuO NPs were reported which were possibly caused by different osmoregulatory strategies between two Amazon fish *Apistogramma agassizii* and *Paracheirodon axelrodi.*

Conclusions

The growing use of nanomaterials, especially in applications from which they are discharged directly, will lead to increased exposure to aquatic organisms. The authors believe that this chapter will provide basic and substantial information on the toxicity of nanomaterials to freshwater microalgae and fish. The nanomaterials as a whole and their dissolved ions contribute to the toxicity. Advances in the toxicity assessment of nanomaterials on microalgae and fish facilitate the understanding of their mode of action. However, a shift from a toxicological approach (stimulating toxicity with extreme concentrations) to an ecotoxicological perspective (assays under environmentally relevant conditions) is required.

References

Alho L de OG, Souza JP, Rocha GS et al (2020) Photosynthetic, morphological and biochemical biomarkers as tools to investigate copper oxide nanoparticle toxicity to a freshwater chlorophyceae. Environ Pollut 265:114856. https://doi.org/10.1016/j.envpol.2020.114856

Amde M, Liu J, Tan ZQ, Bekana D (2017) Transformation and bioavailability of metal oxide nanoparticles in aquatic and terrestrial environments. A Review. Environ Pollut 230:250–267

Aravantinou AF, Andreou F, Manariotis ID (2020) Long-term toxicity of ZnO nanoparticles on Scenedesmus rubescens cultivated in semi-batch mode. Nanomaterials 10(11):2262. https://doi.org/10.3390/nano10112262

Bao S, Tang W, Fang T (2020) Sex-dependent and organ-specific toxicity of silver nanoparticles in livers and intestines of adult zebrafish. Chemosphere 249(7). https://doi.org/10.1016/j.chemosphere.2020.126172

Bar-Ilan O, Albrecht RM, Fako VE et al (2009) Toxicity assessments of multisized gold and silver nanoparticles in zebrafish embryos. Small 5(16):1897–1910. https://doi.org/10.1002/smll.200801716

Bobori D, Dimitriadi A, Karasiali S et al (2020) Common mechanisms activated in the tissues of aquatic and terrestrial animal models after TiO2 nanoparticles exposure. Environ Int 138:105611. https://doi.org/10.1016/j.envint.2020.105611

Boyle D, Clark NJ, Handy RD (2020) Toxicities of copper oxide nanomaterial and copper 2sulphate in early life stage zebrafish: Effects of pH and intermittent pulse exposure. Ecotoxicol Environ Saf 190:109985. https://doi.org/10.1016/j.ecoenv.2019.109985

Braz-Mota S, Campos DF, MacCormack TJ et al (2018) Mechanisms of toxic action of copper and copper nanoparticles in two Amazon fish species: Dwarf cichlid (Apistogramma agassizii) and cardinal tetra (Paracheirodon axelrodi). Sci Total Environ 630:1168–1180. https://doi.org/10.1016/j.scitotenv.2018.02.216

Cameron SJ, Hosseinian F, Willmore WG (2018) A current overview of the biological and cellular effects of nanosilver. Int J Mol Sci 19(7):1–40. https://doi.org/10.3390/ijms19072030

Canli EG, Dogan A, Canli M (2018) Serum biomarker levels alter following nanoparticle (Al2O3, CuO, TiO2) exposures in freshwater fish (Oreochromis niloticus). Environ Toxicol Pharmacol 62:181–187. https://doi.org/10.1016/j.etap.2018.07.009

Chavali MS, Nikolova MP (2019) Metal oxide nanoparticles and their applications in nanotechnology. SN Appl Sci 1(6):607. https://doi.org/10.1007/s42452-019-0592-3

Chen L, Hu C, Guo Y et al (2019a) TiO_2 nanoparticles and BPA are combined to impair the development of offspring zebrafish after parental coexposure. Chemosphere 217:732–741. https://doi.org/10.1016/j.chemosphere.2018.11.052

Chen X, Zhu Y, Yang K et al (2019b) Nanoparticle TiO_2 size and rutile content impact bioconcentration and biomagnification from algae to daphnia. Environ Pollut 247:421–430. https://doi.org/10.1016/j.envpol.2019.01.022

Cheng J, Cheng SH (2012) Influence of carbon nanotube length on toxicity to zebrafish embryos. Int J Nanomedicine 7:3731–3739. https://doi.org/10.2147/IJN.S30459

Chithrani BD, Chan WCW (2007) Elucidating the mechanism of cellular uptake and removal of protein-coated gold nanoparticles of different sizes and shapes. Nano Lett 7(6):1542–1550. https://doi.org/10.1021/nl070363y

Chueh PJ, Liang RY, Lee YH et al (2014) Differential cytotoxic effects of gold nanoparticles in different mammalian cell lines. J Hazard Mater 264(2014):303–312. https://doi.org/10.1016/j.jhazmat.2013.11.031

Chupani L, Niksirat H, Velíšek J et al (2018) Chronic dietary toxicity of zinc oxide nanoparticles in common carp (Cyprinus carpio L.): Tissue accumulation and physiological responses. Ecotoxicol Environ Saf 147:110–116. https://doi.org/10.1016/j.ecoenv.2017.08.024

Coll C, Notter D, Gottschalk F et al (2016) Probabilistic environmental risk assessment of five nanomaterials (nano-TiO2, nano-Ag, nano-ZnO, CNT, and fullerenes). Nanotoxicology 10(4):436–444. https://doi.org/10.3109/17435390.2015.1073812

Cunha RLD da, de Brito-Gitirana L (2020) Effects of titanium dioxide nanoparticles on the intestine, liver, and kidney of Danio rerio. Ecotoxicol Environ Saf 203:111032. https://doi.org/10.1016/j.ecoenv.2020.111032

Das P, Xenopoulos MA, Metcalfe CD (2013) Toxicity of silver and titanium dioxide nanoparticle suspensions to the aquatic invertebrate, Daphnia magna. Bull Environ Contam Toxicol 91(1):76–82. https://doi.org/10.1007/s00128-013-1015-6

Daughton CG (2004) Non-regulated water contaminants: emerging research. Environ Impact Assess Rev 24(7–8):711–732. https://doi.org/10.1016/j.eiar.2004.06.003

de Campos RP, Chagas TQ, da Silva Alvarez TG et al (2019) Analysis of ZnO nanoparticle-induced changes in Oreochromis niloticus behavior as toxicity endpoint. Sci Total Environ 682:561–571. https://doi.org/10.1016/j.scitotenv.2019.05.183

Dedeh A, Ciutat A, Treguer-Delapierre M et al (2015) Impact of gold nanoparticles on zebrafish exposed to a spiked sediment. Nanotoxicology 9(1):71–80. https://doi.org/10.3109/17435390.2014.889238

Dekani L, Johari SA, Joo HS (2019) Comparative toxicity of organic, inorganic and nanoparticulate zinc following dietary exposure to common carp (Cyprinus carpio). Sci Total Environ 656:1191–1198. https://doi.org/10.1016/j.scitotenv.2018.11.474

Diegoli S, Manciulea AL, Begum S et al (2008) Interaction between manufactured gold nanoparticles and naturally occurring organic macromolecules. Sci Total Environ 402(1):51–61. https://doi.org/10.1016/j.scitotenv.2008.04.023

Doerr A (2016) Global metabolomics. Nat Methods 14:32

Dominguez GA, Lohse SE, Torelli MD et al (2015) Effects of charge and surface ligand properties of nanoparticles on oxidative stress and gene expression within the gut of Daphnia magna. Aquat Toxicol 162:1–9. https://doi.org/10.1016/j.aquatox.2015.02.015

Flemming HC, Wingender J (2010) The biofilm matrix. Nat Rev Microbiol 8(9):623–633. https://doi.org/10.1038/nrmicro2415

Freixa A, Acuña V, Sanchís J et al (2018) Ecotoxicological effects of carbon based nanomaterials in aquatic organisms. Sci Total Environ 619–620:328–337. https://doi.org/10.1016/j.scitotenv.2017.11.095

Gao X, Deng R, Lin D (2020) Insights into the regulation mechanisms of algal extracellular polymeric substances secretion upon the exposures to anatase and rutile TiO2 nanoparticles. Environ Pollut 263:114608. https://doi.org/10.1016/j.envpol.2020.114608

Garacci M, Barret M, Mouchet F et al (2017) Few Layer Graphene sticking by biofilm of freshwater diatom Nitzschia palea as a mitigation to its ecotoxicity. Carbon N Y 113:139–150. https://doi.org/10.1016/j.carbon.2016.11.033

Garner KL, Suh S, Keller AA (2017) Assessing the risk of engineered nanomaterials in the environment: development and application of the nanoFate model. Environ Sci Technol 51(10):5541–5551. https://doi.org/10.1021/acs.est.6b05279

Geffroy B, Ladhar C, Cambier S et al (2012) Impact of dietary gold nanoparticles in zebrafish at very low contamination pressure: the role of size, concentration and exposure time. Nanotoxicology 6(2):144–160. https://doi.org/10.3109/17435390.2011.562328

George S, Gardner H, Seng EK et al (2014) Differential effect of solar light in increasing the toxicity of silver and titanium dioxide nanoparticles to a fish cell line and zebrafish embryos. Environ Sci Technol 48(11):6374–6382. https://doi.org/10.1021/es405768n

George S, Lin S, Ji Z et al (2012) Surface defects on plate-shaped silver nanoparticles contribute to its hazard potential in a fish gill cell line and zebrafish embryos. ACS Nano 6(5):3745–3759. https://doi.org/10.1021/nn204671v

Gilroy KD, Hughes RA, Neretina S (2014) Kinetically controlled nucleation of silver on surfactant-free gold seeds. J Am Chem Soc 136(43):15337–15345. https://doi.org/10.1021/ja5081635

Girardi FA, Bruch GE, Peixoto CS et al (2017) Toxicity of single-wall carbon nanotubes functionalized with polyethylene glycol in zebrafish (Danio rerio) embryos. J Appl Toxicol 37(2):214–221. https://doi.org/10.1002/jat.3346

Harper B, Sinche F, Ho WuR et al (2014) The impact of surface ligands and synthesis method on the toxicity of glutathione-coated gold nanoparticles. Nanomaterials 4(2):355–371. https://doi.org/10.3390/nano4020355

Hou J, Liu H, Zhang S et al (2019) Mechanism of toxic effects of Nano-ZnO on cell cycle of zebrafish (Danio rerio). Chemosphere 229:206–213. https://doi.org/10.1016/j.chemosphere.2019.04.217

Hund-Rinke K, Sinram T, Schlich K et al (2020) Attachment efficiency of nanomaterials to algae as an important criterion for ecotoxicity and grouping. Nanomaterials 10(6):1021. https://doi.org/10.3390/nano10061021

Iswarya V, Bhuvaneshwari M, Chandrasekaran N et al (2018) Trophic transfer potential of two different crystalline phases of TiO_2 NPs from Chlorella sp. to Ceriodaphnia dubia. Aquat Toxicol 197:89–97. https://doi.org/10.1016/j.aquatox.2018.02.003

Iswarya V, Johnson JB, Parashar A et al (2017) Modulatory effects of Zn^{2+} ions on the toxicity of citrate- and PVP-capped gold nanoparticles towards freshwater algae, Scenedesmus obliquus. Environ Sci Pollut Res 24(4):3790–3801. https://doi.org/10.1007/s11356-016-8131-x

Janani B, Raju LL, Thomas AM et al (2020) Impact of bovine serum albumin – A protein corona on toxicity of ZnO NPs in environmental model systems of plant, bacteria, algae and crustaceans. Chemosphere :128629. https://doi.org/10.1016/j.chemosphere.2020.128629

Jia YP, Ma BY, Wei XW et al (2017) The in vitro and in vivo toxicity of gold nanoparticles. Chinese Chem Lett 28(4):691–702. https://doi.org/10.1016/j.cclet.2017.01.021

Joonas E, Aruoja V, Olli K et al (2019) Environmental safety data on CuO and TiO_2 nanoparticles for multiple algal species in natural water: Filling the data gaps for risk assessment. Sci Total Environ 647:973–980. https://doi.org/10.1016/j.scitotenv.2018.07.446

Kansara K, Kumar A, Karakoti AS (2020) Combination of humic acid and clay reduce the ecotoxic effect of TiO_2 NPs: a combined physico-chemical and genetic study using zebrafish embryo. Sci Total Environ 698:134133. https://doi.org/10.1016/j.scitotenv.2019.134133

Kansara K, Paruthi A, Misra SK et al (2019) Montmorillonite clay and humic acid modulate the behavior of copper oxide nanoparticles in aqueous environment and induces developmental defects in zebrafish embryo. Environ Pollut 255:113313. https://doi.org/10.1016/j.envpol.2019.113313

Khan SA (2019) Metal nanoparticles toxicity: Role of physicochemical aspects. Elsevier Inc.

Kim KT, Zaikova T, Hutchison JE et al (2013) Gold nanoparticles disrupt zebrafish eye development and pigmentation. Toxicol Sci 133(2):275–288. https://doi.org/10.1093/toxsci/kft081

Kosak née Röhder LA, Brandt T, Sigg L et al (2018) Uptake and effects of cerium(III) and cerium oxide nanoparticles to Chlamydomonas reinhardtii. Aquat Toxicol 197:41–46. https://doi.org/10.1016/j.aquatox.2018.02.004

Kteeba SM, El-Ghobashy AE, El-Adawi HI et al (2018) Exposure to ZnO nanoparticles alters neuronal and vascular development in zebrafish: Acute and transgenerational effects mitigated with dissolved organic matter. Environ Pollut 242:433–448. https://doi.org/10.1016/j.envpol.2018.06.030

Lee KJ, Browning LM, Nallathamby PD et al (2012) In vivo quantitative study of sized-dependent transport and toxicity of single silver nanoparticles using zebrafish embryos. Chem Res Toxicol 25(5):1029–1046. https://doi.org/10.1021/tx300021u

Lee KJ, Browning LM, Nallathamby PD et al (2013) Study of charge-dependent transport and toxicity of peptide-functionalized silver nanoparticles using zebrafish embryos and single nanoparticle plasmonic spectroscopy. Chem Res Toxicol 26(6):904–917. https://doi.org/10.1021/tx400087d

Li J, Ying GG, Jones KC et al (2015) Real-world carbon nanoparticle exposures induce brain and gonadal alterations in zebrafish (Danio rerio) as determined by biospectroscopy techniques. Analyst 140(8):2687–2695. https://doi.org/10.1039/c4an02227k

Liu Y, Tourbin M, Lachaize S et al (2014) Nanoparticles in wastewaters: hazards, fate and remediation. Powder Technol 255:149–156. https://doi.org/10.1016/j.powtec.2013.08.025

Long Z, Ji J, Yang K et al (2012) Systematic and quantitative investigation of the mechanism of carbon nanotubes' toxicity toward Algae. Environ Sci Technol 46(15):8458–8466. https://doi.org/10.1021/es301802g

Lukhele LP, Mamba BB, Musee N et al (2015) Acute toxicity of double-walled carbon nanotubes to three aquatic organisms. J Nanomater 2015. https://doi.org/10.1155/2015/219074

Luongo LA, Zhang X (Jackie) (2010) Toxicity of carbon nanotubes to the activated sludge process. J Hazard Mater 178(1–3):356–362. https://doi.org/10.1016/j.jhazmat.2010.01.087

Ma YB, Lu CJ, Junaid M et al (2018) Potential adverse outcome pathway (AOP) of silver nanoparticles mediated reproductive toxicity in zebrafish. Chemosphere 207:320–328. https://doi.org/10.1016/j.chemosphere.2018.05.019

Maes HM, Stibany F, Giefers S et al (2014) Accumulation and distribution of multiwalled carbon nanotubes in zebrafish (Danio rerio). Environ Sci Technol 48(20):12256–12264. https://doi.org/10.1021/es503006v

Mansano AS, Souza JP, Cancino-Bernardi J et al (2018) Toxicity of copper oxide nanoparticles to Neotropical species Ceriodaphnia silvestrii and Hyphessobrycon eques. Environ Pollut 243:723–733. https://doi.org/10.1016/j.envpol.2018.09.020

Meng Y, Wang S, Wang Z et al (2018) Algal toxicity of binary mixtures of zinc oxide nanoparticles and tetrabromobisphenol A: roles of dissolved organic matters. Environ Toxicol Pharmacol 64:78–85. https://doi.org/10.1016/j.etap.2018.09.010

Middepogu A, Hou J, Gao X et al (2018) Effect and mechanism of TiO 2 nanoparticles on the photosynthesis of Chlorella pyrenoidosa. Ecotoxicol Environ Saf 161:497–506. https://doi.org/10.1016/j.ecoenv.2018.06.027

Moore MN (2006) Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? Environ Int 32(8):967–976. https://doi.org/10.1016/j.envint.2006.06.014

Morozesk M, Franqui LS, Mansano AS et al (2018) Interactions of oxidized multiwalled carbon nanotube with cadmium on zebrafish cell line: the influence of two co-exposure protocols on in vitro toxicity tests. Aquat Toxicol 200(January):136–147. https://doi.org/10.1016/j.aquatox.2018.05.002

Nadeem M, Khan R, Afridi K et al (2020) Green synthesis of cerium oxide nanoparticles (CeO2 NPs) and their antimicrobial applications: a review. Int J Nanomedicine 15:5951–5961. https://doi.org/10.2147/IJN.S255784

Nagai H, Okazaki Y, Chew SH et al (2011) Diameter and rigidity of multiwalled carbon nanotubes are critical factors in mesothelial injury and carcinogenesis. Proc Natl Acad Sci U S A 108(49). https://doi.org/10.1073/pnas.1110013108

Navarro E, Baun A, Behra R et al (2008a) Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. Ecotoxicology 17(5):372–386. https://doi.org/10.1007/s10646-008-0214-0

Navarro E, Piccapietra F, Wagner B et al (2008b) Toxicity of silver nanoparticles to Chlamydomonas reinhardtii. Environ Sci Technol 42(23):8959–8964. https://doi.org/10.1021/es801785m

Nowack B, Bucheli TD (2007) Occurrence, behavior and effects of nanoparticles in the environment. Environ Pollut 150(1):5–22. https://doi.org/10.1016/j.envpol.2007.06.006

Oh WK, Jeong YS, Kim S et al (2012) Fluorescent polymer nanoparticle for selective sensing of intracellular hydrogen peroxide. ACS Nano 6(10):8516–8524. https://doi.org/10.1021/nn204899m

Oukarroum A, Polchtchikov S, Perreault F et al (2012) Temperature influence on silver nanoparticles inhibitory effect on photosystem II photochemistry in two green algae, Chlorella vulgaris and Dunaliella tertiolecta. Environ Sci Pollut Res 19(5):1755–1762. https://doi.org/10.1007/s11356-011-0689-8

Pokhrel LR, Dubey B, Scheuerman PR (2013) Impacts of select organic ligands on the colloidal stability, dissolution dynamics, and toxicity of silver nanoparticles. Environ Sci Technol 47(22):12877–12885. https://doi.org/10.1021/es403462j

Pulido-Reyes G, Briffa SM, Hurtado-Gallego J et al (2019) Internalization and toxicological mechanisms of uncoated and PVP-coated cerium oxide nanoparticles in the freshwater alga:

Chlamydomonas reinhardtii. Environ Sci Nano 6(6):1959–1972. https://doi.org/10.1039/c9en00363k

Qian H, Zhu K, Lu H et al (2016) Contrasting silver nanoparticle toxicity and detoxification strategies in Microcystis aeruginosa and Chlorella vulgaris: new insights from proteomic and physiological analyses. Sci Total Environ 572:1213–1221. https://doi.org/10.1016/j.scitotenv.2016.08.039

Ren B, Jia B, Zhang X et al (2021) Influence of multi-walled carbon nanotubes on enantioselective bioaccumulation and oxidative stress toxicity of indoxacarb in zebrafish (Danio rerio). Chemosphere 267(235):128872. https://doi.org/10.1016/j.chemosphere.2020.128872

Rodrigues DF, Elimelech M (2010) Toxic effects of single-walled carbon nanotubes in the development of E Coli Biofilm. Environ Sci Technol 44(12):4583–4589. https://doi.org/10.1021/es1005785

Röhder LA, Brandt T, Sigg L et al (2014) Influence of agglomeration of cerium oxide nanoparticles and speciation of cerium(III) on short term effects to the green algae Chlamydomonas reinhardtii. Aquat Toxicol 152:121–130. https://doi.org/10.1016/j.aquatox.2014.03.027

oy B, Suresh PK, Chandrasekaran N et al (2020) Antibiotic tetracycline enhanced the toxic potential of photo catalytically active P25 titanium dioxide nanoparticles towards freshwater algae Scenedesmus obliquus. Chemosphere 267:128923. https://doi.org/10.1016/j.chemosphere.2020.128923

Sajid M, Ilyas M, Basheer C et al (2015) Impact of nanoparticles on human and environment: review of toxicity factors, exposures, control strategies, and future prospects. Environ Sci Pollut Res 22(6):4122–4143. https://doi.org/10.1007/s11356-014-3994-1

Sakamoto M, Ha JY, Yoneshima S et al (2015) Free silver ion as the main cause of acute and chronic toxicity of silver nanoparticles to cladocerans. Arch Environ Contam Toxicol 68(3):500–509. https://doi.org/10.1007/s00244-014-0091-x

Sangabathuni S, Murthy RV, Chaudhary PM et al (2017) Mapping the glyco-gold nanoparticles of different shapes toxicity, biodistribution and sequestration in adult zebrafish. Sci Rep 7(1):1–7. https://doi.org/10.1038/s41598-017-03350-3

Saxena P, Harish (2018) Nanoecotoxicological reports of engineered metal oxide nanoparticles on Algae. Curr Pollut Reports 4:128–142

Schwab F, Bucheli TD, Lukhele LP et al (2011) Are carbon nanotube effects on green algae caused by shading and agglomeration? Environ Sci Technol 45(14):6136–6144. https://doi.org/10.1021/es200506b

Sendra M, Sánchez-Quiles D, Blasco J et al (2017) Effects of TiO_2 nanoparticles and sunscreens on coastal marine microalgae: ultraviolet radiation is key variable for toxicity assessment. Environ Int 98:62–68. https://doi.org/10.1016/j.envint.2016.09.024

Sengul AB, Asmatulu E (2020) Toxicity of metal and metal oxide nanoparticles: a review. Environ Chem Lett 18(5):1659–1683. https://doi.org/10.1007/s10311-020-01033-6

Skjolding LM, Kern K, Hjorth R et al (2014) Uptake and depuration of gold nanoparticles in Daphnia magna. Ecotoxicology 23(7):1172–1183. https://doi.org/10.1007/s10646-014-1259-x

Stark WJ, Stoessel PR, Wohlleben W et al (2015) Industrial applications of nanoparticles. Chem Soc Rev 44(16):5793–5805. https://doi.org/10.1039/c4cs00362d

Tang Y, Xin H, Yang S et al (2018) Environmental risks of ZnO nanoparticle exposure on Microcystis aeruginosa: toxic effects and environmental feedback. Aquat Toxicol 204:19–26. https://doi.org/10.1016/j.aquatox.2018.08.010

Tedesco S, Doyle H, Blasco J et al (2010a) Oxidative stress and toxicity of gold nanoparticles in Mytilus edulis. Aquat Toxicol 100(2):178–186. https://doi.org/10.1016/j.aquatox.2010.03.001

Tedesco S, Doyle H, Blasco J et al (2010b) Exposure of the blue mussel, Mytilus edulis, to gold nanoparticles and the pro-oxidant menadione. Comp Biochem Physiol - C Toxicol Pharmacol 151(2):167–174. https://doi.org/10.1016/j.cbpc.2009.10.002

Turan NB, Erkan HS, Engin GO et al (2019) Nanoparticles in the aquatic environment: usage, properties, transformation and toxicity—A review. Process Saf Environ Prot 130:238–249

Van Hoecke K, De Schamphelaere KAC, Ali Z et al (2013) Ecotoxicity and uptake of polymer coated gold nanoparticles. Nanotoxicology 7(1):37–47. https://doi.org/10.3109/17435390.2011.626566

van Pomeren M, Peijnenburg WJGM, Vlieg RC et al (2019) The biodistribution and immunoresponses of differently shaped non-modified gold particles in zebrafish embryos. Nanotoxicology 13(4):558–571. https://doi.org/10.1080/17435390.2018.1564079

Verneuil L, Silvestre J, Mouchet F et al (2015a) Multi-walled carbon nanotubes, natural organic matter, and the benthic diatom Nitzschia palea: "A sticky story." Nanotoxicology 9(2):219–229. https://doi.org/10.3109/17435390.2014.918202

Verneuil L, Silvestre J, Randrianjatovo I et al (2015b) Double walled carbon nanotubes promote the overproduction of extracellular protein-like polymers in Nitzschia palea: An adhesive response for an adaptive issue. Carbon 88:113–125. https://doi.org/10.1016/j.carbon.2015.02.053

Völker C, Boedicker C, Daubenthaler J et al (2013) Comparative toxicity assessment of nanosilver on three Daphnia species in acute, chronic and multi-generation experiments. PLoS One 8(10). https://doi.org/10.1371/journal.pone.0075026

Wang L, Huang X, Sun W et al (2020) A global metabolomic insight into the oxidative stress and membrane damage of copper oxide nanoparticles and microparticles on microalga Chlorella vulgaris. Environ Pollut 258:113647. https://doi.org/10.1016/j.envpol.2019.113647

Wu F, Harper BJ, Crandon LE et al (2020) Assessment of Cu and CuO nanoparticle ecological responses using laboratory small-scale microcosms. Environ Sci Nano 7(1):105–115. https://doi.org/10.1039/c9en01026b

Xin Q, Rotchell JM, Cheng J et al (2015) Silver nanoparticles affect the neural development of zebrafish embryos. J Appl Toxicol 35(12):1481–1492. https://doi.org/10.1002/jat.3164

Xiong JQ, Ru S, Zhang Q et al (2020) Insights into the effect of cerium oxide nanoparticle on microalgal degradation of sulfonamides. Bioresour Technol 309:123452. https://doi.org/10.1016/j.biortech.2020.123452

Ye N, Wang Z, Wang S et al (2018) Toxicity of mixtures of zinc oxide and graphene oxide nanoparticles to aquatic organisms of different trophic level: particles outperform dissolved ions. Nanotoxicology 12(5):423–438. https://doi.org/10.1080/17435390.2018.1458342

Yin E, Zhao Z, Chi Z et al (2020) Effect of Chlamydomonas reinhardtii on the fate of CuO nanoparticles in aquatic environment. Chemosphere 247:125935. https://doi.org/10.1016/j.chemosphere.2020.125935

Yoo MH, Rah YC, Choi J et al (2016) Embryotoxicity and hair cell toxicity of silver nanoparticles in zebrafish embryos. Int J Pediatr Otorhinolaryngol 83:168–174. https://doi.org/10.1016/j.ijporl.2016.02.013

Zhang H, Chen Z, Huang Q (2020a) Study of the toxicity of ZnO nanoparticles to Chlorella sorokiniana under the influence of phosphate: spectroscopic quantification, photosynthetic efficiency and gene expression analysis. Environ Sci Nano 7(5):1431–1443. https://doi.org/10.1039/C9EN01464K

Zhang J, Jiang L, Wu D et al (2020b) Effects of environmental factors on the growth and microcystin production of Microcystis aeruginosa under TiO_2 nanoparticles stress. Sci Total Environ 734:139443. https://doi.org/10.1016/j.scitotenv.2020.139443

Zhao CM, Wang WX (2010) Biokinetic uptake and efflux of silver nanoparticles in Daphnia magna. Environ Sci Technol 44(19):7699–7704. https://doi.org/10.1021/es101484s

Zhao CM, Wang WX (2011) Comparison of acute and chronic toxicity of silver nanoparticles and silver nitrate to Daphnia magna. Environ Toxicol Chem 30(4):885–892. https://doi.org/10.1002/etc.451

Zhao J, Cao X, Wang Z et al (2017) Mechanistic understanding toward the toxicity of graphene-family materials to freshwater algae. Water Res 111:18–27. https://doi.org/10.1016/j.watres.2016.12.037

Chapter 7
Bridging the Gap Between Nanotoxicological Data and the Critical Structure–Activity Relationships

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Abstract The rapid development of nanotoxicology research has led to an exponential increase in data being accumulated and the urgent need of developing computational methods for extracting and processing critical nanostructure-activity relationships from large data sets. During the past several years, artificial intelligence, especially deep learning, has emerged as a powerful method to mine useful information from complex big data, which has been widely used for face recognition, autonomous driving, and medical diagnosis. Inspired by these successes, researchers have successfully applied these technologies in the areas of toxicology. Compared with small molecules, the complexity and diversity of nanostructures lead to many challenges in the application of artificial intelligence to nanotoxicology. Here, we focus on the current status of nanomaterial databases and the applications of artificial intelligence to nanotoxicology research and provide future perspectives on developments that are likely or need to occur in the near future that allow big data and artificial intelligence to make a deeper contribution to nanosafety.

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Introduction

As early as 2003 (Colvin 2003), scientists started to pay attention to the toxicity of nanomaterials. Since then, a massive amount of nanotoxicological data has been generated. However, the underlying mechanisms and the crucial nanostructure-toxicity relationship information have not been fully understood till today. Facing enormous nanotoxicological data and massive emerging data, a logical next step is data mining, which makes use of available and emerging data to analyze the structure–activity relationships and predict the hazards of engineered nanomaterials (Basei et al. 2019; Shatkin 2020).

The rapid development of artificial intelligence (AI) provides an effective way for data mining. In fact, big data and AI complement each other, i.e., the more data, the more sophisticated AI will become. On the other hand, big data is simply useless without AI to analyze it. AI approaches, represented by machine learning and deep learning methods, give the AI system a powerful learning ability, and enable it to quickly and effectively extract useful information from big data in various formats. In recent years, the applications of AI are represented by face recognition (Iqbal et al. 2019; Schofield et al. 2019), self-driving cars (Daily et al. 2017; Grigorescu et al. 2020) and accurate medical diagnosis (Chan et al. 2020; Esteva et al. 2019). Similarly, AI also plays important role in the field of toxicology.

In the past decade, the development of new experimental protocols, especially high-throughput screening (HTS) assays (Barrick et al. 2017; Huang et al. 2016), and the progress of combinatorial chemistry have generated various biological data for millions of compounds (Brenner and Lerner 1992; Corbett et al. 2006). Data sharing projects, such as PubChem (Kim et al. 2016), have made chemical big data publicly available, which have advanced modern toxicology studies into a big data era. The available massive amount of public data brings urgent requests for the development of innovative modeling approaches, driven by the recent progress of AI, which can fulfill the current needs of chemical risk assessment. Combined with the nanotoxicology data, machine learning and deep learning approaches have been applied to construct quantitative nanostructure-activity relationships (QNAR) for risk assessment of nanomaterials (Fourches et al. 2010). Unlike traditional quantitative structure–activity relationships (QSAR) for small molecules, nanomaterials are more complex as they are less well-defined and feature distributions of size, shape and other properties. As a result, the high structural diversity and complexity of nanomaterials typically lead to specific challenges, especially when it comes to the choice of molecular descriptors. More importantly, although massive amounts of nanotoxicology data have been generated, the available nanomaterial databases are extremely scarce and not suitable for machine learning or deep learning (Tropsha et al. 2017). The two main factors mentioned above created a huge gap between the nanotoxicology data and the critical structure–activity relationships.

In this chapter, we describe how to use publicly available databases and AI technologies to convert nanotoxicology big data into critical structure–activity relationship information. We start with a discussion on fundamental concepts of big data,

AI, and QSAR. We then review the current and emerging toxicity databases for small molecules and nanomaterials, as well as the applications of various machine learning and deep learning approaches for toxicity prediction using these databases. Finally, we discuss the developments that are likely to occur or need to occur in the near future that allow big data science and AI technologies to make a deeper contribution to nanosafety.

Big Data, Artificial Intelligence and QSAR

The relationships between big data, AI and QSAR are shown in Fig. 7.1. As described in the introduction, big data and AI are merging into a synergistic relationship. Nowadays, it is increasingly evident that the two areas complement each other in various subdisciplines of chemistry and toxicology. For example, QSAR can be regarded as the application of big data and AI in the area of cheminformatics.

What is big data? Based on the definition of Sagiroglu et al. (Sagiroglu and Sinanc 2015), the term "big data" refers to data sets that are too large or complex, with associated difficulties of storing, analyzing, and visualizing them using traditional data-processing application software. The emergence of big data is accompanied by

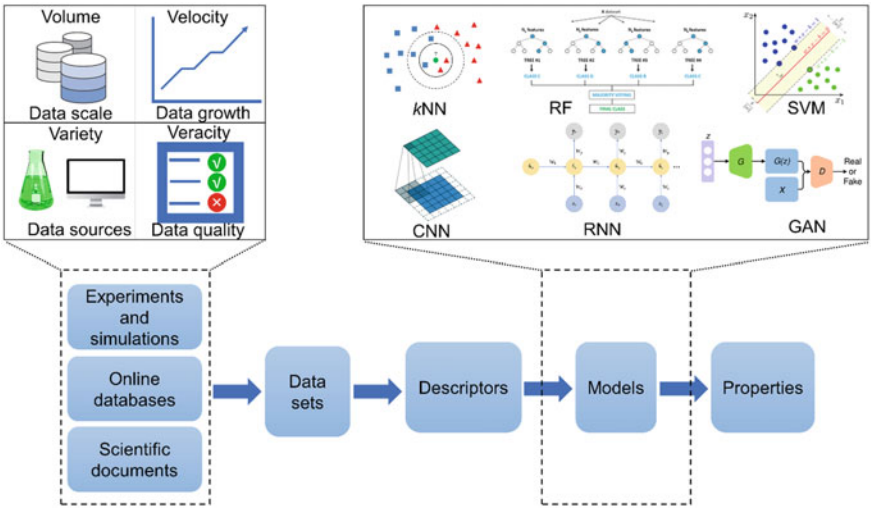

Fig. 7.1 The relationships between big data, artificial intelligence (AI) and QSAR. Big data and AI complement each other, the more data, the more sophisticated AI will become. On the other hand, big data cannot be analyzed without AI. QSAR can be regarded as the application of big data and AI in cheminformatics. Many AI approaches, such as kNN (k-nearest neighbor), RF (random forest), SVM (support vector machine), CNN (convolutional neural network), RNN (recurrent neural network), and GAN (generative adversarial network) can be applied to extract critical structure–activity relationships from chemical toxicity data

the popularization of the Internet, which has been widely used in almost all science and engineering domains at present. The rational use and effective mining of large data sets have become important factors in promoting the development of the related domains. For example, by comparing 50 million common queries entered weekly in the United States with CDC's (Centers for Disease Control and Prevention) data on the seasonal flu spread between 2003 and 2008, the Google engineers successfully predicted the spread of the winter influenza a few weeks before the H1N1 influenza outbreak in 2009 (Ginsberg et al. 2009). Through technological innovation, as well as the comprehensive perception, collection, arrangement, analysis and sharing of data, big data in combination with AI provides people with a new way to see and transform the world.

While bringing opportunities, the available big data for related domains also brings new challenges. The challenges raised by big data are known as the "four Vs": volume (scale of data), velocity (growth of data), variety (diversity of sources), and veracity (uncertainty of data). Compilation of large amounts of data generated daily and shared through public databases, such as PubChem and ChEMBL, represent the diversity of data. For example, as of June 2021, the PubChem database included around 110 million compounds, 271 million substances, and 293 million bioactivities. It is not feasible to apply traditional computational approaches or even personal computers (PCs) to deal with data with this kind of volume for modeling purposes. Currently, most data depository portals (e.g., PubChem) gather data from diverse sources, such as journal publishers, academia, and government agencies, which define the variety of data. Furthermore, veracity reflects the degree of uncertainty inherent to data from different sources and requires novel technologies for data curation and management.

The challenges to using big data discussed above and the involvement of new types of data (e.g., audio and images) have given birth to the rapid development of computer hardware and new AI technologies. The term "artificial intelligence" was first proposed by John McCarthy at the Dartmouth Conference in 1956. In this conference, AI was described as "the science and engineering of making intelligent machines" (Buchanan 2006). Machine learning is a subset of AI and it provides the ability to automatically learn and improve from experience without being explicitly programmed (Ongsulee 2017). Deep learning is a specific subset of machine learning. It consists of neural networks of hidden layers and complex architectures (LeCun et al. 2015). The most important advantage that the deep learning methods have over traditional machine learning methods is not their ability to generate superior models, but their ability to automatically extract useful representations from training data, a step called feature engineering. Traditional machine learning methods contain random forest (RF), support vector machine (SVM), and k-nearest neighbor (kNN), while the deep learning methods are represented by a convolutional neural network (CNN), recurrent neural network (RNN) and generative adversarial network (GAN). Nowadays, machine learning and deep learning are the main approaches to achieve AI, which has been widely used to extract useful scientific information from various big data of drug discovery, material design and other areas.

Impacts have been continuously made by AI. On February 21, 2020, the journal Cell published a research paper by James Collins' team of the Massachusetts Institute of Technology (MIT) Institute of Medical Engineering and Science (Stokes et al. 2020). The team used deep learning to build an AI prediction platform, which screened out potential antibiotics from more than 107 million molecules in the ZINC15 database. One of the resulting hits was a potent antibiotic, which they named halicin, and which showed broad-spectrum antibiotic activity in mice, effectively killing superbugs that are resistant to all known antibiotics. On May 5, 2016, a research paper about machine-learning-assisted materials discovery using failed experiments appeared on the cover of the Nature journal (Raccuglia et al. 2016). In this study, the authors used data of failed or unsuccessful hydrothermal syntheses collected from archived laboratory notebooks from their laboratory, to train a machine learning model to predict reaction success. They found that the resulting accuracy exceeded that of experienced chemists. As a result, a question was asked: "Can AI create the next wonder material?" (Nosengo 2016). Some researchers believe that machine learning will change traditional material discovery methods, and AI will bring revolutionary changes to material science. Toxicological research has also greatly benefited from the development of big data science and AI technologies (Cherkasov et al. 2014).

QSAR models are developed by establishing linear or non-linear relationships that connect experimentally measured properties or biological activities with a set of chemical descriptors that encode the molecular structures. Once a series of predicted models are obtained, they can be used for database mining to identify novel chemical compounds with desired properties. Historically, QSAR modeling has been applied to computer-aided drug discovery since it first appeared in the scientific literature in 1962 (Hansch et al. 1962). Since then, the field has grown and evolved substantially, building on the successful use of QSAR modeling in environmental chemistry, protein design, organic synthesis, and material discovery. QSAR modeling has proved to be very useful in cases of the "classic" chemicals, such as small organic molecules, inorganic crystals, peptides, and even proteins. However, the development of the QNAR, also called "nano-QSAR", is greatly hindered by the diversity and complexity of nanostructures. The concept of QNAR was first proposed by Fourches et al. in 2010 (Fourches et al. 2010). Like traditional QSAR, the QNAR models are based on the assumption that similar nanomaterials will induce similar biological effects. Similar to the traditional QSAR method, QNAR mainly comprises the following steps: compiling a dataset, calculating the nanodescriptors, constructing machine learning models, and validating the models. As a result, the QNAR approach enables the encoding of existing knowledge from nanomaterial data sets into predictive models, which directly correlate the nanostructures with bioactivities or toxicities of nanomaterials. The role of QNAR in predictive nanotoxicology can be summarized as follows: (1) to predict the potential toxicity of nanomaterials, (2) to provide HTS with predicted nanomaterials with desired properties, and (3) to help understand the underlying toxicity mechanisms.

Small Molecule Databases and Data Mining

Current Small Molecule Databases

Over the past decades, there has been a rapid growth in the amount of toxicity data based on the developments of combinatorial chemistry and HTS. As a novel synthesis strategy (Brenner and Lerner 1992), combinatorial chemistry provides a powerful tool for the rapid creation of large numbers of organic or inorganic compounds, which have been used as valuable sources for the discovery of drugs and advanced functional materials. The progress of combinatorial chemistry has also stimulated the development of HTS technologies. HTS is the use of automated equipment to rapidly test thousands to millions of compounds for bioactivity, or toxicity at organism, cellular, or molecular levels (Hertzberg and Pope 2000). Recently, HTS has become increasingly popular in toxicological research because it tests the toxicity of substances rapidly and greatly reduces the cost of experimental testing (Macarron et al. 2011).

A significant HTS effort in toxicology is the research program entitled Toxicity Forecaster (ToxCast), which was initiated in the United States Environmental Protection Agency (EPA) in 2006 (Dix et al. 2007). The main purpose of this program is to employ a battery of in vitro HTS assays to quickly evaluate the toxicity of compounds and prioritize limited testing resources toward chemicals that likely represent the greatest hazard to human health and the environment. Although ToxCast contains more than 700 bioassays, the number of chemicals is limited. To this end, another big collaborative program called Tox21, was launched by the National Toxicology Program (NTP), the EPA National Center for Computational Toxicology (NCCT), and the National Institutes of Health (NIH) Chemical Genomics Center (NCGC) in 2008. As of 2018, the Tox21 program generated over 120 million data entries for approximately 8500 chemicals.

Data collected and curated from existing data are another type of big toxicity data source. As of June 2021, 479,398 results were retrieved from the Web of Science using "chemical*" and "toxicity*" as keywords. Together with scientific publications, patents, conference reports, and dissertations also constitute valuable information for chemical toxicity. The extracted information can be used to create and greatly enrich databases of chemical structures, properties, and bioactivities. However, data sets from multiple sources are called unstructured data and they may differ significantly regarding chemical names, experimental conditions, and even endpoints. Thus, it is essential to ensure the machine learning or deep learning models are built with high-quality data.

Current publicly available databases relevant to chemical toxicity, obtained from HTS programs, scientific documents, and other sources are summarized in Table 7.1. Based on the applications, these databases can be classified into two main categories: (1) comprehensive databases of chemical collections (e.g., PubChem and ChEMBL), and (2) databases designed for a specific purpose (e.g., LTBK and EcoTox). Most databases are being updated frequently and regularly maintained by specialized agencies, and the amount of data is also increasing rapidly with the above-mentioned HTS

Table 7.1 Selected available toxicity databases for small molecules

Name	Description	Size (as of June 2021)	Web site
PubChem	The world's largest public repository for information on chemical substances and their biological activities	Over 110 million compounds	https://pubchem.ncbi.nlm.nih.gov/
ChEMBL	A manually curated database of bioactive molecules with drug-like properties	Over 2.1 million compounds	https://www.ebi.ac.uk/chembl/
EcoTox	A comprehensive, publicly available database providing single chemical environmental toxicity data on aquatic life, terrestrial plants and wildlife	12,326 compounds	https://cfpub.epa.gov/ecotox/
T3DB	Toxin and Toxin Target Database or Toxic Exposome Database combines detailed toxin data with comprehensive toxin target information	3678 compounds	http://www.t3db.ca/
ACToR	Aggregated Computational Toxicology Online Resource is the warehouse for EPA's web applications which can be used to explore and visualize complex computational toxicology information	8,932,820 compounds	https://actor.epa.gov/actor/home.xhtml
CTD	Comparative Toxicogenomics Database, a robust, publicly available database that aims to advance understanding about how environmental exposures affect human health	13,378 compounds	https://ctdbase.org/

(continued)

Table 7.1 (continued)

Name	Description	Size (as of June 2021)	Web site
DrugMatrix	One of the world's largest toxicogenomics reference resource containing large-scale gene expression data	Over 600 different compounds	https://ntp.niehs.nih.gov/data/drugmatrix/
CEBS	Chemical Effects in Biological Systems, a free public database of toxicology and toxicogenomics studies involving genetic, carcinogenic, and short-term toxicity	Over 11,000 compounds	https://manticore.niehs.nih.gov/cebssearch
LTKB	Liver Toxicity Knowledge Base, a project at the FDA's National Center for toxicological research to study drug-induced liver injury	1036 compounds	https://www.fda.gov/science-research/bioinformatics-tools/liver-toxicity-knowledge-base-ltkb
WITHDRAW	A database of withdrawn and discontinued drugs that were pulled out of global markets due to safety concerns	578 compounds	http://cheminfo.charite.de/withdrawn/
BindingDB	A public database of measured binding affinities, mainly focusing on the interactions of proteins considered to be drug-targets with small molecules	989,383 compounds	https://www.bindingdb.org/bind/index.jsp

assays and data collection. For example, from 2008 to June 2021, the number of compounds in PubChem increased over four-fold from 25.6 million to 110 million. Combined with the fast-growing toxicity data, various machine learning and deep learning approaches are being employed to advance the development of computational toxicology, which can complement experimental toxicity tests to predict toxicity, prioritize chemicals, and also minimize late-stage failures in drug design. Next, we will mainly review machine learning using the above-mentioned big data in computational toxicology, which has built prediction models for cytotoxicity, organ damage, acute toxicity, ecotoxicity and other subareas of toxicology.

The Applications for Toxicity Prediction Using AI Approaches

The cytotoxicity of chemicals often results in cell death, cell lysis or cell growth inhibition. Several machine learning approaches have been applied for the prediction of compound cytotoxicity based on in vitro data. For example, Svensson et al. (2017) applied the conformal prediction with random forest to model the cytotoxicity of compounds collected from PubChem. The results showed that conformal prediction can be a useful tool for modeling the large-scale imbalanced data, i.e., the toxic compounds only accounted for an average of 0.8% of the whole dataset of the chemicals. In another study, the authors developed machine learning models to predict the 50% growth inhibition bioassay endpoint of 17,142 compounds by integrating chemical structure and cell line information (Cortés-Ciriano et al. 2016), and the model performance significantly outperformed those of previous studies. Similarly, several studies also showed that the addition of bioactivity descriptors can increase the performance of QSAR modeling and the model can also be more interpretable (Irwin et al. 2020; Norinder et al. 2020; Whitehead et al. 2019).

Various machine learning models have been developed to predict organ damage, such as cardiotoxicity and hepatotoxicity. Blockage of the hERG potassium channel is the main adverse effect related to cardiotoxicity. As a result, several in silico models were developed to assess the drug cardiotoxicity according to the in vitro hERG potassium channel assays. For instance, Zhang et al. (2016) developed various machine learning models to discriminate hERG blockers from non-blockers, in which 1570 unique compounds were collected from ChEMBL database and scientific publications. In addition, the combination of pharmacophores and machine learning methods improved the predictive capabilities of models for evaluating hERG blockage and also provided insights into the mechanisms (Wang et al. 2016).

In drug discovery, chemical hepatotoxicity, also termed "drug-induced liver injury", is the most common reason for a drug to be withdrawn from the market. Thus, many in silico tools have been proposed to improve the hepatotoxicity prediction of drug candidates. In 2016, Mulliner et al. (2016) constructed so far the most extensive dataset comprising 3712 compounds with liver-related toxicity findings in humans and animals, which were collected from publications. Based on this dataset, various machine learning and deep learning models have been developed for chemical hepatotoxicity prediction (Asilar et al. 2020; Feng et al. 2019; Liu et al. 2018; Mora and Marrero-ponce 2020; Zhao et al. 2020). A deep learning model based on gene expression data was developed to accurately predict drug-induced liver injury. Animal experiments were also conducted to verify the predicted results, which provided safety information for drug discovery in the early stage (Feng et al. 2019).

In addition, based on the datasets of acute toxicity (e.g., acute oral toxicity and acute dermal toxicity) (Xu et al. 2017; Zhu et al. 2009), ecotoxicity (e.g., biodegradation half-life and aquatic toxicity) (Cheng et al. 2012; Sun et al. 2015) and endocrine disruption (e.g., estrogen and androgen receptor binding) (Du et al. 2017; Russo et al. 2018), various machine learning and deep learning methods have been developed

and applied to construct classification and regression models to predict the corresponding endpoints and analyze the related toxicity mechanisms. More importantly, recent efforts are directed to building quantitative approaches to translate in vitro toxicity potencies to equivalent in vivo doses using in vitro−in vivo extrapolation (IVIVE) techniques (Huang et al. 2016; Sipes et al. 2017), aiming to predict the potential human health risk.

Nanomaterial Databases and Nanotoxicity Prediction

Current Status of Nanomaterial Databases

As described in the introduction, data-driven machine learning in nanotoxicology is greatly hindered by the lack of available nanomaterial databases and suitable nanodescriptors. There is a huge gap between nanotoxicology big data and critical nanostructure-activity relationships. Despite the great promise of data-driven methods, the limitation in most studies is the small size of data sets of nanomaterials (e.g., usually less than 50). The limited availability of larger nanotoxicological data sets has in a significant number of studies using the same data sets generated by a few labs. In fact, there are only dozens of data sets currently widely used for machine learning. This gap between data generation and available data sets calls for urgent action to accelerate data collection and deposition into public databases. Recently developed EU-US Nanoinformatics Roadmap 2030 envisages a flow of data from experimentalists into structured databases that can be used by computational modelers to predict nanomaterial properties, exposure and hazard values that will support regulatory actions (Haase and Klaessig 2018).

Several other efforts have been made to create curated databases for storing emerging research results regarding nanotoxicology. As early as 2006, a data portal named the Cancer Nanotechnology Laboratory (caNanoLab) (Gaheen et al. 2013) was initiated by the U.S. National Cancer Institute (NCI) as a collaborative effort between the NCI Center for Biomedical Informatics and Information Technology (CBIIT) and the NCI Office of Cancer Nanotechnology Research (OCNR). The data portal was designed to address the needs of nanotechnology in cancer diagnostics and therapeutics through the collection and analysis of nanotechnology data. The caNanoLab includes information on nanomaterial composition (e.g., carbon, metal oxide, and lipid-based nanomaterials), physicochemical characterization (e.g., size, solubility, and zeta potential), in vitro toxicity (e.g., cytotoxicity, immunotoxicity, and oxidative stress) and in vivo toxicity (e.g., blood clearance, organ weight measurement, and half-lethal concentration or LC_{50} values) for about 1,378 nanomaterials. However, the caNanoLab is not fully accessible to the public due to some proprietary data. Recently, as a forum of EU-funded projects addressing the safety of nanomaterials and nanotechnology, the EU NanoSafety Cluster proposed to construct large-scale nanomaterial databases for transparent data sharing and data analysis, and

create computational nanotoxicology models to support the nanosafety community (Oomen et al. 2014). These efforts are also aligned with other international research efforts including those in the United States, through the EU-US nanoEHS (environment, health and safety) platform (Haase and Klaessig 2018). Under this platform, several nanomaterial databases have been developed, such as eNanoMapper (Jeliazkova et al. 2015), and NANoREG (Jantunen et al. 2018). The eNanoMapper is a comprehensive database, which was constructed by the 7th framework program for European research (EU FP7) project (Lewandowski et al. 2016) to develop a computational framework for nanotoxicity data management. The eNanoMapper contains three major parts: (1) the ontology for common vocabulary terms used in nanosafety research; (2) the database for data sharing and search which covered 7,189 nanomaterial entities until August 2020; and (3) various nanomaterial modeling tools (e.g., RRegrs (Tsiliki et al. 2015), Jaqpot Quattro (Chomenidis et al. 2017) and Nano-Lazar (Helma et al. 2017)) for toxicity prediction. Many other nanomaterial databases, such as NANoREG, NanoReg2 and caLIBRAte are also publicly available in eNanoMapper. Nanomaterial databases and their specific research goals are summarized in Table 7.2. For instance, the Safe and Sustainable Nanotechnology (S2NANO) database was designed to develop and commercialize safe and sustainable nano-products (Trinh et al. 2018). However, compared with the above-mentioned toxicity databases for small molecules, the current nanomaterial databases are still quite small. This is mainly due to the fact that high throughput synthesis and characterization of nanomaterials have not been adopted in the areas of nanotoxicology due to the complexity and diversity of nanomaterials. As a result, most of the data are from "one-nanomaterial-at-a-time" research. Due to the lack of uniform experimental standards from laboratory to laboratory, the data quality is poor, resulting in contradictory or self-contradictory results. These data are difficult to be used for machine learning.

More importantly, nanomaterial entities (e.g., composition, physicochemical properties, and biological activities of the nanomaterials) in these databases exist as text outputs extracted directly from publications, ignoring nanostructure digitalization that is critical for modeling studies. Nanostructure digitalization or nanostructure annotation is a process that converts nanostructures into digital information stored in electronic files. For example, as shown in Fig. 7.2, the PubChem and Protein Data Bank databases apply SDF and PDB file formats respectively to encode the structure information (e.g., atomic types, atomic coordinates and chemical bonds) of small molecules and biological macromolecules. However, none of the above-mentioned nanomaterial databases contains such electronic files. To this end, in our recent study (Yan et al. 2020a), we adopted PDB files for nanostructure digitalization. With these electronic files, the detailed three-dimensional nanostructures can be visualized through special software (e.g., VMD, MOE and Jmol). In addition, the nanodescriptors representing the whole nanostructures can be calculated from the PDB files, while variables (e.g., physicochemical properties) used in previous modeling studies are mostly experimentally generated. The electronic files for more than 700 unique nanomaterials, combined with the corresponding physicochemical

Table 7.2 Selected available nanomaterial databases

Name	Description	Size (as of June 2021)	Website
caNanoLab	A public repository for the storage and sharing of well-characterized biomedical nanomaterial data and information	6036 data points	https://cananolab.nci.nih.gov/
eNanoMapper	A Database and Ontology Framework for Nanomaterials Design and Safety Assessment	7189 data points	http://www.enanomapper.net/
S^2NANO	Core Database: data collected from S^2NANO's measurement and analysis protocol-based experiments for physicochemical properties and in vitro cytotoxicity, Extended Database: literature-collected data	33,393 rows of raw data obtained from literature mining and protocol-based experiments	http://portal.s2nano.org/
Nanomaterial registry	A database that captures the minimal information about nanomaterial physicochemical characteristics	2031 data points	http://nanohub.org/
NANoREG Toolbox	Output of the project "A common European approach to the regulatory testing of nanomaterials", includes tools for the safety assessment of nanomaterials	2940 data points	http://www.nanoreg.eu/
NanoDatabank	Contains information about nanomaterial properties, experimental and simulation datasets of nanomaterial fate and transport, as well as toxicity data	Over 1000 data points	http://nanoinfo.org/nanodatabank/

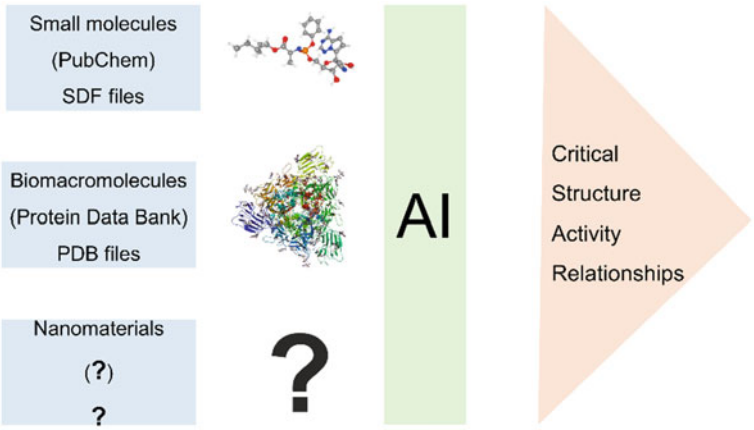

Fig. 7.2 Nanostructure digitalization is indispensable for the nanomaterial database. Electronic files storing the molecular structure information (e.g., atom type and atomic coordinates) can be used for structure visualization, machine learning and molecular simulation

properties and biological activities, and also the detailed information about experimental protocols, are publicly available in the PubVINAS database (http://www.pubvinas.com/). Currently, more than half of the data are generated from in-house nano-combinatorial libraries and HTS of these libraries. These technologies ensure the data quantity and quality, which will be discussed in detail in the Conclusions and Perspectives. Other data comes from strict data curation of massive scientific publications. Users can also share their new data (e.g., new nanomaterials synthesized and/or tested in bioassays) by uploading them as a text file. After reviewing the uploaded files, the system administrator generates the PDB files and adds the new data sets to the PubVINAS database.

The Inadequacy of Appropriate Nanodescriptors

Whether it is traditional QSAR modeling for small molecules or QNAR modeling for nanomaterials, the descriptors are the core factors affecting the model performance. The nanodescriptors, which have been used in machine learning models for nanomaterials, can be classified as experimental, empirical, and structural descriptors. Important experimental variables to describe the characteristics of nanomaterials, such as nanomaterial size (Liu et al. 2011), zeta potential (Cho et al. 2012), and magnetic properties (Fourches et al. 2010), have been used as nanodescriptors. Other experimental descriptors extracted from transmission electron microscopy (TEM) (Bigdeli et al. 2014) and spectral images (Borders et al. 2013) have also been used in previous studies. However, these experimental descriptors relied on some specific laboratory conditions that may not be reproducible. More importantly, it is not feasible to acquire

experimental properties for new nanomaterials not yet synthesized. Therefore, these descriptors cannot be used for virtual screening.

Empirical nanodescriptors, usually involving the applications of quantum chemistry (Puzyn et al. 2011) or molecular simulations (Ahmed et al. 2017), have also been used in previous machine learning models. However, the calculation of empirical nanodescriptors requires strong expertise. One needs to be familiar with various structural parameters (e.g., space group and unit cell parameters) of nanomaterials to select appropriate force fields (e.g., AMBER, CHARMM and OPLS) and calculation methods (e.g., ab initio, semi-empirical and density functional theory). In addition, the calculation of these empirical descriptors often involves the use of complex quantum chemistry (e.g., VASP and Gaussian) or molecular simulation (e.g., GROMACS and NAMD) software. These, combined with the high demand for computational resources, limit the wide application of these empirical nanodescriptors. Chemical structural descriptors mainly calculated from surface ligands of nanomaterials (Fourches et al. 2016), cannot fully represent the entire nanostructures, such as ligand densities, nanomaterial types, shapes and sizes. Recently, we developed novel nanodescriptors by employing Delaunay tessellation and atomic properties (Yan et al. 2019). The novel nanodescriptors can not only represent the nanostructure diversity but also have fast and high-throughput calculation characteristics. For 5-nm gold nanoparticles with more than 26,000 atoms, the calculation can be completed in 10 s on a regular PC. The utility has been validated by accurately predicting various physicochemical properties and biological activities of nanoparticles (Yan et al. 2019).

The Applications of AI for Nanotoxicity Prediction

Despite the above challenges, several machine learning and deep learning models have been developed to uncover the critical nanostructure-activity relationships from nanotoxicology data. As described above, most predictive models are built on very small data sets for one material type. For example, Puzyn et al. (2011) used multiple linear regression and quantum chemical descriptors to predict the toxicity of 17 different metal oxide nanoparticles to *Escherichia coli*. By analyzing the model results, they found that the descriptor representing the enthalpy of formation of a gaseous cation had the greatest impact on the model performance, which can be used to explore the potential toxicity mechanism. Similarly, Liu et al. (2013) applied six different machine learning methods to build QNAR models to predict the cytotoxicity of 24 metal oxides nanoparticles using a series of experimental and calculated descriptors. They identified the ionic index (Z2/r, in which Z and r are charge number and ionic radius of metal cations in the nanoparticle crystals, respectively) and the conduction energy descriptors as toxicity-relevant correlating parameters for metal oxide nanoparticles. Undoubtedly, the predictive ability of the models is limited, and can only predict the properties of one material type (e.g., metal oxide nanomaterials). Although some studies built predictive models based on

relatively larger data sets, the usage of inappropriate nanodescriptors also limited the applications of the models. For instance, in 2010, Fourches et al. (2010) developed kNN and SVM classification models to predict cytotoxicity of 51 nanomaterials using experimental descriptors. This is also one of the first studies to use machine learning for nanotoxicity prediction. In 2014, Walkey et al. (2014) synthesized a library of 105 surface-modified gold nanoparticles and characterized the serum protein corona fingerprint formed around these nanoparticles. Using these fingerprint descriptors and multiple linear regression methods, the authors developed QNAR models to accurately predict the cell association of the gold nanoparticles. Obviously, these models cannot be used for virtual screening as described above. More importantly, these studies did not implement external experiments to verify the predictive abilities of the models. Therefore, the actual application value of these models is debatable.

In recent years, our laboratory synthesized a series of carbon nanotube and gold nanoparticle libraries through nano-combinatorial chemistry methods. These datasets of physicochemical properties and biological activities have been widely used for machine learning. For example, Fourches et al. (2016) used RF, kNN, and SVM methods and descriptors calculated from surface ligands to build QNAR models for classifying various toxicity endpoints (i.e., protein binding, cell viability and cell proliferation) of 80 carbon nanotubes. Based on these machine learning models, 10 putatively active and 10 putatively inactive carbon nanotubes with the ligands were prioritized by virtual screening and were synthesized and tested. Similarly, Wang et al. (2017) developed novel nanodescriptors using surface chemistry simulation of virtual nanostructures to build machine learning models for predicting cellular uptake, ability to induce oxidative stress and hydrophobicity of 34 gold nanoparticles. The models were also used to predict the properties of seven newly designed virtual nanostructures, and the predicted results were validated experimentally. Recently, the deep neural network has been used for the first time to predict the in vivo fate (i.e., half-life, spleen accumulation and liver accumulation) of nanomaterials (Lazarovits et al. 2019).

In summary, compared with small molecules, the application of AI to nanotoxicity prediction is still in its infancy. The future tasks include in vivo toxicity prediction and mixtures' toxicity prediction, among others. In the next part, we will discuss future directions to accelerate the transformation of nanotoxicity data to critical nanostructure-activity relationships.

Conclusion and Perspectives

To truly realize the transformation from nanotoxicology big data to useful information, the roadblocks (e.g., paucity of data sets and inadequacy of nanodescriptors) to the application of machine learning to nanosafety should be removed or reduced. We propose that, by extending the development of machine learning and deep learning methods used in the field of small molecule toxicity to nanotoxicity, the field can be advanced in several directions in the future (Fig. 7.3). We envision that these

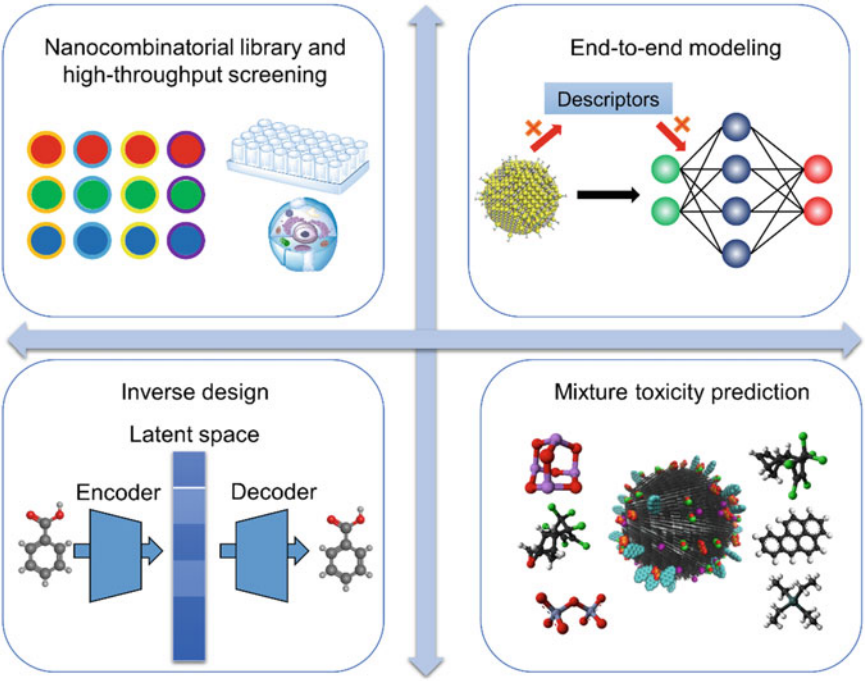

Fig. 7.3 The future development directions proposed for computational nanotoxicology. Upper left panel: the nano-combinatorial libraries and high-throughput screening of these libraries can provide high-quality nanomaterial data for machine learning and deep learning. Upper right panel: the end-to-end deep learning can build direct relationships between nanomaterial properties and the nanostructures without complicated nanodescriptors calculation. Lower left panel: the inverse design can make nanomaterial design more targeted. Lower right panel: the toxicity predictions of mixtures have practical significance

suggestions will prompt the fast development of machine learning and deep learning methods to generate impact in this area.

Continuous Generation of Modeling-Friendly Data Sets Using Nano-combinatorial Chemistry and High Throughput Screening

Nanomaterials with structural diversity and unified experimental standard biological effects are important prerequisites for ensuring the construction of a machine learning model with good robustness and generalization ability, which can be achieved through nano-combinatorial libraries and HTS of these libraries. The nano-combinatorial

library of nanomaterials can be achieved by programming diverse physicochemical parameters, including morphology, composition, and surface properties. In the past decade, researchers have employed high-throughput methodologies to investigate correlations between the physicochemical properties of nanomaterials and their biological activities. These methodologies include the synthesis of a large collection of nanomaterials (Weissleder et al. 2005) or well-designed combinatorial libraries (Zhou et al. 2008). Our laboratory is one of the earliest research groups that carried out nano-combinatorial chemistry research: (1) In 2008, Zhou et al. (2008) designed a nanotube library containing 80 multiwalled carbon nanotubes with surface chemistry modified by small molecules with diverse physicochemical properties. The nanotube library has been used to modulate cytotoxicity, immunotoxicity, autophagy, cell differentiation, and perturbations to the CYP3A4-enzyme in the liver in systematic ways. (2) Similarly, in 2010, Zhou et al. (2011) developed a library of 30 gold nanoparticles that display a diverse array of surface chemistries and used these to probe cell recognition. 3) By gradual alteration of only one property while keeping other properties essentially unchanged, a library containing 17 gold nanoparticles with gradually increasing positive or negative charges was designed by Su et al. (2012). 4) In 2018, 41 gold nanoparticles with a univariant gradient change in single physicochemical property, such as charge density, hydrophobicity, or hydrogen bond density, were designed by Sun et al. (2018). 5) Recently, Bai et al. (2020) synthesized a comprehensive library of 36 nanoparticles with all combinations of three types of core materials (Au, Pt and Pd), two sizes (6 and 26 nm), and each conjugated with one of six surface ligands of different hydrophobicity. Other nanomaterial libraries can be seen in our previous studies or the PubVINAS nanomaterial database (http://www.pubvinas.com/), and these nano-combinatorial library data sets have been widely used for machine learning (Fourches et al. 2016; Le et al. 2015; Wang et al. 2017).

Development of End-to-End Deep Learning Models for Nanotoxicity Prediction

Compared with the traditional machine learning methods, a major advantage of the deep learning methods is automatic feature extraction, which greatly promotes the development of AI. For example, the convolutional neural network can be used to automatically extract image features for face recognition (Lawrence et al. 1997). The word2vec algorithm uses neural networks to automatically learn word associations for natural language processing (Ma and Zhang 2015). Inspired by these accomplishments, researchers are now applying these technologies to automatically extract features from molecular representations (e.g., images and SMILES) without descriptor calculation. For instance, Cortés-Ciriano and Bender (2019) developed a set of convolutional neural network architectures for the prediction of compound cytotoxicity from their Kekulé structure representations that require no generation of compound descriptors or use of sophisticated image processing techniques. Similarly,

Asilar et al. (2020) applied a convolutional neural network to predict liver toxicity with chemical images as input, which built direct correlations between chemical structures and their properties without the calculation of molecular descriptors. We can learn from these methods to build end-to-end deep learning models for nanotoxicity prediction. In our recent study (Yan et al. 2020b), we applied nanostructure images and a convolutional neural network for end-to-end modeling of nanoparticle activities and properties. However, we need to keep in mind that only with the above-mentioned nanostructure digitalization can the nanostructures be transformed into images or other suitable forms of data.

Inverse Design of New Nanomaterials

One of the important aims of machine learning models is to identify novel nanomaterials that have certain desirable properties, such as low toxicity or desirable bioactivities. Currently, the main way machine learning achieves this is by predicting the properties of a large number of virtual nanomaterials, a process that is also called "high-throughput virtual screening" (Pyzer-Knapp et al. 2015). Although this approach has led to beneficial new materials, it still faces great challenges, such as: (1) the construction and screening of a large virtual database are time-consuming and inefficient as the number of resources required is disproportionally large compared to the small number of hits discovered; (2) many materials in the database are far from the domains of applicability of the models and this leads to inaccurate predictions. Recent advances in deep learning (i.e., deep generative models) have changed the above landscape allowing the generation of new molecules and materials for efficient exploration. Deep generative models mainly contain two parts, an encoder converting the discrete representation of a molecule or material (e.g., 3D geometry structure, SMILES or images) into latent variables or descriptors, and a decoder allowing the latent descriptions to be inverted to generate structures for new molecules or materials with improved properties (Gómez-Bombarelli et al. 2018). The molecular inverse design method is different from the traditional method in that it no longer derives properties from the structure, but pre-selects the property parameters, and infers unknown molecules that meet these properties through the reverse mapping between the structure and the properties. Recently, researchers have started to apply these techniques to target user-desired properties before molecules or materials synthesis. For example, Kim et al. (2020) developed a generative adversarial network, called ZeoGAN to implement the inverse design of crystalline porous materials with desired heat of adsorption of methane. Recently, Kotsias et al. (2020) developed a conditional recurrent neural network for molecular generation, which tackles the inverse design problem by directly shaping the properties of the generated molecules. However, there are so far rare examples of the application of inverse design for nanomaterials.

Toxicity Prediction of Nanomaterials Combined with Emerging Contaminants

In recent years, anthropogenic nanomaterials have been detected in different environmental compartments, such as in the atmosphere and surface water. Inevitably, the occurrence and distribution of nanomaterials in the environment has led to interactions with chemical substances and may produce mixed effects in environmental organisms by acting as carriers of various organic chemical substances and heavy metals. Therefore, studies focusing on the combined toxicity of nanomaterials with chemical substances, especially emerging contaminants, are indispensable. As mentioned above, although various machine learning models have been built for toxicity prediction, almost all studies focus on individual compounds or nanomaterials and there are still no mature methodologies that could be directly used to model properties of mixtures. The application of machine learning in mixture toxicity prediction is mainly limited by the following factors. Firstly, lack of reliable data poses one of the biggest challenges even for chemical mixtures of small molecules; the limited and sparse data has been summarized in a previous study (Cherkasov et al. 2014). Secondly, there is a lack of appropriate descriptors to describe the properties of mixtures. Cherkasov et al. (2014) summarized all previously used descriptors, which can be classified as experimental descriptors and three different calculated descriptors. The shortcomings of the experimental descriptors are not repeated here, but the major limitation is the aspect that all calculated descriptors characterize individual compounds in the mixture independently. The third limitation is the lack of rigorous external validation. Commonly, both training and test sets include data points of the same mixture with different molar fractions. As a result, the model's true predictive performance is not estimated properly. Thus, the main tasks to overcome these limitations in the prediction of mixture toxicity of nanomaterials and other chemical substances, are: (1) to generate more toxicity data regarding nanomaterials combined with emerging contaminants, (2) to develop novel descriptors representing the combined properties of mixtures, such as in our previous study (Liu et al. 2020), where we applied the tetrahedral descriptor to describe the properties of carbon nanoparticles combined with various contaminants, and (3) to implement more rigorous external validation, for example, (i) the prediction of the investigated properties for any composition of the mixture from the training set, and (ii) the prediction of the investigated properties for mixtures formed by novel pure compounds absent in the training set.

In summary, we discussed how to apply AI approaches to bridge the gap between nanotoxicology data and critical nanostructure-activity relationships. The main issues holding the field back are the relative paucity of high-quality data that can be used to train and validate models and the need for better descriptors to encode nanomaterials properties. We advocate generating data sets suitable for machine learning through nano-combinatorial libraries and HTS of the libraries. In addition, we propose to

construct universal nanodescriptors for nanostructure representation or to build end-to-end deep learning models without nanodescriptors calculation. For this, nanostructure digitalization is a necessary prerequisite. In a recent study (Yan et al. 2020a), we constructed a proof-of-concept nanomaterial database containing electronic files of various nanostructures, which were designed for machine learning. In the future, we plan to construct a more comprehensive nanomaterial database that will contain more electronic files of nanostructures, including cell images, physicochemical properties, biological activity data as well as omics data. We believe that the combination of a comprehensive nanomaterial database and AI approaches will accelerate the conversion of nanotoxicity data to useful information.

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References

Ahmed L, Rasulev B, Kar S et al (2017) Inhibitors or toxins? Large library target-specific screening of fullerene-based nanoparticles for drug design purpose. Nanoscale 9:10263–10276

Asilar E, Hemmerich J, Ecker GF (2020) Image based liver toxicity prediction. J Chem Inf Model 60:1111–1121

Bai X, Wang S, Yan X et al (2020) Regulation of cell uptake and cytotoxicity by nanoparticle core under the controlled shape, size, and surface chemistries. ACS Nano 14:289–302

Barrick A, Châtel A, Bruneau M et al (2017) The role of high-throughput screening in ecotoxicology and engineered nanomaterials. Environ Toxicol Chem 36:1704–1714

Basei G, Hristozov D, Lamon L et al (2019) NanoImpact making use of available and emerging data to predict the hazards of engineered nanomaterials by means of in silico tools: a critical review. NanoImpact 13:76–99

Bigdeli A, Hormozi-Nezhad MR, Jalali-Heravi M et al (2014) Towards defining new nano-descriptors: extracting morphological features from transmission electron microscopy images. RSC Adv 4:60135–60143

Borders TL, Fonseca AF, Zhang H et al (2013) Developing descriptors to predict mechanical properties of nanotubes. J Chem Inf Model 53:773–782

Brenner S, Lerner RA (1992) Encoded combinatorial chemistry. Proc Natl Acad Sci USA 89:5381–5383

Buchanan BG (2006) A (very) brief history of artificial intelligence. AI Mag 26:53–60

Chan HP, Samala RK, Hadjiiski LM et al (2020) Deep learning in medical image analysis. Adv Exp Med Biol 1213:3–21

Cheng F, Ikenaga Y, Zhou Y et al (2012) In silico assessment of chemical biodegradability. J Chem Inf Model 52:655–669

Cherkasov A, Muratov EN, Fourches D et al (2014) QSAR modeling: where have you been? Where are you going to? J Med Chem 57:4977–5010

Cho WS, Duffin R, Thielbeer F et al (2012) Zeta potential and solubility to toxic ions as mechanisms of lung inflammation caused by metal/metal oxide nanoparticles. Toxicol Sci 126:469–477

Chomenidis C, Drakakis G, Tsiliki G et al (2017) Jaqpot Quattro: a novel computational web platform for modeling and analysis in nanoinformatics. J Chem Inf Model 57:2161–2172

Colvin VL (2003) The potential environmental impact of engineered nanomaterials. Nat Biotechnol 21:1166–1171
Corbett PT, Leclaire J, Vial L et al (2006) Dynamic combinatorial chemistry. Chem Rev 106:3652–3711
Cortés-Ciriano IC, Bender A (2019) KekuleScope: prediction of cancer cell line sensitivity and compound potency using convolutional neural networks trained on compound images. J Cheminform 11:41
Cortés-Ciriano I, Van Westen GJP, Bouvier G et al (2016) Improved large-scale prediction of growth inhibition patterns using the NCI60 cancer cell line panel. Bioinformatics 32:85–95
Daily M, Medasani S, Behringer R et al (2017) Self-driving cars. Computer 50:18–23
Dix DJ, Houck KA, Martin MT et al (2007) the ToxCast program for prioritizing toxicity testing of environmental chemicals. Toxicol Sci 95:5–12
Du H, Cai Y, Yang H et al (2017) In silico prediction of chemicals binding to aromatase with machine learning methods. Chem Res Toxicol 30:1209–1218
Esteva A, Robicquet A, Ramsundar B et al (2019) A guide to deep learning in healthcare. Nat Med 25:24–29
Feng C, Chen H, Yuan X et al (2019) Gene expression data based deep learning model for accurate prediction of drug-induced liver injury in advance. J Chem Inf Model 59:3240–3250
Fourches D, Pu D, Li L et al (2016) Computer-aided design of carbon nanotubes with the desired bioactivity and safety profiles. Nanotoxicology 10:374–383
Fourches D, Pu D, Tassa C et al (2010) Quantitative nanostructure - Activity relationship modeling. ACS Nano 4:5703–5712
Gaheen S, Hinkal GW, Morris SA et al (2013) CaNanoLab: data sharing to expedite the use of nanotechnology in biomedicine. Comput Sci Discov 6:014010.
Ginsberg J, Mohebbi MH, Patel RS et al (2009) Detecting influenza epidemics using search engine query data. Nature 457:1012–1014
Gómez-Bombarelli R, Wei JN, Duvenaud D et al (2018) Automatic chemical design using a data-driven continuous representation of molecules. ACS Cent Sci 4:268–276
Grigorescu S, Trasnea B, Cocias T et al (2020) A survey of deep learning techniques for autonomous driving. J Field Robot. 37:362–386
Haase and Klaessig (eds) (2018) EU US Roadmap Nanoinformatics 2030. https://doi.org/10.5281/zenodo.1486012
Hansch C, Maloney P, Fujita T et al (1962) Correlation of biological activity of phenoxyacetic acids with Hammett substituent constants and partition coefficients. Nature 194:178–180
Helma C, Rautenberg M, Gebele D (2017) Nano-Lazar: read across predictions for nanoparticle toxicities with calculated and measured properties. Front Pharmacol 8:377
Hertzberg RP, Pope AJ (2000) High-throughput screening: new technology for the 21st century. Curr Opin Chem Biol 4:445–451
Huang R, Xia M, Sakamuru S et al (2016) Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization. Nat Commun 7:1–10
Iqbal M, Sameem MSI, Naqvi N et al (2019) A deep learning approach for face recognition based on angularly discriminative features. Pattern Recognit Lett 128:414–419
Irwin BWJ, Mahmoud S, Whitehead TM et al (2020) Imputation versus prediction: applications in machine learning for drug discovery. Future Drug Discov 2:2
Jantunen APK, Gottardo S, Rasmussen K et al (2018) An inventory of ready-to-use and publicly available tools for the safety assessment of nanomaterials. NanoImpact 12:18–28
Jeliazkova N, Chomenidis C, Doganis P et al (2015) The eNanoMapper database for nanomaterial safety information. Beilstein J Nanotechnol 6:1609–1634
Kim B, Lee S, Kim J (2020) Inverse design of porous materials using artificial neural networks. Sci Adv 6:eaax9324.
Kim S, Thiessen PA, Bolton EE et al (2016) PubChem substance and compound databases. Nucleic Acids Res 44:D1202–D1213

Kotsias PC, Arús-Pous J, Chen H et al (2020) Direct steering of de novo molecular generation with descriptor conditional recurrent neural networks. Nat Mach Intell 2:254–265
Lawrence S, Giles CL, Tsoi AC et al (1997) Face recognition: a convolutional neural-network approach. IEEE Trans Neural Networks 8:98–113
Lazarovits J, Sindhwani S, Tavares AJ et al (2019) Supervised learning and mass spectrometry predicts the in vivo fate of nanomaterials. ACS Nano 13:8023–8034
Le TC, Yan B, Winkler DA (2015) Robust prediction of personalized cell recognition from a cancer population by a dual targeting nanoparticle library. Adv Funct Mater 25:6927–6935
LeCun Y, Bengio Y, Hinton G (2015) Deep learning. Nature 521:436–444
Lewandowski I, Clifton-Brown J, Trindade LM et al (2016) Progress on optimizing miscanthus biomass production for the European bioeconomy: results of the EU FP7 project OPTIMISC. Front Plant Sci 7:1–23
Liu G, Yan X, Sedykh A et al (2020) Analysis of model PM2.5-induced inflammation and cytotoxicity by the combination of a virtual carbon nanoparticle library and computational modeling. Ecotoxicol Environ Saf 191:110216.
Liu L, Fu L, Zhang J et al (2018) Three-level hepatotoxicity prediction system based on adverse hepatic effects. Mol Pharm 16:393–408
Liu R, Rallo R, George S et al (2011) Classification NanoSAR development for cytotoxicity of metal oxide nanoparticles. Small 7:1118–1126
Liu R, Zhang HY, Ji ZX et al (2013) Development of structure-activity relationship for metal oxide nanoparticles. Nanoscale 5:5644–5653
Ma L, Zhang Y (2015) Using Word2Vec to process big text data. 2015 IEEE International Conference on Big Data (Big Data), pp 2895–2897.
Macarron R, Banks MN, Bojanic D et al (2011) Impact of high-throughput screening in biomedical research. Nat Rev Drug Discov 10:188–195
Mora JR, Marrero-Ponce Y, García-Jacas CR et al (2020) Ensemble models based on QuBiLS-MAS features and shallow learning for the prediction of drug-induced liver toxicity: improving deep learning and traditional approaches. Chem Res Toxicol 33:1855–1873
Mulliner D, Schmidt F, Stolte M et al (2016) Computational models for human and animal hepatotoxicity with a global application scope. Chem Res Toxicol 29:757–767
Norinder U, Spjuth O, Svensson F (2020) Using predicted bioactivity profiles to improve predictive modeling. J Chem Inf Model 60:2830–2837
Nosengo N (2016) The material code. Nature 533:22–25
Ongsulee P (2017) Artificial intelligence, machine learning and deep learning. 2017 15th International Conference on ICT and Knowledge Engineering (ICT&KE), pp 1–6.
Oomen AG, Bos PMJ, Fernandes TF et al (2014) Concern-driven integrated approaches to nanomaterial testing and assessment-report of the NanoSafety Cluster Working Group 10. Nanotoxicology 8:334–348
Puzyn T, Rasulev B, Gajewicz A et al (2011) Using nano-QSAR to predict the cytotoxicity of metal oxide nanoparticles. Nat Nanotechnol 6:175–178
Pyzer-Knapp EO, Suh C, Gómez-Bombarelli R et al (2015) What is high-throughput virtual screening? A perspective from organic materials discovery. Annu Rev Mater Res 45:195–216
Raccuglia P, Elbert KC, Adler PDF et al (2016) Machine-learning-assisted materials discovery using failed experiments. Nature 533:73–76
Russo DP, Zorn KM, Clark AM et al (2018) Comparing multiple machine learning algorithms and metrics for estrogen receptor binding prediction. Mol Pharm 15:4361–4370
Sagiroglu S, Sinanc D (2015) Big data - A review. Int J Appl Eng Res 10:1294–1298
Schofield D, Nagrani A, Zisserman A et al (2019) Chimpanzee face recognition from videos in the wild using deep learning. Sci Adv 5:eaaw0736.
Shatkin JA (2020) The future in nanosafety. Nano Lett 20:1479–1480
Sipes NS, Wambaugh JF, Pearce R et al (2017) An intuitive approach for predicting potential human health risk with the Tox21 10k library. Environ Sci Technol 51:10786–10796

Stokes JM, Yang K, Swanson K et al (2020) A deep learning approach to antibiotic discovery. Cell 180:688-702.e13

Su G, Zhou H, Mu Q et al (2012) Effective surface charge density determines the electrostatic attraction between nanoparticles and cells. J Phys Chem C 116:4993–4998

Sun H, Liu Y, Bai X et al (2018) Induction of oxidative stress and sensitization of cancer cells to paclitaxel by gold nanoparticles with different charge densities and hydrophobicities. J Mater Chem B 6:1633–1639

Sun L, Zhang C, Chen Y et al (2015) In silico prediction of chemical aquatic toxicity with chemical category approaches and substructural alerts. Toxicol Res 42:452–463

Svensson F, Norinder U, Bender A (2017) Modelling compound cytotoxicity using conformal prediction and PubChem HTS data. Toxicol Res 6:73–80

Trinh TX, Ha MK, Choi JS et al (2018) Curation of datasets, assessment of their quality and completeness, and nanoSAR classification model development for metallic nanoparticles. Environ Sci Nano 5:1902–1910

Tropsha A, Mills KC, Hickey AJ (2017) Reproducibility, sharing and progress in nanomaterial databases. Nat Nanotechnol 12:1111–1114

Tsiliki G, Munteanu CR, Seoane JA et al (2015) RRegrs: an R package for computer-aided model selection with multiple regression models. J Cheminform 7:1–16

Walkey CD, Olsen JB, Song F et al (2014) Protein corona fingerprinting predicts the cellular interaction of gold and silver nanoparticles. ACS Nano 8:2439–2455

Wang S, Sun H, Liu H et al (2016) ADMET evaluation in drug discovery. 16. Predicting hERG blockers by combining multiple pharmacophores and machine learning approaches. Mol Pharm 13:2855–2866

Wang W, Sedykh A, Sun H et al (2017) Predicting nano-bio interactions by integrating nanoparticle libraries and quantitative nanostructure activity relationship modeling. ACS Nano 11:12641–12649

Weissleder R, Kelly K, Sun EY et al (2005) Cell-specific targeting of nanoparticles by multivalent attachment of small molecules. Nat Biotechnol 23:1418–1423

Whitehead TM, Irwin BWJ, Hunt P et al (2019) Imputation of assay bioactivity data using deep learning. J Chem Inf Model 59:1197–1204

Xu Y, Pei J, Lai L (2017) Deep learning based regression and multiclass models for acute oral toxicity prediction with automatic chemical feature extraction. J Chem Inf Model 57:2672–2685

Yan X, Sedykh A, Wang W et al (2019) In silico profiling nanoparticles: predictive nanomodeling using universal nanodescriptors and various machine learning approaches. Nanoscale 11:8352–8362

Yan X, Sedykh A, Wang W et al (2020) Construction of a web-based nanomaterial database by big data curation and modeling friendly nanostructure annotations. Nat Commun 11:1–10

Yan X, Zhang J, Russo DP et al (2020) Prediction of nano-bio interactions through convolutional neural network analysis of nanostructure images. ACS Sustain Chem Eng 8:19096–19104

Zhang C, Zhou Y, Gu S et al (2016) In silico prediction of hERG potassium channel blockage by chemical category approaches. Toxicol Res 5:570–582

Zhao L, Zhu H, Russo DP et al (2020) Mechanism-driven read-across of chemical hepatotoxicants based on chemical structures and biological data. Toxicol Sci 174:178–188

Zhou H, Jiao P, Yang L et al (2011) Enhancing cell recognition by scrutinizing cell surfaces with a nanoparticle array. J Am Chem Soc 133:680–682

Zhou H, Mu Q, Gao N, Liu A, Xing Y, Gao S, Zhang Q, Qu G, Chen Y, Liu G, Zhang B, Yan B (2008). A nano-combinatorial library strategy for the discovery of nanotubes with reduced protein-binding, cytotoxicity, and immune response. *Nano Lett* 8(3):859–865. https://doi.org/10.1021/nl0730155

Zhu H, Martin TM, Ye L, Sedykh A, Young DM, Tropsha A (2009). Quantitative structure−activity relationship modeling of rat acute toxicity by oral exposure. *Chem Res Toxicol* 22(12):1913–1921. https://doi.org/10.1021/tx900189p

Chapter 8
Knowledge Gained from Co-exposure Studies of Nanomaterials and Chemicals

Lingxiangyu Li and Zhenlan Xu

Abstract With the rapid development of nanotechnology, engineered nanomaterials have been extensively produced and used in diverse fields, resulting in an inevitable release of engineered nanomaterials into the natural environment where various chemicals including organic pollutants and toxic metals are widely detected. Possible interactions between engineered nanomaterials and chemicals have aroused public concerns in recent years. The combined toxicity of engineered nanomaterials and chemicals is closely species-specific, related to environmental media and can be either synergistic, additive or antagonistic. The "Trojan horse" type pathway has been identified as an important mechanism for the synergistic, additive or antagonistic effects of engineered nanomaterials and chemicals on organisms, whereas complexation might be related to antagonistic effects. Further studies should be conducted in the future to fully understand the mixture effects of engineered nanomaterials and chemicals in the environment and better assess the potential risks of co-exposures.

Coexistence of Engineered Nanomaterials and Chemicals in the Environment

Along with the rapid development of nanotechnology, a series of engineered nanomaterials have been designed, produced and used in recent years. More than 1800 commercial products on the market use or contain engineered nanomaterials such as silver nanoparticles (Ag NPs), titanium dioxide nanoparticles (TiO_2 NPs), zinc oxide nanoparticles (ZnO NPs), copper oxide nanoparticles (CuO NPs) and carbon

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nanotubes (CNTs). These engineered nanomaterials would be inevitably released during their production, usage and disposal, resulting in engineered nanomaterials in different environmental compartments, e.g., the surface waters and soils (Oyelami and Semple 2015; Jr. Hochella et al. 2019). For instance, the predicted surface water concentrations of Ag NPs and TiO_2 NPs in the E.U. were reported to be in the range of 0.5–0.9 ng/L and 0.4–1.4 μg/L, respectively (Sun et al. 2014a, b). Indeed, the measured concentrations of Ag NPs in a German river, at the locations distant from wastewater treatment plant (WWTP) discharge points, were determined to be between 0.9 and 2.3 ng/L (Li et al. 2016). Also, Gondikas et al. (2014) confirmed the occurrence of TiO_2 NPs in the Olde Danube Lake in a 12-month field survey. Further, the levels of engineered nanomaterials in the environment are expected to increase in the following years due to their extensive usage in diverse fields.

Besides engineered nanomaterials, chemicals including organic pollutants and heavy metals are widely present in the natural environment; persistent organic pollutants (POPs) such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) and toxic metals like cadmium (Cd) and lead (Pb) are often detected in surface waters (Samecka-Cymerman and Kempers 2004; Zareitalabad et al. 2013). The coexistence of engineered nanomaterials and chemicals in the environment is thus highly possible, which is a serious issue not to be ignored neither by the scientific community nor the engineers and politicians.

The occurrence and build-up of engineered nanomaterials in the environment lead to their interactions with organic pollutants and heavy metals, possibly exerting mixture effects on organisms by acting as carriers or catalysts for chemicals due to the specific properties of nanomaterials (Hartmann and Baun 2010; Naasz et al. 2018). The interactions between engineered nanomaterials and chemicals have attracted extensive attention in recent years (Fig. 8.1) and several studies have shown that possible interactions and risks are highly dependent on the environmental factors and individual properties of the components (Ginzburg et al. 2018; Teng et al. 2020). Mechanisms for mixture effects of nanomaterials and chemicals have also been proposed. For example, the 'Trojan horse' type action has been identified as an important pathway for the synergistic effect of nanomaterials and chemicals on organisms (Naasz et al. 2018). Besides synergistic effects, additive and antagonistic effects of nanomaterials and chemicals are related to the 'Trojan horse' pathway (Fig. 8.2). Therefore, interactions and potential risks, as well as mechanisms of action of engineered nanomaterials and chemicals, are summarized in this chapter, to provide a systematic understanding of nanomaterial-chemical interactions and their biological effects.

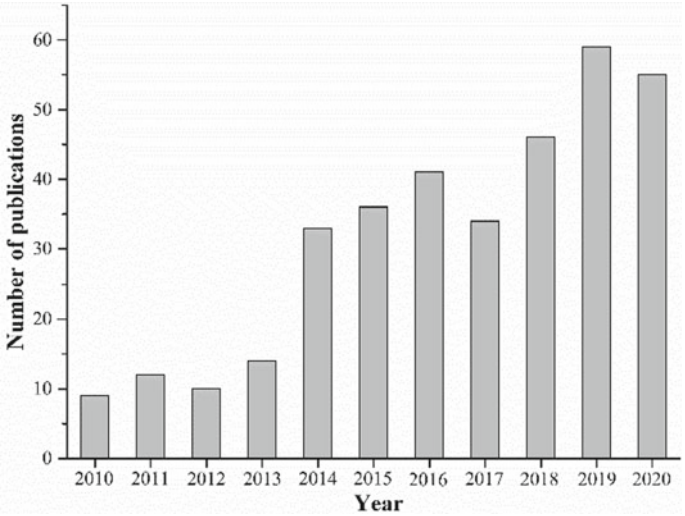

Fig. 8.1 Number of studies on co-exposures of engineered nanomaterials and chemicals by year. The data were collected from Web of Science by using "co-exposure and nanomaterials" as keywords

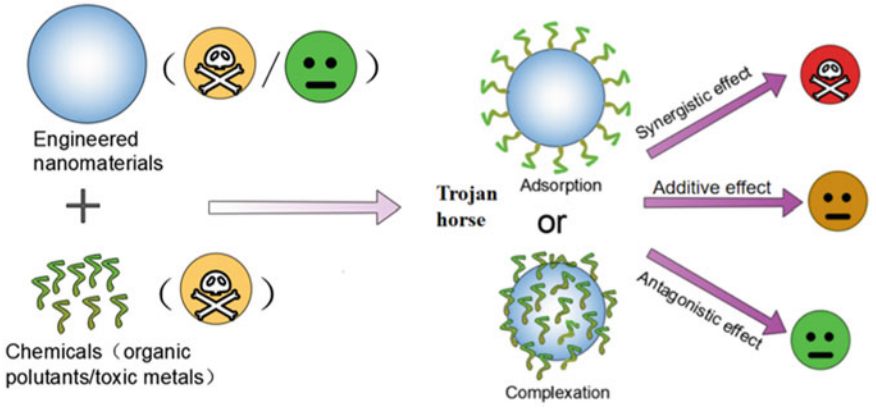

Fig. 8.2 Scheme of the potential mechanisms of interaction and resulting binary effects of engineered nanomaterials and chemicals. The Trojan-horse effect includes adsorption and complexation. Here the *yellow circle* with skull and bones represents toxicity, the *red circle* with skull and bones represents enhanced toxicity, the *brown circle* with face represents moderated toxicity, and the *green circle* with face represents safety

Risks of Co-exposure to Nanomaterials and Organic Pollutants

Binary Effects of TiO_2 Nanomaterials and Organic Pollutants

Among the metal-based engineered nanomaterials, TiO_2 is one of the most widespread, with uses in industrial and commercial applications, including photoelectric devices, paints, sunscreens, cosmetics, textiles, and food packages (Botta et al. 2011; Azimzada et al. 2020). With increasing production and use, leakage of TiO_2 NPs into the natural environment is inevitable. The concentration of TiO_2 NPs in aquatic ecosystems can be as high as 1 mg/L (Shi et al. 2016), which is about three orders of magnitude higher than the predicted level in the water (Sun et al. 2014a, b). Clearly, TiO_2 NPs inevitably interact with organic pollutants in the environment, affecting the behavior and toxicity of organic pollutants.

Bisphenol A (BPA) is one of the most highly produced and widely used industrial chemicals, primarily applied in the production of polycarbonate plastics and epoxy resins. Commercial products such as baby bottles, water containers, and medical devices contain BPA, resulting in high global demand and production capacity of BPA (Huang et al. 2012). Due to its high production levels, BPA has been detected in various environmental matrices, including surface water. For instance, BPA accumulation in water samples from several rivers in China has been detected to be 4 μg/L (Guo et al. 2019), and much higher concentrations (up to 28 μg/L) were detected in contaminated rivers in Germany (Heisterkamp et al. 2004). Previous studies have demonstrated that BPA can disrupt normal physiological function by acting as an estrogen agonist, exerting weak estrogenic, anti-androgenic and anti-thyroid activities (Alonso-Magdalena et al. 2012; Delfosse et al. 2012). Recently, co-exposure to TiO_2 NPs and BPA in the aquatic environment has attracted attention and prompted studies regarding the effects of TiO_2 NPs on the uptake, bioaccumulation and toxicity of BPA in aquatic organisms. Model organisms such as zebrafish (*Danio rerio*) were co-exposed to TiO_2 NPs and BPA, showing that TiO_2 NPs at a concentration of 100 μg/L enhanced bioaccumulation of BPA in zebrafish adults through acting as a carrier for BPA and thus led to endocrine disruption and impairment of reproduction (Fang et al. 2016). After 21-day co-exposure, increased tissue burdens of both TiO_2 NPs and BPA were observed in the zebrafish adults, and a reduction in plasma concentrations of estradiol, testosterone, follicle-stimulating hormone, and luteinizing hormone was detected. In another study, fertilized zebrafish eggs were exposed to TiO_2 NPs, BPA or their binary mixtures up to 6 days post fertilization (dpf) (Fu et al. 2020). The results showed no significant change in hatching, malformations, survival and weight of the larvae among all groups. Yet, TiO_2 NPs significantly increased the body burden of BPA in the co-exposure groups and suppressed the expression of neurodevelopmental marker genes (e.g., α1-tubulin, mbp and syn2α) as well as the locomotor behavior. In addition, co-exposure was found to reciprocally facilitate bioaccumulation of BPA in zebrafish adults while enhancing maternal

transfer to offspring, wherein thyroid endocrine disruption and developmental neurotoxicity were observed in larval offspring by parental exposure to BPA in combination with TiO_2 NPs (Guo et al. 2019). In particular, offspring larvae derived from the co-exposed parents showed lethargic swimming behavior. The enhanced bioaccumulation and bioconcentration of BPA in zebrafish were primarily attributed to the enhanced bioavailability of BPA due to the specific adsorptive effect of BPA on TiO_2-NPs. Besides zebrafish, bivalve mollusks were co-exposed to BPA and TiO_2 NPs for 14 days, resulting in elevated levels of metallated metallothionein (Zn, Cu-MT), up-regulated lipid peroxidation and lipofuscin accumulation, and unstable DNA (Gnatyshyna et al. 2019).

Another endocrine disruptor, tris(1,3-dichloro-2-propyl) phosphate (TDCIPP, used as a flame retardant) also exhibited enhanced bioaccumulation in zebrafish due to the presence of TiO_2 NPs (Ren et al. 2018). Compared to TDCIPP exposure alone, increased tissue burdens of both TDCIPP and TiO_2 were observed in the liver, brain, and gonads, which indicates that TiO_2 NPs adsorbed TDCIPP and acted as a carrier facilitating the uptake and translocation of TDCIPP in tissues. Moreover, co-exposure inhibited egg production and further caused serious developmental toxicity in F1 larvae. In another study, mixture effects of TiO_2 NPs and TDCIPP on the invertebrate (earthworm *Eisenia fetida*) in soil were examined (Zhu et al. 2021a, b). Compared to single exposures, co-exposure greatly reduced the weight gain rate of *E. fetida*, indicating synergistic toxicity between TiO_2 NPs and TDCIPP. This may be caused by the superimposition of toxicity pathways of TiO_2 NPs and TDCIPP, because no significant effects on the concentrations of TDCIPP and TiO_2 in soil or *E. fetida* during co-exposure were detected. Also, co-exposure significantly inhibited genes related to digestion and nutrient adsorption based on transcriptomic analysis of *E. fetida* intestinal region.

POPs, being emerging pollutants in the environment, have been of great concern during the last decades because of their properties, including persistence, long-range transport, bioaccumulation potential, and possible toxicity to humans and wildlife (Meng et al. 2017). The occurrence of POPs in the environment has been extensively reported; for example, PFOS, PFOA, polybrominated diphenyl ethers (PBDEs), and phenanthrene have been detected in the environmental matrices from water to sediment. Studies of co-exposures to TiO_2 NPs and POPs were thus performed, to investigate possible effects of TiO_2 NPs on uptake, translocation and bioaccumulation of POPs in organisms. TiO_2 NPs of two crystalline phases (i.e., anatase and rutile) enhanced the whole-body PFOS concentration in zebrafish due to PFOS adsorption on TiO_2 NPs, resulting in the formation of TiO_2–PFOS complexes which enhanced the bioavailability of PFOS to zebrafish (Qiang et al. 2015). The bioaccumulation of PFOS was more promoted by anatase, which was attributed to the greater adsorption capacity of PFOS to anatase, the slower migration of their complex in water, and the slower elimination rate of anatase from fish. TiO_2 NPs acting as carriers by adsorbing phenanthrene and forming TiO_2–phenanthrene complexes was also observed in another study, where uptake of phenanthrene by marine bivalve clam was enhanced by the presence of TiO_2 NPs, with the bioaccumulation factor (BAF) being 1.7 times higher than that of phenanthrene alone (Tian et al. 2014). The enhanced

uptake can be explained by ingestion of TiO_2 NP complexes into the gut of clam and subsequent desorption of phenanthrene there. Besides enhanced bioaccumulation, the presence of TiO_2 NPs can affect the toxicity of organic chemicals. TiO_2 NPs at a concentration of 100 μg/L enhanced the metabolism of pentachlorophenol (PCP, a restricted-use pesticide) in zebrafish and caused oxidative damage as well as developmental toxicity (Fang et al. 2015). Also, TiO_2 NPs were reported to enhance the PCP-induced thyroid endocrine disruption but not the neurobehavioral defects in zebrafish larvae (Lei et al. 2020).

Interestingly, negligible effects of TiO_2 NPs on the translocation and bioaccumulation of PFOA in hydroponically grown pumpkin seedlings were observed, which might be due to different pathways for translocation of TiO_2 NPs and PFOA in the seedling and negligible adsorption of PFOA on TiO_2 NPs (Xu et al. 2019). A similar phenomenon was observed for marine bivalve (*Scapharca subcrenata*) co-exposed to TiO_2 NPs and PBDEs; TiO_2 NPs acted as carriers and enhanced the ingestion of PBDEs, while the bioaccumulation of PBDEs was not significantly increased in the presence of TiO_2 NPs (Tian et al. 2015). Thus, a similar accumulation kinetics pattern was observed after exposure to PBDEs in the presence and absence of TiO_2 NPs. Besides pumpkin seedlings and marine bivalves, the European sea bass (*Dicentrarchus labrax*) is another species that has been co-exposed to POPs and TiO_2 NPs. The results showed that TiO_2 NPs did not interfere with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) detoxification and bioconcentration due to negligible interactions between TiO_2 NPs and 2,3,7,8-TCDD in the seawater (Torre et al. 2015). Moreover, TiO_2 NPs were shown to not interfere with 2,3,7,8-TCDD-dependent expression of biotransformation-related genes in the liver of European sea bass (Vannuccini et al. 2015). Nevertheless, gene expression in the digestive gland of 2,3,7,8-TCDD-exposed marine mussel *Mytilus galloprovincialis* was affected by co-exposure to TiO_2 NPs (Banni et al. 2016). 2,3,7,8-TCDD up-regulated endocrine and signal transduction-related processes, while TiO_2 NPs mainly up-regulated cytoskeletal genes. Although co-exposure induced transcriptional changes common to individual treatments, also a newly generated process -response to chemical stimulus, was identified. In a recent study, mixture toxicity effects of TiO_2 NPs and 3,3′,4,4′-tetrachlorobiphenyl (PCB77) in juvenile brown trout following co-exposure via the diet were investigated (Lammel et al. 2019). The study demonstrated that genes encoding for proteins and enzymes essential for tight junction function and ROS elimination were upregulated in the intestine of fish in the co-exposure groups but not in the individual exposure groups.

In contrast to the above-discussed TiO_2 NP-induced enhancement or negligible changes in the uptake and bioaccumulation of organic chemicals, the presence of TiO_2 NPs in the exposure system reduced the uptake of benzo(α)pyrene (B(α)P, a type of polycyclic aromatic hydrocarbons or PAHs, which are widespread environmental contaminants) in the blue mussel (*Mytilus edulis*), since TiO_2 NPs significantly reduced the bioavailability of B(α)P (Farkas et al. 2015). Interestingly, most biomarker responses (e.g., superoxide dismutase, catalase and glutathione peroxidase) did not decrease despite the lower B(α)P uptake in combined exposure.

Pesticides and herbicides are also widely detected in the environment, resulting in a possible co-existence with engineered nanomaterials. Previous studies have investigated the mixture effects of TiO_2 NPs and pesticides or herbicides on organisms. For example, cypermethrin (CYP) uptake in zebrafish was greatly increased by TiO_2 NPs at a concentration of 1 mg/L, resulting in the generation of reactive oxygen species (ROS) (Li et al. 2018). Also, compared to the single exposure, co-exposure significantly decreased locomotion activity and enhanced the down-regulation of mRNA expression of specific genes and the levels of the neurotransmitter. Similarly, co-exposure to TiO_2 NPs and atrazine led to a synergistic effect on the toxicity of TiO_2 NPs-atrazine to algae (*Chlorella pyrenoidosa*), enhancing bioaccumulation of atrazine and affecting biophysicochemical properties of algal cells (Zhang et al. 2017).

Several studies have focused on the effects of TiO_2 NPs on the uptake, bioaccumulation and toxicity of organic chemicals. Nevertheless, several studies have also investigated the possible impacts of organic chemicals on the uptake and toxicity of TiO_2 NPs in aquatic organisms. For example, the joint toxicity of TiO_2 NPs and a broad-spectrum antibiotic florfenicol to alga *C. pyrenoidosa* was investigated and the observed toxicity associated with TiO_2 NP exposure was found to be enhanced by adding florfenicol into the system (Wang et al. 2016). In contrast, an antagonistic or additive effect on alga *C. pyrenoidosa* was observed in the co-exposure of TiO_2 NPs and organochlorine chemicals such as PCB77, hexachlorobenzene (HCB), and pentachlorobenzene (PCB) (Zhang et al. 2017). Specifically, antagonistic effects for TiO_2–PCB77 and TiO_2–HCB and an additive effect for TiO_2–PCB were determined. The bioaccumulation of TiO_2 NPs in algal cells significantly decreased when these organic chemicals were added to the system. The study illustrated well the aspect that mixture effects are highly dependent on the nature of organic chemicals. Similarly, the presence of tetracycline (TC) decreased the uptake of TiO_2 NPs by alga *Scenedesmus obliquus* (Iswarya et al. 2017). The toxicity of TC and TiO_2 NPs was either additive or antagonistic, depending on the concentration of the chemicals. It was also noted that at the higher stress level, i.e., in the case of additive toxicity of TiO_2–TC, the algal cells produced higher levels of extracellular polymeric substances (EPS) as a means of alleviating the toxicity.

Since algal growth is highly dependent on the presence of nutrients like phosphorus, binary effects of TiO_2 NPs and phosphorus on algae *Chlorella ellipsoides* were investigated (Matouke et al. 2018). Co-exposure resulted in decreased phosphorus bioconcentration in the algal cells with a significant increase in chlorophyll a/b and total chlorophyll contents. Moreover, a significant decrease in specific growth rate and optical density were observed, and activities of antioxidant enzymes like malondialdehyde, superoxide dismutase, peroxidase and glutathione-S-transferase were significantly increased, suggesting that the addition of TiO_2 NPs to phosphorus affected the physiology of algae. Algal blooms accompanied by eutrophication in freshwater have aroused public concerns, so joint effects of microcystin-LR (MCLR), a toxin produced by blue-green algae, and TiO_2 NPs on zebrafish were investigated (Wu et al. 2019a, b). The results showed that the presence of TiO_2 NPs aggravated MCLR-induced abnormity of swimming and social behavior of zebrafish. The

underlying mechanism was attributed to the increased ROS content in zebrafish brain upon co-exposure compared to MCLR single exposure, confirmed by the significant changes in the expression of genes related to the antioxidant enzymes.

Based on these findings, it is clear that TiO_2 NPs can influence the translocation, fate and toxicity of organic chemicals in the natural environment including surface water and soil, and vice versa. Yet, the mixture effects are highly dependent on the type of organic chemical and their modes of action in organisms.

Binary Effects of ZnO Nanomaterials and Organic Pollutants

Nanosized zinc oxide is among the nanomaterials produced in the highest volumes. Thus, due to concerns over high predicted and measured environmental concentrations, binary effects of ZnO NPs and organic pollutants have been evaluated in aquatic and soil organisms as well as plants. For example, the effects of co-exposure of PFOS and ZnO NPs on the hypothalamic-pituitary-thyroid (HPT) axis in zebrafish were examined and it was shown that the co-exposure decreased the body length and increased the malformation rates compared to PFOS exposure alone (Du et al. 2016, 2017). In particular, the expression of proteins and genes were significantly altered by the co-exposure compared with the exposure to PFOS alone, whereas co-exposure caused more serious thyroid disruption effects in zebrafish. Further investigations revealed that co-exposure to PFOS and ZnO NPs adversely impacted zebrafish development, reproduction in the F0 generation, and offspring embryonic growth (Du et al. 2018). More importantly, lower fertilization and hatching rates, greater mortality and higher malformation occurrence were observed in the F1 generation after long-term co-exposure of the parental zebrafish (i.e., F0 generation. In contrast, co-exposure to an organophosphate pesticide chlorpyrifos and ZnO NPs showed a positive effect on earthworm (*Eisenia andrei*) reproduction, which was up to 84% higher than the theoretically predicted values (Garcia-Gomez et al. 2019). A recent study about binary effects of ZnO NPs and phenanthrene, a PAH derived from coal tar, on wheat growth showed that co-exposure relative to single exposure caused serious damage to seedling roots, accompanied by a significant decrease in superoxide dismutase and catalase activities and disruption of the antioxidant system (Zhu et al. 2019). Altogether, the studies that have assessed the individual and combined toxicity of ZnO NPs and organic chemicals have provided valuable information about the range of different responses of organisms to co-exposures.

Binary Effects of Ag Nanomaterials and Organic Pollutants

Ag NPs have received much attention due to their antibacterial activity. The extensive use of Ag NPs has led to concerns over their release into the environment, which would result in possible interactions between Ag NPs and organic chemicals. Several

studies have focused on co-exposure effects of Ag NPs and pesticides in various plant species. For example, *Arabidopsis thaliana* was co-exposed to Ag NPs and chiral herbicide imazethapyr (IM) and it was shown that silver concentrations in roots and shoots dramatically increased compared with Ag NPs exposure alone (Wen et al. 2016). Also, co-exposure of Ag NPs and (R)-IM accelerated uptake and bioaccumulation of silver in roots and shoots relative to the co-exposure of Ag NPs and (S)-IM, because (R)-IM increased the levels of amino acids in roots which resulted in the formation of adducts with silver ions and consequent release of higher amounts of silver ions from Ag NPs and thus higher ecotoxicity. Recently, bioaccumulation, translocation, and toxicity of insecticide imidacloprid (IMDA) to *Cucurbita pepo* L (zucchini) were evaluated upon co-exposure to Ag NPs under soil-grown conditions (Torre-Roche et al. 2018). The study showed that co-exposure relative to a single exposure to IMDA significantly suppressed IMDA accumulation in the shoots (stem and leaves) of zucchini by 33%, while IMDA decreased silver accumulation compared to single Ag NP exposure. However, the expression pattern of the seven selected genes did not indicate significant Ag NP-IMDA interactions in toxicity to *C. pepo*. Similarly, Ag NPs decreased dichlorodiphenyldichloroethylene (*p,p′*-DDE, a major breakdown product of DDT) accumulation in *Glycine max* L. (soybean) tissue by 40%, with co-exposure to Ag NPs resulting in lower DDE uptake than co-exposure to bulk Ag (Torre-Roche et al. 2013). In addition, the concentration of Ag NPs in the co-exposure system played an important role in the binary effects in *C. pepo*; Ag NPs at 500 mg/L suppressed DDE uptake in zucchini by 21–29%, whereas Ag NPs at 2000 mg/L showed no impact on DDE uptake (Torre-Roche et al. 2013).

Besides plants, co-exposure effects of the binary mixtures of Ag NPs and organic chemicals have been studied in bacteria. The antibiotics enoxacin, kanamycin, neomycin, and tetracycline showed synergistic growth inhibition against the drug-resistant bacteria *Salmonella typhimurium* DT 104 when combined with Ag NPs, yet ampicillin and penicillin did not (Deng et al. 2016). This was due to the formation of Ag NP-antibiotic complexes with the four antibiotics, resulting in higher silver ion release, which did not occur with ampicillin and penicillin. The presence of tetracycline enhanced the binding of Ag to *S. typhimurium* by 21% and the release of silver ions by 26%. Compared with Ag NPs alone, the tetracycline-Ag NP complex interacted more strongly with the *S. typhimurium* cells and caused more silver ions release, creating a temporal high level of silver ions near the bacteria cell wall that resulted in bacteria growth inhibition (Deng et al. 2016). This is consistent with the findings reported in previous studies where silver ions released from Ag NPs have been identified as the key agents causing toxicity. In addition, it has been shown that the enhanced toxicity of Ag NPs with antibiotics to bacteria varies with the surface coating of the NPs. For example, antibiotic-mediated increased toxicity was more prominent with polyvinylpyrrolidone (PVP)-capped Ag NPs as compared to citrate and sodium dodecyl sulfate (SDS)-capped NPs (Kora and Rastogi 2013).

Binary Effects of Carbon-based Nanomaterials and Organic Pollutants

Carbon-based nanomaterials such as carbon nanotubes, fullerene and graphene are produced and used in many industrial sectors, resulting in inevitable release during their usage and disposal. In the environment, the released nanomaterials would interact with organic chemicals and pose potential risks, which need to be identified and characterized by conducting studies on the binary effects of carbon nanomaterials and organic pollutants. The results of the relevant studies available to date have been summarized in this section. A study about the effects of single-wall carbon nanotubes (SWCNT) on the uptake, bioaccumulation and antioxidant defense of PFOS in zebrafish showed that bioaccumulation of PFOS in the liver, intestines, gills and brain decreased with increasing dosage of SWCNT, yet an opposite trend was observed in fish skin (Li et al. 2017). Also, co-exposure induced more ROS than PFOS single exposure, and the effects of PFOS on superoxide dismutase, catalase and acetylcholinesterase activities were enhanced. In addition to the concentration of SWCNTs the surface charge can affect the accumulation of organic pollutants in organisms. This was demonstrated in a study investigating the co-exposures of neutrally or negatively charged SWCNTs and phenanthrene in Japanese medaka (Su et al. 2013). Coexposure to SWCNT was shown to facilitate the accumulation of phenanthrene in the digestive tract of the fish and therefore enhance the whole-body phenanthrene concentration by 2.1 fold after 72 h. Phenanthrene associated with neutrally charged SWCNTs stayed in the digestive tract of fish longer than phenanthrene associated with the negatively charged SWCNTs.

Besides SWCNT, multi-wall carbon nanotubes (MWCNT) can affect the uptake, accumulation and toxicity of organic pollutants in organisms. Following co-exposure to MWCNT, the accumulation of antidepressant drug fluoxetine and its main metabolite norfluoxetine in zebrafish larvae was significantly enhanced, suggesting a synergistic effect of MWCNT and fluoxetine or norfluoxetine (Yan et al. 2019). On the contrary, antagonistic effects of MWCNT and 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47, an extensively used brominated flame retardant) in zebrafish were observed when compared with BDE-47 exposure alone (Wang et al. 2020). Namely, the growth inhibition induced by BDE-47 was mitigated in the presence of MWCNT. Also, the levels of oxidative stress biomarkers (e.g., ROS, superoxide, catalase) and DNA damage decreased in the presence of MWCNT compared to the exposures to BDE-47 alone. Similarly, bioaccumulation of PCP in goldfish liver, gills, muscle, intestine and gut was inhibited after co-exposure with MWCNT (Kan et al. 2021). MWCNTs were shown to reduce PCP-induced toxicity to liver tissues by the alleviation of hepatic oxidative damage. Interestingly, another study documented that MWCNTs potentiated the acute toxicity of the pesticide carbofuran in freshwater fish Nile tilapia (*Oreochromis niloticus*), leading to a more than fivefold increase in the half-lethal concentration (LC_{50}) values (Campos-Garcia et al. 2015). A synergistic effect of MWCNTs and a macrolide antibiotic roxithromycin was also observed; an addition of MWCNTs significantly facilitated the bioaccumulation of roxithromycin

in the liver (32–80%), gill (15–74%), intestine (51–113%) and bile (15–67%) of crucian carp (*Carassius auratus*), while a 0.3-fold increase in the metabolic enzyme activity and oxidative stress in the liver were observed (Yan et al. 2017). In addition, MWCNT facilitated the transport of PCP into carp (*C. auratus* red var.) (Sun et al. 2014a, b). These results imply that the species of the aquatic organism and the type of organic chemical can both affect the biological responses to co-exposures with MWCNTs. In addition, similar to SWCNT, surface properties can influence the binary effects of MWCNT and organic pollutants. Pristine MWCNT significantly aggravated the toxicity of carbamazepine, an anti-epileptic drug, (to diatom *Navicula* sp., whereas hydroxyl-functionalized MWCNT exhibited an insignificant effect on carbamazepine toxicity (Ding et al. 2019). A synergetic effect of MWCNT and sodium pentachlorophenate (PCP–Na) on the invertebrate *E. fetida* was observed, regardless of whether the two chemicals were both added separately or after the adsorption of PCP–Na to MWCNT (Hu et al. 2014). Yet, exposure concentration was shown to be an important factor for the binary effect; there was no difference between the single and combined toxicity of MWCNT and PCP–Na when low concentrations of the two chemicals were used.

Binary effects of CNTs and organic pollutants on cell lines have been also extensively investigated to gain insights into the underlying molecular mechanisms. No significant differences in mitochondrial activity, membrane damage, and cell apoptosis were detected when human breast adenocarcinoma cell line (MCF-7 cells) was exposed to SWCNT with and without adsorbed 17α-ethinylestradiol (EE2, a synthetic estrogen used in oral contraceptive pills) (Song et al. 2014). However, the bioactivity of SWCNT-adsorbed EE2, i.e., activation of the estrogen receptors, was significantly inhibited, suggesting that SWCNTs could be used for adsorption of EE2 in the environment and, thus, for mitigation of the toxicity of environmental estrogens. Additive toxicity of MWCNT and B(α)P was demonstrated in A549 lung cells, with oxidative stress identified as the main mechanism of cytotoxicity (Azari et al. 2020). The interaction of B(α)P with MWCNTs and metal impurities of MWCNTS were proposed to play a role in the combined toxicity of the nanomaterials and PAHs.

An important aspect of the combined effects of nanomaterials and organic compounds is the ability of nanomaterials to impact the efficiency of antibiotics against bacteria. Several studies have explored the impact of MWCNTs on the antibacterial efficiency of antibiotics. Oxidized MWCNTs reduced the antibacterial action of a quinolone antibiotic ciprofloxacin (CIP) to Gram-negative *E. coli*, which manifested in reduced cell membrane disruption and oxidative stress upon co-exposure (Deng et al. 2020). Moreover, MWCNTs alleviated the CIP-induced interference with the global gene expression and metabolite profile in bacteria. A similar antagonistic effect was also observed when *E. coli* was co-exposed to PCP and oxidized MWCNTs (Deng et al. 2019). Nevertheless, MWCNT–PCP and MWCNT–CIP binary exposures induced distinct additive and synergistic toxicities, respectively, in *Bacillus subtilis*, a Gram-positive bacterial strain (Deng et al. 2021). It was shown that MWCNTs increased bacterial bioaccumulation of PCP and CIP via destabilizing and damaging the cell membrane. Similarly, pre-treatment of *Pseudomonas aeruginosa*, a clinically relevant bacterial strain, with MWCNTs, increased

the sensitivity of the bacteria to last-resort carbapenem antibiotics—meropenem and imipenem (Mortimer et al. 2018). The interactions between nanomaterials and antibiotics and understanding the underlying mechanisms driving the combined effects are especially relevant in combating the globally concerning antibiotic resistance issue.

Binary effects of other types of carbon-based nanomaterials, such as fullerene and graphene, and organic pollutants have also been investigated in several studies. Antagonistic interactions between B(α)P and fullerene in toxicological response at genotoxic and proteomic level were demonstrated in a marine mussel (*M. galloprovincialis*) (Barranger et al. 2019). Similarly, co-exposure to graphene led to reduced bioavailability of B(α)P to brine shrimp (*Artemia franciscana*) as graphene with high surface area adsorbed significant amounts of B(α)P (Rodd et al. 2018). Antagonistic toxicity, manifesting through DNA damage, was also confirmed in the co-exposure of graphene and triphenyl phosphate (a flame retardant) to mussel *M. galloprovincialis* (Meng et al. 2020). However, several studies have indicated that fullerenes, carbon nanomaterials that can readily be internalized by cells, facilitate the transport of PAHs in vivo and in vitro and can enhance the toxicity of organic chemicals. For example, C_{60} and B(α)P exhibited synergistic effects in zebrafish hepatocyte cell line by inducing a significant loss in cellular viability and inducing an increase in B(α)P accumulation in the cells, impairing the detoxification response by phase II enzymes (Ferreira et al. 2014). Also, carbon nanopowder was shown to increase the cytotoxicity of B(α)P in zebrafish embryos and the co-exposure resulted in a higher incidence of necrotic and apoptotic cells (Torre et al. 2017). Yet, the presence of C_{60} did not induce changes in the bioaccumulation and biotransformation of antidepressant venlafaxine, a herbicide diuron or antibacterial agent triclosan in biofilms, suggesting microorganisms in the biofilms are not affected by the mixture of C_{60} and these microcontaminants (Santos et al. 2019). This illustrates the differences in toxicological responses of eukaryotic and prokaryotic cells, in part influenced by the extent of uptake of nanomaterials, expected to be lower for prokaryotic organisms. Thus, adsorption of organic compounds to nanomaterials can increase the bioaccumulation and toxicity of organic compounds if nanomaterials are readily taken up by the cells, which is expected to occur at a higher rate in the case of eukaryotic than prokaryotic organisms.

Binary Effects of Other Engineered Nanomaterials and Organic Pollutants

Among the engineered nanomaterials available on the market, SiO_2 NPs, Fe_2O_3 NPs and CeO_2 NPs are gradually attracting attention due to their superior properties. Accordingly, combined toxicities of these engineered nanomaterials and organic pollutants have also been investigated. Especially, the binary effects of SiO_2 NPs and B(α)P have been extensively tested. In a recent study, severe synergistic and additive toxic effects induced by SiO_2 NP and B(α)P long-term co-exposure were

observed in the BEAS-2B cell line even at a low dose (Wu et al. 2016, 2019a, b). The synergistic toxicity of SiO_2 NPs and B(α)P was also observed in zebrafish embryos where the molecular actions of co-exposure on the immune system, inflammatory process and cardiovascular development were more severe than in the case of single exposures (Asweto et al. 2018). Moreover, oxidative stress, cardiac toxicity and inflammation response of B(α)P, BDE-209 and tetrabromobisphenol A (TBBPA) in zebrafish embryos were enhanced by SiO_2 NPs (Duan et al. 2016; Chao et al. 2018; Zhu et al. 2021a, b). Since B(α)P is recognized as a reprotoxic compound, its combined toxicity with engineered nanomaterials to reproductive cells in vitro has been investigated. Considering that B(α)P is produced in the process of incomplete combustion of fossil fuels and CeO_2 is used as a diesel additive, the health effects of B(α)P and CeO_2 NP co-exposures are especially relevant. It was shown, using human and rat gametes, that CeO_2 NP and B(α)P mixture induced additive DNA damage in sperm and cumulus cells, while no additive effect was induced in rat oocytes (Cotena et al. 2021). A study of the combined effects of B(α)P and Bi_2O_3 NPs, attractive for biomedical and cosmetics applications, demonstrated that the two chemicals synergistically reduced cell viability, induced lactate dehydrogenase leakage, caspases (−3 and −9) and mitochondrial membrane potential loss in mouse spermatogonia cells (Ahamed et al. 2021). Oxidative stress was proposed as a plausible mechanism for synergistic toxicity of B(α)P and Bi_2O_3 NPs. Also, binary effects of Fe_2O_3 NPs and glyphosate-based herbicide in fish (*Poecilia reticulata*) were studied, showing that co-exposure resulted in high DNA damage relative to a single exposure, which suggested synergic effects for the mixture (Trigueiro et al. 2021).

Risks of Co-exposure to Nanomaterials and Toxic Metals

The wide occurrence of toxic metals in the environment has prompted studies about the impacts of co-exposure of engineered nanomaterials and toxic metals on organisms. In the next sections, the findings of these studies have been summarized for different types of nanomaterials.

Binary Effects of TiO_2 Nanomaterials and Toxic Metals

Among toxic metals, cadmium (Cd) pollution is considered one of the major global environmental issues due to its adverse effects on organisms and human health. For this reason, Cd and TiO_2 NP co-exposure effects on a wide range of model systems from cell lines to invertebrates, plants and vertebrates have been studied. For example, the non-cytotoxic concentration of TiO_2 NPs (15 mg/L) effectively enhanced the oxidative stress response of Cd^{2+}, indicated by pro-oxidant agent generation and antioxidant depletion in human liver (HepG2) and breast cancer (MCF-7)

cells (Ahamed et al. 2020). The study demonstrated that non-cytotoxic concentrations of TiO_2 NPs can enhance the toxicological potential of Cd^{2+} in human cells by facilitating the internalization of Cd into the cells. Yet, co-exposure to TiO_2 NPs and Cd^{2+} did not lead to increased adverse effects in a marine bivalve *M. galloprovincialis* when 0.1 mg/L of TiO_2 NPs were used in the co-exposure (Balbi et al. 2014). Clearly, the dose of TiO_2 NPs appears to be an important factor affecting in the binary effects of TiO_2 NPs and Cd^{2+}. Also, organic matter was shown to influence the effect of TiO_2 NPs on the uptake, accumulation and toxicity of Cd^{2+} by freshwater snails *Bellamya aeruginosa* (Ma et al. 2017). TiO_2 NPs did not promote Cd accumulation at the organic matter level of 4.8%, while at higher organic matter levels (7.1 or 11.6%) TiO_2-NPs significantly enhanced Cd^{2+} accumulation and toxicity as evidenced by aggravated DNA damage and decreased Na^+/K^+-ATPase activities. Interestingly, negligible and even antagonistic effects of TiO_2 NPs on the accumulation and toxicity of Cd^{2+} in plants such as radish (*Raphanus astivus*) and rice (*Oryzasativa* L.) have been reported. Morphological changes in nuclei, vacuoles and shape of radish root cells were observed upon single Cd^{2+} exposure and these effects were not abolished in the presence of TiO_2 NPs (Manesh et al. 2018). Also, the height, biomass and root length of rice seedlings indicated significant toxicity of Cd^{2+} to plant growth, whereas TiO_2 NPs had the potential to alleviate the Cd^{2+} toxicity (Ji et al. 2017). A recent study showed that exposure mode can affect the binary effects of TiO_2 NPs and Cd^{2+} (Lian et al. 2020). Specifically, root co-exposure to TiO_2 NPs and Cd^{2+} enhanced Cd uptake and caused greater phytotoxicity in maize (*Zea mays* L.) than foliar exposure to TiO_2 NPs. Moreover, co-exposure effects of TiO_2 NPs and Cd^{2+} have also been studied from the perspective of using TiO_2 NPs as potential bioremediation agents in mitigating the environmental toxicity of Cd. Promising prospects of this approach were recently demonstrated by Mottola et al. who showed that adding TiO_2 NPs into the Cd^{2+}-containing aquatic system induced no statistically significant loss of DNA integrity and a great reduction of the apoptotic cell percentage in zebrafish (Mottola et al. 2021).

Besides Cd, lead (Pb) is one of the major toxic pollutants of environmental concern. Thus, the combined toxicity of TiO_2 NPs and Pb has been investigated in several studies. In contrast to Cd, accumulation and toxicity of Pb in zebrafish were enhanced by adding TiO_2 NPs, because TiO_2 NPs acted as effective carriers of Pb to enhance its uptake, bioavailability, and toxicity in zebrafish (Hu et al. 2019). Moreover, co-exposure to TiO_2 NPs and Pb was shown to affect the feeding behavior of cyclopoid copepods by inhibiting the ingestion and filtration of microalgae and inducing the increase of carbohydrate and lipid levels (Matouke and Mustapha 2018). In another study with A549 cell lines, free Pb ions were not bioavailable inside the cells due to strong adsorption to TiO_2 NPs, resulting in decreased toxicity of Pb (Ahamed et al. 2019). It is possible that the enhancement or inhibition of the accumulation and toxicity of Pb in the presence of TiO_2 NPs is species-dependent.

TiO_2 NPs have also been proposed for environmental remediation of another toxic metal, arsenic (As) which has motivated studies on the combined biological effects of the two chemicals. It has been established that co-exposure to TiO_2 NPs (1 mg/L) and

As (50 μg/L) induces oxidative stress in an estuarine polychaeta *Laeonereis acuta* (Nunes et al. 2017) and golden mussel *Limnoperna fortunei* (Nunes et al. 2020). Moreover, the TiO_2 NPs affected metabolization capacity, favoring the accumulation of more toxic As compounds, and it was independent of the crystalline form (anatase or rutile) of the TiO_2 NPs (Nunes et al. 2020). In a separate study, it was also demonstrated that TiO_2 NPs increase As accumulation in mussels (Qian et al. 2020). TiO_2 NPs enhanced the toxicity of As by disturbing the osmotic adjustment system in mussels by reducing arsenobetaine and Na^+-K^+-ATPase activity. Moreover, the dynamic balance of adsorption/desorption of As by TiO_2 NPs led to the disruption of As distribution and affected As biotransformation in mussels (Qian et al. 2020). Altogether, the studies warrant careful environmental risk assessment of combined toxicity when considering the application of TiO_2 NPs for remediation of the metal-contaminated environment.

Binary Effects of Carbon-based Nanomaterials and Toxic Metals

Binary effects of carbon-based nanomaterials and toxic metals have been extensively investigated. For example, co-exposure effects of oxidized MWCNTs and Cd were studied using a zebrafish liver cell line (Morozesk et al. 2018, 2020). It was proposed that MWCNTs (10 mg/L) increased Cd toxicity at low concentration by a Trojan horse pathway and/or synergistic effect, resulting in apoptosis and necrosis and an increase in the Cd content in the zebrafish liver cell line. Besides MWCNTs, graphene oxide increased the acute toxicity (96 h) of Cd in shrimp (*Palaemon pandaliformis*), impairing the routine metabolism of shrimp (Melo et al. 2019). Graphene oxide also enhanced accumulation and toxicity (ROS, oxidative stress, gene expression) of As and hexavalent chromium (Cr^{6+}) in plants (*Triticum aestivum* L. and *Solamun lycopersicum*) and aquatic organisms (zebrafish) (Cao et al. 2021; Chen et al. 2021). Wang et al. assessed the impacts of differently functionalized MWCNTs and pristine SWCNTs on the aquatic microbial communities in the absence and presence of Cu or Cr (Wang et al. 2015). Interestingly, adding Cu or Cr into the water with CNT significantly enhanced the toxicity of CNT and transiently affected microbial communities, while functionalized CNTs were more toxic than the pristine ones. Although microbial communities recovered after 40 days, total microbial numbers continued to decrease, suggesting that metals aggravate the impacts of CNTs on ecosystems.

Binary Effects of Other Engineered Nanomaterials and Toxic Metals

Engineered nanomaterials such as Fe_3O_4 NPs, Ag NPs, ZnO NPs, and CeO_2 NPs have also attracted the attention about their combined effects with toxic metals. The mixture effects of NPs and metals can either be beneficial, i.e., resulting in reduced toxicity, or can exert higher toxicity than single exposures. For example, Cd^{2+}-induced damage in the mice liver was reduced by adding Fe_3O_4 NPs through reduction of oxidative stress (Zhang et al. 2016). Moreover, the study established that Fe_3O_4 NPs exhibited antagonistic effects on the biodistribution of Fe and Cd in mice because of mutually competitive inhibition of Fe and Cd uptake. In contrast, the combined toxic effect of As and Fe_3O_4 NPs on the ciliated protozoa *Tetrahymena pyriformis* was significantly enhanced and the survival rates decreased from 92.3 to 45.3% after 30 h co-exposure (Zou et al. 2013). A recent study investigated the interactions between As and Ag NPs in *Caenorhabditis elegans* and found that co-exposure resulted in higher toxicity than the individual exposure to As, showing deleterious effects in the development and reproduction of the animals throughout the generations (Josende et al. 2019). Interestingly, binary effects of ZnO NPs and Pb in mice were dependent on the weight of mice; ZnO NPs enhanced the deposition of Pb in all major organs in the overweight mice compared with normal-weight mice (Jia et al. 2017). Co-exposures resulted in higher levels of hepatic ROS, proinflammatory cytokines, and liver injury in the overweight mice but not in the normal weight mice. The study highlighted the potential human health risks of oral consumption of common food-related NPs (ZnO NPs) and heavy metals, particularly in the susceptible overweight population.

Conclusions

Facing the rapid development of nanotechnology, the discharge and build-up of engineered nanomaterials in the environment, where the occurrence of various chemicals including organic pollutants and toxic metals is inevitable, can result in interactions between engineered nanomaterials and chemicals that may exert harmful environmental and health impacts. Binary effects of engineered nanomaterials and chemicals on organisms have been extensively investigated, documenting that adsorption and complexation of chemicals with engineered nanomaterials are primary modes of interaction. Overall, synergistic, additive, and antagonistic effects have been reported for the co-exposures of engineered nanomaterials and chemicals. The studies conducted to date indicate that the potential risks of co-exposures to engineered nanomaterials and chemicals are species-specific and related to environmental media. Further studies are needed to completely understand the mixture effects of engineered nanomaterials and chemicals in the environment.

References

Ahamed M, Akhtar MJ, Alaizeri ZM et al (2020) TiO_2 nanoparticles potentiated the cytotoxicity, oxidative stress and apoptosis response of cadmium in two different human cells. Environ Sci Pollut Res 27:10425–10435

Ahamed M, Akhtar MJ, Alhadlaq HA (2019) Preventive effect of TiO_2 nanoparticles on heavy metal Pb-induced toxicity in human lung epithelial (A549) cells. Toxicol in Vitro 57:18–27

Ahamed M, Akhtar MJ, Khan MAM et al (2021) Co-exposure of Bi_2O_3 nanoparticles and bezo[α]pyrene-enhanced in vitro cytotoxicity of mouse spermatogonia cells. Environ Sci Pollut Res 28:17109–17118

Alonso-Magdalena P, Ropero AB, Soriano S et al (2012) Bisphenol-A acts as a potent estrogen via non-classical estrogen triggered pathways. Mol Cell Endocrinol 355:201–207

Asweto CO, Hu H, Liang S et al (2018) Gene profiles to characterize the combined toxicity induced by low level co-exposure of silica nanoparticles and benzo[α]pyrene using whole genome microarrays in zebrafish embryos. Ecotoxicol Environ Saf 163:47–55

Azari MR, Mohammadian Y, Pourahmad J et al (2020) Additive toxicity of co-exposure to pristine multi-walled carbon nanotubes and benzo α pyrene in lung cells. Environ Res 183:109219

Azimzada A, Farner JM, Hadioui M et al (2020) Release of TiO_2 nanoparticles from painted surfaces in cold climates: characterization using a high sensitivity single-particle ICP-MS. Environ Sci: Nano 7:139–148

Balbi T, Smerilli A, Fabri R et al (2014) Co-exposure to n-TiO_2 and Cd^{2+} results in interactive effects on biomarker responses but not in increased toxicity in the marine bivalve *M. galloprovincialis*. Sci Total Environ 493:355–364

Banni M, Sforzini S, Balbi T et al (2016) Combined effects of n-TiO_2 and 2,3,7,8-TCDD in *Mytilus galloprovincialis* digestive gland: a transcriptomic and immunohistochemical study. Environ Res 145:135–144

Barranger A, Langan LM, Sharma V et al (2019) Antagonistic interactions between benzo[α]pyrene and fullerene (C60) in toxicological response of marine mussels. Nanomaterials 9:987

Botta C, Labille J, Auffan M et al (2011) TiO_2-based nanoparticles released in water from commercialized sunscreens in a life-cycle perspective: structures and quantities. Environ Pollut 159:1543–1550

Campos-Garcia J, Martinez DST, Alves OL et al (2015) Ecotoxicological effects of carbofuran and oxidised multiwalled carbon nanotubes on the freshwater fish Nile tilapia: nanotubes enhance pesticide ecotoxicity. Ecotoxicol Environ Saf 111:131–137

Cao X, Ma C, Chen F et al (2021) New insights into the mechanism of graphene oxide-enhanced phytotoxicity of arsenic species. J Hazard Mater 410:124959

Chao S, Huang CP, Chen P et al (2018) Uptake of BDE-209 on zebrafish embryos as affected by SiO_2 nanoparticles. Chemosphere 205:570–578

Chen Y, Li J, Zhou Q et al (2021) Hexavalent chromium amplifies the developmental toxicity of graphene oxide during zebrafish embryogenesis. Ecotoxicol Environ Saf 208:111487

Cotena M, Auffan M, Tassistro V et al (2021) In vitro co-exposure to CeO_2 nanomaterials from diesel engine exhaust and benzo(α)pyrene induces additive DNA damage in sperm and cumulus cells but not in oocytes. Nanomaterials 11:478

De Melo CB, Coa F, Alves OL et al (2019) Co-exposure of graphene oxide with trace elements: effects on acute ecotoxicity and routine metabolism in *Palaemon pandaliformis* (shrimp). Chemosphere 223:157–164

Delfosse V, Grimaldi M, Pons J et al (2012) Structural and mechanistic insights into bisphenols action provide guidelines for risk assessment and discovery of bisphenol A substitutes. Proc Natl Acad Sci USA 109:14930–14935

Deng R, Gao X, Hou J et al (2020) Multi-omics analyses reveal molecular mechanisms for the antagonistic toxicity of carbon nanotubes and ciprofloxacin to *Escherichia coli*. Sci Total Environ 726:138288

Deng H, McShan D, Zhang Y et al (2016) Mechanistic study of the synergistic antibacterial activity of combined silver nanoparticles and common antibiotics. Environ Sci Technol 50:8840–8848
Deng R, Yang K, Lin D (2021) Pentachlorophenol and ciprofloxacin present dissimilar joint toxicities with carbon nanotubes to *Bacillus subtilis*. Environ Pollut 270:116071
Deng R, Zhu Y, Hou J et al (2019) Antagonistic toxicity of carbon nanotubes and pentachlorophenol to *Escherichia coli*: physiological and transcriptional responses. Carbon 145:658–667
Ding T, Li W, Li J (2019) Influence of multi-walled carbon nanotubes on the toxicity and removal of carbamazepine in diatom *Navicula* sp. Sci Total Environ 697:134104
Du J, Cai J, Wang S et al (2017) Oxidative stress and apoptosis to zebrafish (*Danio rerio*) embryos exposed to perfluorooctane sulfonate (PFOS) and ZnO nanoparticles. Int J Occup Med Environ Health 30:213–229
Du J, Tang J, Xu S et al (2018) Parental transfer of perfluorooctane sulfonate and ZnO nanoparticles chronic co-exposure and inhibition of growth in F1 offspring. Regul Toxicol Pharmacol 98:41–49
Du J, Wang S, You H et al (2016) Effects of ZnO nanoparticles on perfluorooctane sulfonate induced thyroid-disrupting on zebrafish larvae. J Environ Sci 47:153–164
Duan J, Yu Y, Li Y et al (2016) Inflammatory response and blood hypercoagulable state induced by low level co-exposure with silica nanoparticles and benzo[α]pyrene in zebrafish (*Danio rerio*) embryos. Chemosphere 151:152–162
Fang Q, Shi Q, Guo Y et al (2016) Enhanced bioconcentration of bisphenol A in the presence of nano-TiO_2 can lead to adverse reproductive outcomes in zebrafish. Environ Sci Technol 50:1005–1013
Fang Q, Shi X, Zhang L et al (2015) Effect of titanium dioxide nanoparticles on the bioavailability, metabolism, and toxicity of pentachlorophenol in zebrafish larvae. J Hazard Mater 283:897–904
Farkas J, Bergum S, Nilsen EW et al (2015) The impact of TiO_2 nanoparticles on uptake and toxicity of benzo(α)pyrene in the blue mussel (*Mytilus edulis*). Sci Total Environ 511:469–476
Ferreira JLR, Lonne MN, Franca TA et al (2014) Co-exposure of the organic nanomaterial fullerene C60 with benzo[α]pyrene in *Danio rerio* (zebrafish) hepatocytes: evidence of toxicological interactions. Aquat Toxicol 147:76–83
Fu J, Guo Y, Yang L et al (2020) Nano-TiO_2 enhanced bioaccumulation and developmental neurotoxicity of bisphenol A in zebrafish larvae. Environ Res 187:109682
Garcia-Gomez C, Babin M, Garcia S et al (2019) Joint effects of zinc oxide nanoparticles and chlorpyrifos on the reproduction and cellular stress responses of the earthworm *Eisenia andrei*. Sci Total Environ 688:199–207
Ginzburg AL, Truong L, Tanguay RL et al (2018) Synergistic toxicity produced by mixtures of biocompatible gold nanoparticles and widely used surfactants. ACS Nano 12:5312–5322
Gnatyshyna L, Falfushynska H, Horyn O et al (2019) Biochemical responses of freshwater mussel *Unio tumidus* to titanium oxide nanoparticles, bisphenol A, and their combination. Ecotoxicology 28:923–937
Gondikas AP, von der Kammer F, Read RB et al (2014) Release of TiO_2 nanoparticles from sunscreens into surface waters: a one-year survey at the Old Danube recreational lake. Environ Sci Technol 48:5415–5422
Guo Y, Chen L, Wu J et al (2019) Parental co-exposure to bisphenol A and nano-TiO_2 causes thyroid endocrine disruption and developmental neurotoxicity in zebrafish offspring. Sci Total Environ 650:557–565
Hartmann NB, Baun A (2010) The nano cocktail: ecotoxicological effects of engineered nanoparticles in chemical mixtures. Integr Environ Assess Manag 6:311–313
Heisterkamp I, Gandrass J, Ruck W (2004) Bioassay-directed chemical analysis utilizing LC-MS: a tool for identifying estrogenic compounds in water samples? Anal Bioanal Chem 378:709–715
Hu S, Han J, Yang L et al (2019) Impact of co-exposure to titanium dioxide nanoparticles and Pb on zebrafish embryos. Chemosphere 233:579–589
Hu CW, Zhang LJ, Wang WL et al (2014) Evaluation of the combined toxicity of multi-walled carbon nanotubes and sodium pentachlorophenate on the earthworm *Eisenia fetida* using avoidance bioassay and comet assay. Soil Biol Biochem 70:123–130

Huang YQ, Wong CK, Zheng JS et al (2012) Bisphenol A (BPA) in China: a review of sources, environmental levels, and potential human health impacts. Environ Int 42:91–99

Iswarya V, Sharma V, Chandrasekaran N et al (2017) Impact of tetracycline on the toxic effects of titanium dioxide (TiO_2) nanoparticles towards the freshwater algal species, *Scenedesmus obliquus*. Aquat Toxicol 193:168–177

Ji Y, Zhou Y, Ma C et al (2017) Jointed toxicity of TiO_2 NPs and Cd to rice seedlings: NPs alleviated Cd toxicity and Cd promoted NPs uptake. Plant Physiol Bioch 110:82–93

Jia J, Li F, Zhai S et al (2017) Susceptibility of overweight mice to liver injury as a result of the ZnO nanoparticle-enhanced liver deposition of Pb^{2+}. Environ Sci Technol 51:1775–1784

Jr. Hochella M, Mogk D, Ranville JF et al (2019) Natural, incidental, and engineered nanomaterials and their impacts on the Earth system. Science 363:eaau8299

Josende ME, Nunes SM, Muller L et al (2019) Multigenerational effects of ecotoxicological interaction between arsenic and silver nanoparticles. Sci Total Environ 696:133947

Kan H, Zhang H, Lu M et al (2021) Effects of carboxylated multi-walled carbon nanotubes on bioconcentration of pentachlorophenol and hepatic damages in goldfish. Ecotoxicology 30:1389–1398

Kora AJ, Rastogi L (2013) Enhancement of antibacterial activity of capped silver nanoparticles in combination with antibiotics, on model gram-negative and gram-positive bacteria. Bioinorg Chem Appl 2013:871097

Lammel T, Wassmur B, Mackevica A et al (2019) Mixture toxicity effects and uptake of titanium dioxide (TiO_2) nanoparticles and 3,3′,4,4′-tetrachlorobiphenyl (PCB77) in juvenile brown trout following co-exposure via the diet. Aqua Toxicol 213:105195

Lei L, Qiao K, Guo Y et al (2020) Titanium dioxide nanoparticles enhanced thyroid endocrine disruption of pentachlorophenol rather than neurobehavioral defects in zebrafish larvae. Chemosphere 249:126536

Li Y, Men B, He Y et al (2017) Effect of single-wall carbon nanotubes on bioconcentration and toxicity of perfluorooctane sulfonate in zebrafish (*Danio rerio*). Sci Total Environ 607–608:509–518

Li L, Stoiber M, Wimmer A et al (2016) To what extent can full-scale wastewater treatment plant effluent influence the occurrence of silver-based nanoparticles in surface waters? Environ Sci Technol 50:6327–6333

Li M, Wu Q, Wang Q et al (2018) Effect of titanium dioxide nanoparticles on the bioavailability and neurotoxicity of cypermethrin in zebrafish larvae. Aquat Toxicol 199:212–219

Lian J, Zhao L, Wu J et al (2020) Foliar spray of TiO_2 nanoparticles prevails over root application in reducing Cd accumulation and mitigating Cd-induced phytotoxicity in maize (*Zea mays* L.). Chemosphere 239:124794

Ma T, Wang M, Gong S et al (2017) Impacts of sediment organic matter content and pH on ecotoxicity of coexposure of TiO_2 nanoparticles and cadmium to freshwater snails *Bellamya aeruginosa*. Arch Environ Contam Toxicol 72:153–165

Manesh RR, Grassi G, Bergami E et al (2018) Co-exposure to titanium dioxide nanoparticles does not affect cadmium toxicity in radish seeds (*Raphanus sativus*). Ecotoxicol Environ Saf 148:359–366

Matouke MM, Elewa DT, Abdullahi K (2018) Binary effect of titanium dioxide nanoparticles ($nTiO_2$) and phosphorus on microalgae (Chlorella 'Ellipsoides Gerneck, 1907). Aquat Toxicol 198:40–48

Matouke MM, Mustapha M (2018) Bioaccumulation and physiological effects of copepods sp. (*Eucyclop* sp.) fed Chlorella ellipsoids exposed to titanium dioxide (TiO_2) nanoparticles and lead (Pb^{2+}). Aquat Toxicol 198:30–39

Meng J, Hong S, Wang T et al (2017) Traditional and new POPs in environments along the Bohai and Yellow Seas: an overview of China and South Korea. Chemosphere 169:503–515

Meng X, Li F, Wang X et al (2020) Toxicological effects of graphene on mussel *Mytilus galloprovincialis* hemocytes after individual and combined exposure with triphenyl phosphate. Mar Pollut Bull 151:110838

Morozesk M, Franqui LS, Mansano AS et al (2018) Interactions of oxidized multiwalled carbon nanotube with cadmium on zebrafish cell line: the influence of two co-exposure protocols on in vitro toxicity tests. Aquat Toxicol 200:136–147

Morozesk M, Franqui LS, Pinheiro FC et al (2020) Effects of multiwalled carbon nanotubes co-exposure with cadmium on zebrafish cell line: metal uptake and accumulation, oxidative stress, genotoxicity and cell cycle. Ecotoxicol Environ Saf 202:110892

Mortimer M, Devarajan N, Li D et al (2018) Multiwall carbon nanotubes induce more pronounced transcriptomic responses in Pseudomonas aeruginosa PG201 than graphene, exfoliated boron nitride, or carbon black. ACS Nano 12:2728–2740

Mottola F, Santonastaso M, Iovine C et al (2021) Adsorption of Cd to TiO_2-NPs forms low genotoxic aggregates in zebrafish cells. Cells 10:310

Naasz S, Altenburger R, Kuhnel D (2018) Environmental mixtures of nanomaterials and chemicals: The Trojan-horse phenomenon and its relevance for ecotoxicity. Sci Total Environ 635:1170–1181

Nunes SM, Josende ME, Ruas CP et al (2017) Biochemical responses induced by co-exposition to arsenic and titanium dioxide nanoparticles in the estuarine polychaete *Laeonereis acuta.* Toxicology 376:51–58

Nunes SM, Muller L, Simioni C et al (2020). Impact of different crystalline forms of $nTiO_2$ on metabolism and arsenic toxicity in *Limnoperna fortunei.* Sci Total Environ 728:138318

Oyelami AO, Semple KT (2015) Impact of carbon nanomaterials on microbial activity in soil. Soil Biol Biochem 86:172–180

Qian W, Chen CC, Zhou S et al (2020) TiO_2 nanoparticles in the marine environment: enhancing bioconcentration, while limiting biotransformation of arsenic in the mussel *Perna viridis*. Environ Sci Technol 54:12254–12261

Qiang L, Shi X, Pan X et al (2015) Facilitated bioaccumulation of perfluorooctanesulfonate in zebrafish by nano-TiO_2 in two crystalline phases. Environ Pollut 206:644–651

Ren X, Zhao X, Duan X et al (2018) Enhanced bio-concentration of tri(1,3-dichloro-2-propyl) phosphate in the presence of nano-TiO_2 can lead to adverse reproductive outcomes in zebrafish. Environ Pollut 233:612–622

Rodd AL, Castilho CJ, Chaparro CEF et al (2018) Impact of emerging, high-production-volume graphene-based materials on the bioavailability of benzo(α)pyrene to brine shrimp and fish liver cells. Environ Sci: Nano 5:2144–2161

Samecka-Cymerman A, Kempers AJ (2004) Toxic metals in aquatic plants surviving in surface water polluted by copper mining industry. Ecotoxicol Environ Saf 59:64–69

Santos LHMLM, Freixa A, Insa S et al (2019) Impact of fullerenes in the bioaccumulation and biotransformation of venlafaxine, diuron and triclosan in river biofilms. Environ Res 169:377–386

Shi X, Li Z, Chen W et al (2016) Fate of TiO_2 nanoparticles entering sewage treatment plants and bioaccumulation in fish in the receiving streams. NanoImpact 3–4:96–103

Song M, Wang F, Zeng L et al (2014) Co-exposure of carboxyl-functionalized single-walled carbon nanotubes and 17α-ethinylestradiol in cultured cells: effects on bioactivity and cytotoxicity. Environ Sci Technol 48:13978–13984

Su Y, Yan X, Pu Y et al (2013) Risks of single-walled carbon nanotubes acting as contaminants-carriers: potential release of phenanthrene in Japanese medaka (*Oryzias latipes*). Environ Sci Technol 47:4704–4710

Sun TY, Gottschalk F, Hungerbuhler K et al (2014b) Comprehensive probabilistic modelling of environmental emissions of engineered nanomaterials. Environ Pollut 185:69–76

Sun H, Ruan Y, Zhu H et al (2014a) Enhanced bioaccumulation of pentachlorophenol in carp in the presence of multi-walled carbon nanotubes. Environ Sci Pollut Res 21:2865–2875

Teng C, Jia J, Wang Z et al (2020) Oral co-exposures to zinc oxide nanoparticles and $CdCl_2$ induced maternal-fetal pollutant transfer and embryotoxicity by damaging placental barriers. Ecotoxicol Environ Saf 189:109956

Tian S, Zhang Y, Song C et al (2014) Titanium dioxide nanoparticles as carrier facilitate bioaccumulation of phenanthrene in marine bivalve, ark shell (*Scapharca subcrenata*). Environ Pollut 192:59–64

Tian S, Zhang Y, Song C et al (2015) Bioaccumulation and biotransformation of polybrominated diphenyl ethers in the marine bivalve (*Scapharca subcrenata*): influence of titanium dioxide nanoparticles. Mar Pollut Bull 90:48–53

Torre CD, Buonocore F, Frenzilli G et al (2015) Influence of titanium dioxide nanoparticles on 2,2,7,8-tetrachlorodibenzo-*p*-dioxin bioconcentration and toxicity in the marine fish European sea bass (*Dicentrarchus labrax*). Environ Pollut 196:185–193

Torre CD, Parolini M, Giacco LD et al (2017) Adsorption of B(α)P on carbon nanopowder affects accumulation and toxicity in zebrafish (*Danio rerio*) embryos. Environ Sci Nano 4:1132–1146

Torre-Roche RDL, Hawthorne J, Musante C et al (2013) Impact of Ag nanoparticle exposure on p,p′-DDE bioaccumulation by *Cucurbita pepo* (zucchini) and *Glycine max* (soybean). Environ Sci Technol 47:718–725

Torre-Roche RDL, Pagano L, Majumdar S et al (2018) Co-exposure of imidacloprid and nanoparticle Ag or CeO_2 to *Cucurbita pepo* (zucchini): contaminant bioaccumulation and translocation. NanoImpact 11:136–145

Trigueiro NSS, Goncalves BB, Dias FC et al (2021) Co-exposure of iron oxide nanoparticles and glyphosate-based herbicide induces DNA damage and mutagenic effects in the guppy (*Poecilia reticulata*). Environ Toxicol Pharmacol 81:103521

Vannuccini ML, Grassi G, Leaver MJ et al (2015) Combination effects of nano-TiO_2 and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on biotransformation gene expression in the liver of European sea bass *Dicentrarchus labrax*. Comp Biochem Physiol C 176–177:71–78

Wang Z, Wang S, Peijnenburg WJGM (2016) Prediction of joint algal toxicity of nano-CeO_2/nano-TiO_2 and florfenicol: independent action surpasses concentration addition. Chemosphere 156:8–13

Wang F, Yao J, Liu H et al (2015) Cu and Cr enhanced the effects of various carbon nanotubes on microbial communities in an aquatic environment. J Hazard Mater 292:137–145

Wang W, Zhao X, Ren X et al (2020) Antagonistic effects of multi-walled carbon nanotubes and BDE-47 in zebrafish (*Danio rerio*): oxidative stress, apoptosis and DNA damage. Aquat Toxicol 225:105546

Wen Y, Zhang L, Chen Z et al (2016) Co-exposure of silver nanoparticles and chiral herbicide imazethapyr to *Arabidopsis thaliana*: enantioselective effects. Chemosphere 145:207–214

Wu J, Shi Y, Asweto CO et al (2016) Co-exposure to amorphous silica nanoparticles and benzo[α]pyrene at low level in human bronchial epithelial BEAS-2B cells. Environ Sci Pollut Res 23:23134–23144

Wu Q, Yan W, Liu C et al (2019b) Co-exposure with titanium dioxide nanoparticles exacerbates MCLR-induced brain injury in zebrafish. Sci Total Environ 693:133540

Wu J, Zhang J, Nie J et al (2019a) The chronic effect of amorphous silica nanoparticles and benzo[α]pyrene co-exposure at low dose in human bronchial epithelial BEAS-2B cells. Toxicol Res 8:731

Xu Z, Tang T, Cheng H et al (2019) Negligible effects of TiO_2 nanoparticles at environmentally relevant concentrations on the translocation and accumulation of perfluorooctanoic acid and perfluorooctanesulfonate in hydroponically grown pumpkin seedlings (*Cucurbita maxima* × *C. moschata*). Sci Total Environ 686:171–178

Yan Z, Lu G, Sun H et al (2017) Influence of multi-walled carbon nanotubes on the effects of roxithromycin in crucian carp (*Carassius auratus*) in the presence of natural organic matter. Chemosphere 178:165–172

Yan Z, Lu G, Sun H et al (2019) Comparison of the accumulation and metabolite of fluoxetine in zebrafish larva under different environmental conditions with or without carbon nanotubes. Ecotoxicol Environ Saf 172:240–245

Zareitalabad P, Siemens J, Hamer M et al (2013) Perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) in surface waters, sediments, soils and wastewater—a review on concentrations and distribution coefficients. Chemosphere 91:725–732

Zhang S, Deng R, Lin D et al (2017) Distinct toxic interactions of TiO_2 nanoparticles with four coexisting organochlorine contaminants on algae. Nanotoxicology 11:1115–1126

Zhang Y, Xu X, Zhu S et al (2016) Combined toxicity of Fe_3O_4 nanoparticles and cadmium chloride in mice. Toxicol Res 5:1309–1317

Zhu B, Han J, Lei L et al (2021a) Effects of SiO_2 nanoparticles on the uptake of tetrabromobisphenol A and its impact on the thyroid endocrine system in zebrafish larvae. Ecotoxicol Environ Saf 209:111845

Zhu Y, Wu X, Liu Y et al (2021b) Synergistic growth inhibition effect of TiO_2 nanoparticles and tris(1,3-dichloro-2-propyl) phosphate on earthworm in soil. Ecotoxicol Environ Saf 208:111462

Zou X, Xu B, Yu C et al (2013) Combined toxicity of ferroferric oxide nanoparticles and arsenic to the ciliated protozoa *Tetrahymena pyriformis.* Aquat Toxicol 134–135:66–73

Zhu J, Zou Z, Shen Y et al (2019) Increased ZnO nanoparticle toxicity to wheat upon co-exposure to phenanthrene. Environ Pollut 247:108–117

Chapter 9
Micro- and Nanoplastic Pollution in Terrestrial Ecosystems

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Abstract Plastic pollution is ubiquitous and has become a global challenge due to its slow degradation. Especially, small-sized plastics less than 5 mm, known as microplastics (MPs) and nanoplastics (NPs), have attracted wide attention. Although the majority of plastics are produced, used, and discarded on land, there are huge knowledge gaps concerning MPs and NPs in terrestrial ecosystems. In this chapter, we summarize: (1) different methods for floatation, digestion, identification, and quantification of MPs in convoluted environmental substrates; (2) the possible sources of MPs entering into the soil, and the characterizations of MPs in various types of soil; (3) how MPs may affect soil properties and plants. Finally, we also highlight the shortcomings of existing studies and outline the directions for future studies.

Introduction

Plastics are one of the most omnipresent, versatile, and economical materials in the modern age. The global production of plastics has been growing rapidly to address expanding economy. There is an estimated 6300 Mt of plastic waste produced

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between 1950 and 2015, and this number may increase to 12,000 Mt by 2050 (Geyer et al. 2017). The weight of plastics in the ocean may eventually exceed that of fish (Xu et al. 2020). Plastic waste is now so ubiquitous that it can be found in remote areas, including Arctic and Antarctic ice sheets and deep-sea sediments (Horton et al. 2017; Waller et al. 2017). Plastic pollution has become a critical environmental issue, receiving extensive attention due to its long-lasting nature, recalcitrance to degradation, and low recovery rate.

Plastics are made from organic materials such as crude oil, natural gas, salt, coal, or bio-matter, which eventually end up in the environment (de Souza Machado et al. 2018a). When plastics enter the environment, the larger items tend to break up into small plastic particles. Those particles less than 5 mm in diameter are categorized as microplastics (MPs) (Arthur et al. 2009; Thompson et al. 2004). MPs can both originate from engineered micron-sized plastics (primary MPs) (Cole et al. 2011; Fendall and Sewell 2009) such as cosmetics or personal care products, and fragments of large plastic products such as agricultural mulch film (secondary MPs) (Barnes et al. 2009; Huang et al. 2020). These MPs are intentionally or unintentionally discharged into terrestrial environments by littering, landfill, or sewage water (Mason et al. 2016; Wang et al. 2019). Over 90% of MPs in sewage water are maintained in sludge during wastewater treatment (Long et al. 2019; Talvitie et al. 2017b). The widespread utilization of sewage sludge compost products can directly or indirectly result in the introduction of large quantities of MPs into farmland (He et al. 2018; Zhang et al. 2020). Once entering soils, MPs can have significant long-term impacts on soil processes and organisms (Rillig et al. 2019; Zang et al. 2020). For example, MPs can be adsorbed onto the roots of farm plants by a crack-entry mode and then transported and accumulated in aboveground tissues (Li et al. 2020). MPs accumulated in food plants may be ingested by humans and pose risk for human health (Rillig 2020, Wang et al. 2020a).

To date, the literature describing the fate of MPs in terrestrial environments is still scarce. This may be due to the lack of efficient methods to extract and detect MPs in the complex soil matrix, especially those MPs with small size (Bläsing and Amelung 2018; Chai et al. 2020). Currently, the impact of MPs on soil properties and terrestrial plants remains poorly understood. This chapter reviews the current methods to detect MPs in the soil matrix and primary sources of MPs in terrestrial ecosystems. We also summarize the current knowledge on the occurrence of MPs in soils and the effects of MPs on soil properties and plants.

Analytical Procedures for Characterization of Microplastics

Different methods have been proposed in recent years for characterizing MPs in environmental samples. However, these methods vary according to the sample matrix and no standard protocols have been established. Generally, a typical analytical procedure of MPs includes flotation, digestion, identification, and quantification. Here,

we summarize the available methods in each step and evaluate their advantages and limitations.

Flotation

To separate the MPs from the soil matrix, density fractionation is the most frequently used method. The density of common MPs (0.8–1.4 g cm^{-3}) is generally less than that of soils (2.6–2.7 g cm^{-3}). This density difference allows us to separate MPs from soil particles. However, considerable amounts of MPs in soils are confined in soil aggregates (Zhang and Liu 2018). This reduces the recovery of MPs in the samples. Additional procedures, including ultrasonication, stirring, aeration, and constant flow, are needed to assist the extraction of MPs from soils.

Several salt solutions (e.g., NaCl, $CaCl_2$, $ZnCl_2$, $ZnBr_2$, NaBr, and NaI) have been utilized to separate MPs from the soil matrix. Among these, NaCl is cheap, non-toxic, and easily available. Meanwhile, Na^+ promotes the dispersion of soil particles, releasing the MPs that are trapped in the soil aggregates. However, the density of saturated NaCl solution is only 1.2 g cm^{-3}, which may result in a low recovery of high-density MPs such as polyoxymethylene ($\rho = 1.41$–1.43 g cm^{-3}). The density of the $CaCl_2$ solution is higher than that of the NaCl solution. But Ca^{2+} easily flocculates with organic matter (OM), which interferes with later identification (Scheurer and Bigalke 2018). Compared with NaCl, $ZnCl_2$, $ZnBr_2$, and NaBr are toxic chemicals which limits their use (Liu et al. 2019; Zarfl 2019). NaI is more environmentally friendly and has a higher density ($\rho = 1.8$ g cm^{-3}) to ensure most MPs float. Unfortunately, NaI is more expensive than other options. To reduce expenses without compromising flotation efficiency, some researchers have adopted a two-step method. Saturated NaCl solution is used to pretreat the sample and then NaI solution is applied for further extraction (Chai et al. 2020; Nuelle et al. 2014; Zhou et al. 2020a).

In addition to density separation, pressurized fluid extraction, oil extraction, and electrostatic charges are used to separate MPs from the environmental matrix (Zarfl 2019). But these methods are less popular in the MPs analysis.

Digestion

Soil samples have a highly variable OM content that ranges from 0.02% in desert sands to nearly 100% in bog soils (da Costa et al. 2019). OM density generally ranges between 1.0 and 1.4 g cm^{-3}, which is similar to those of plastics such as polystyrene (PS, $\rho = 1.05$ g cm^{-3}) and polycarbonate (PC, $\rho = 1.18$ g cm^{-3}). It is challenging to identify MPs under microscopy in the presence of OM (da Costa et al. 2019). Meanwhile, OM interferes with the analysis by Fourier transform infrared (FTIR) and Raman spectroscopy (Bläsing and Amelung 2018). Hence, various kinds of

acidic, alkaline, and oxidizing treatments and enzymatic digestion are frequently used to remove OM in the samples (Hurley et al. 2018; Möller et al. 2020).

Acid digestion using hydrochloric acid (HCl) and nitric acid (HNO_3) is effective to remove OM. However, they may also digest MPs, e.g., PS and polyamide (PA) (Avio et al. 2015; Catarino et al. 2017; Cole et al. 2014; Dehaut et al. 2016; Nuelle et al. 2014; Rocha-Santos and Duarte 2015,). Similarly, alkaline digestion with sodium hydroxide (NaOH) can dissolve PC, PA, polyethylene terephthalate (PET), and polyethylene (PE) fibers, and result in melding or discoloration of MPs (Cole et al. 2014; Dehaut et al. 2016; Herrera et al. 2018). Sample digestion with 10% potassium hydroxide (KOH) solution at room temperature is most popular and has been shown nondestructive to MPs. But this method is time-consuming and may not be suitable for samples rich in plant materials or stabilized soil OM (Bläsing and Amelung 2018; Herrera et al. 2018). Overall, using strong acid or alkaline could generate unexpected bias in the results when analyzing MPs (Löder et al. 2017).

Oxidation with hydrogen peroxide (H_2O_2) is another routinely used technique to remove OM in the sample preparations (Pansu and Gautheyrou 2006). However, H_2O_2 is much less effective to remove OM in the samples (Nuelle et al. 2014). For example, treating a sample with 35% H_2O_2 at ambient temperature for 7 days only dissolves 25% of the biogenic materials (Cole et al. 2014). Moreover, H_2O_2 oxidation might cause discoloration or damage to MPs like polypropylene (PP), PC, and PE (Nuelle et al. 2014). To save time, some researchers used elevated temperatures during H_2O_2 oxidation. However, high temperatures can cause a complete loss of some plastics (Sujathan et al. 2017). Fenton's reagent, which uses ferrous cations to catalyze the oxidization of organic components with H_2O_2, is a promising alternative to peroxide oxidation. This procedure is relatively low in cost, time-saving, and has little influence on the MPs recovery rate. It has been successfully applied in the isolation of MPs from organic-rich wastewater without significant alterations of the MPs surface (Tagg et al. 2017). Hurley et al. showed that Fenton's reagent is effective to remove organic compounds which are generally recalcitrant to H_2O_2 digestion (Hurley et al. 2018). However, certain biogenic matter is not easily removed by Fenton's reagent (Möller et al. 2020).

In contrast to the chemical treatments mentioned above, enzymatic digestion is useful for removing OM without degrading the MPs. However, enzymatic digestion is much more expensive than chemical treatments. Meanwhile, an enzyme may be matrix-specific and different enzymes are needed to degrade different sample matrices (Hurley et al. 2018; Möller et al. 2020; Zarfl 2019). This limits the wide application of enzymatic digestion in MPs analysis.

Identification and Quantification

Identification and quantification of MPs in a complex environmental matrix is rather challenging. Often, MPs can be visually inspected under microscopy, after which the plastics can be discriminated from other materials by attenuated total reflection

(ATR)-FTIR spectroscopy. After ruling out the non-plastics, further characterizations can be carried out in the plastic particles (Chai et al. 2020; Zhou et al. 2018; Zhou et al. 2020a). This method is low in cost but is also time-consuming. Moreover, the microscopic inspection method is less effective for identifying micron-sized plastics when interference is strong (Shim et al. 2017).

Two instruments commonly used for the identification of micron-sized plastics are Raman and FTIR spectroscopy, which identify MPs via their vibrational spectrum that is unique for every polymer type. But both technologies have some limitations (Cabernard et al. 2018; Chai et al. 2020; Horton et al. 2017). The standard spectra of Raman spectroscopy cannot encompass all types of MPs. Meanwhile, the additives contained in MPs and the autofluorescence produced by soils can generate interference (Löder and Gerdts 2015). Raman spectroscopy setting (e.g., laser intensity, exposure time) needs to be adjusted frequently according to the sample matrix, making the analysis also labor-intensive. Thus, a high level of professional skills is required when analyzing samples by Raman spectroscopy.

Like FTIR spectroscopy, ATR-FTIR cannot efficiently identify particles of small size. It is difficult to cover the diamond internal reflection element with the sample, leading to fluctuations of the necessary surface contact of the specimen with the diamond to obtain a valid spectrum (Horton et al. 2017). Small MPs in soils can be inspected individually by μ-ATR and transmission mode using μ-FTIR (Liu et al. 2018; Scheurer and Bigalke 2018; Zhou et al. 2018; Zhou et al. 2020a). But these techniques still cannot quickly, accurately, and comprehensively identify a mass of particles in soil samples. Before detection, target MP particles need to be manually selected from the sample. Unfortunately, this is slow when processing soil samples that have a complex matrix background and contain numerous granular particles (Zarfl 2019; Zhang and Liu 2018).

Our recent study has adapted a novel Wizards function when using μ-FTIR (Nicolet iN 10 MX, Themo Fisher, USA). This process can quickly target all particles, and at the same time determine the optimal aperture size and spectra for each particle (Chai et al. 2020). It can automatically detect, analyze, and classify MPs, giving a detailed report of the length, width, number, and quality of MPs in the samples (Fig. 9.1). This is a quick and effective method for the identification and quantification of almost all types of MPs in soils. Nevertheless, μ-FTIR spectroscopy is generally limited to MPs with a size larger than 10 μm. In addition, irregularly-shaped particles may generate reflection errors and interfere with spectra matching (Zarfl 2019).

One of the major differences between FTIR and Raman spectroscopy is the spatial resolution. As mentioned above, μ-FTIR analysis is suitable for MPs greater than 10 μm, while Raman spectroscopy has a detection limit as low as 300 nm. Thus, the two spectroscopy techniques can be used as complementary techniques for probing MPs. Raman spectroscopy is sensitive to homo-nuclear and non-polar bonds, while FTIR spectroscopy is more useful for the identification of hetero-nuclear functional group vibrations and polar bonds (Silva et al. 2018). When used together, FTIR and Raman spectroscopy enable identification of a broader range of MPs.

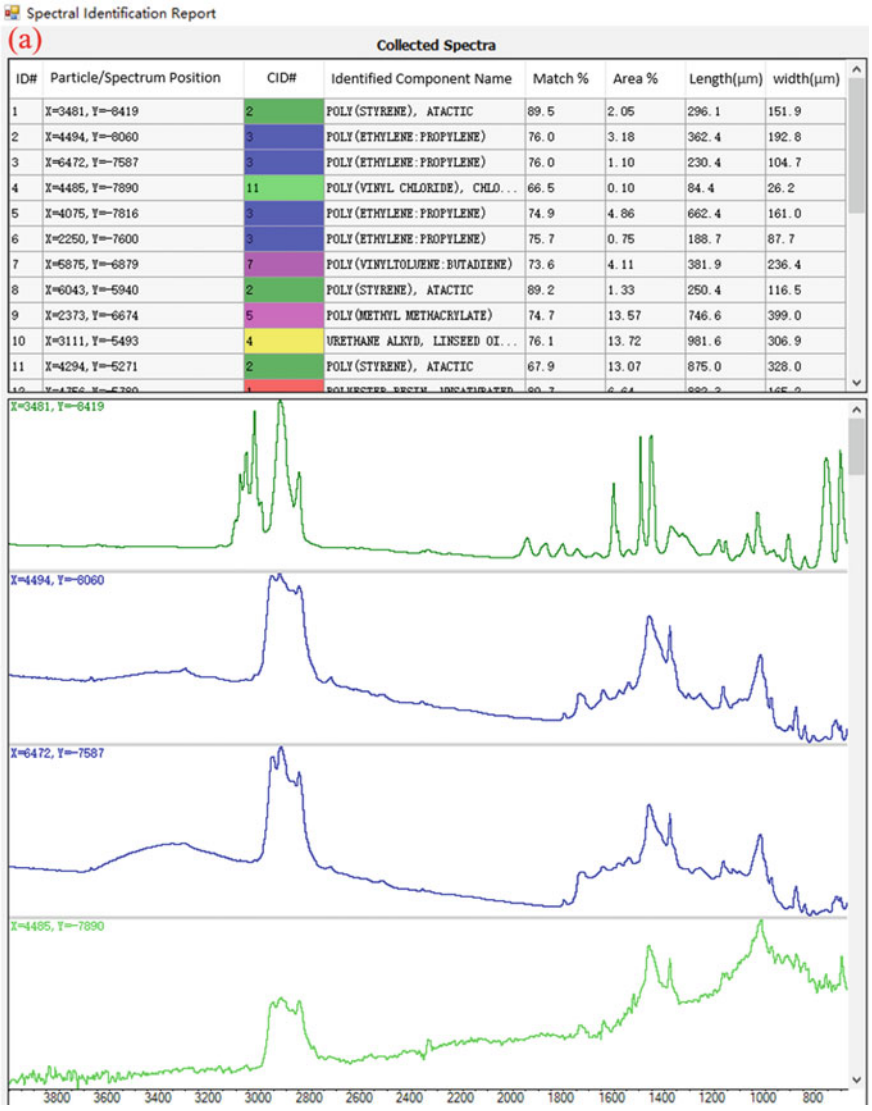

Fig. 9.1 Analysis of micron-sized plastics by μ-FTIR (Nicolet iN 10 MX, Themo Fisher, USA). The same color denotes the same particle properties. **a** Detailed information showing the particulate position, identified component name, length, and width for each particulate; **b** identified library components of the particulates, reporting the number of particulates and the best library match; and **c** scan results for a gold-coated microslide with particulates

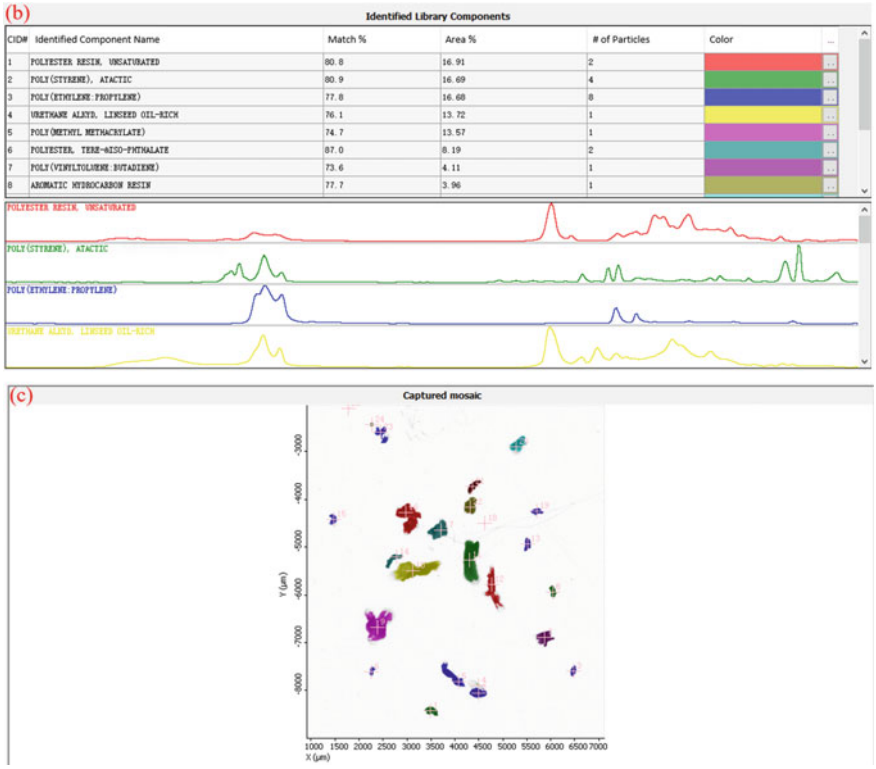

Fig. 9.1 (continued)

Thermal analyses such as pyrolysis gas chromatography–mass spectrometry and thermogravimetric analysis are two emerging analytic techniques for characterizing MPs (David et al. 2018; Dümichen et al. 2017). However, due to the destructive nature of the methods, information on the number, size, and forms of the MPs are not available. This information is critical in the context of evaluating the influence of MPs on organisms and ecosystems (Möller et al. 2020). Moreover, they are not suitable for routine surveys or large-scale studies, because these techniques are also time-consuming (da Costa et al. 2019).

Sources of Microplastics in Terrestrial Environments

Compared with aquatic environments, MPs in terrestrial ecosystems are much more poorly understood. Evidence has shown that a majority of global plastic waste is retained in landfills. Therefore, soils can be regarded as a large MPs sink (Geyer et al. 2017; He et al. 2019; Ng et al. 2018). Besides landfills, sewage sludge, compost,

and organic fertilizer treatment, wastewater irrigation, atmospheric precipitation, and residual mulch degradation are considered the dominant contributors to MPs in soils (Table 9.1). Other sources such as plastic blasting during industrial processes, surface detachment of plastic coating, illegal dumping, and littering also significantly contribute to MPs in terrestrial environments (Zhou et al. 2020b).

Microplastics in Sewage Sludge and Wastewater

Wastewater treatment plants (WWTPs) are receptors of MPs originating from surface water, wastewater, and stormwater (Zhang et al. 2020). MPs from personal care products, cosmetic products, and synthetic clothing can be released into domestic wastewater, which will eventually enter WWTPs. Microbeads and fibers from laundry could be a main source of MPs in domestic wastewater.

The most common color and shape of the sludge-based MPs were white (59.6%) and fiber (63%), respectively. The major types of MPs were polyolefin, acrylic fibers, PE, and PA. It was also found that the MPs levels varied with season and that MPs pollution was more serious in eastern China than in western China (Li et al. 2018). Another study suggested that the MPs content in sludge from Chile ranged from 18 to 41 particles g^{-1} (median value: 34 particles g^{-1}), with fibers as the major MPs detected in sludge (Corradini et al. 2019). Li et al. (2018) measured the MPs concentrations in 79 sludge samples collected from 28 WWTPs in China, which ranged from 1.60 to 56.4 $\times$ 10^3 particles kg^{-1} dry sludge (average value: 22.7 $\pm$ 12.1 $\times$ 10^3 particles kg^{-1} dry sludge). Based on the total sludge production in China, it was calculated that the average amount of sludge-based MPs released into the environment was 1.56 $\times$ 10^{14} particles $year^{-1}$.

Despite WWPTs having high removal efficiencies (95–99%) of MPs (Blair et al. 2019; Talvitie et al. 2017a), MPs remaining in the effluent can be carried into soils by agricultural irrigation. Wang et al. (2020b) characterized MPs in the influents and effluents of nine domestic WWTPs, five industrial WWTPs, and the wastewater from ten industrial plants, four livestock farms, and four fish ponds in Changzhou, China. They found that the MPs in the influents and effluents of domestic WWTPs ranged from 18 to 890 and 6 to 26 particles L^{-1}, respectively. The industrial WWTP effluents contained 6–12 particles L^{-1}, and the MPs levels in wastewater from industrial plants, livestock farms, and fish ponds ranged from 8 to 23, 8 to 40, and 13 to 27 particles L^{-1}, respectively. PE, PP, and PS accounted for up to almost 83% of the total MPs. Fragments and films were the major shapes of MPs detected in wastewater, and a major portion of the MPs were smaller than 500 μm. Another study estimated the annual MPs emissions from WWTP effluents into 10 major river basins in Germany (Schmidt et al. 2020), reporting mean and median effluent concentrations of 2.8 $\times$ 10^4 and 6.3 $\times$ 10^3 items m^{-3}, respectively. The mean and median total annual MPs emissions from WWTPs into German rivers were estimated to be 7.3 $\times$ 10^{12} and 6.8 $\times$ 10^{12} items $year^{-1}$, respectively. These data suggest that WWTPs can act as transport vectors of MPs to the environment.

Table 9.1 Major sources of MPs in terrestrial environments

Location	Occurrence	MPs concentration	Commonly detected polymer	References
China	5 Years of continuous mulching	80.3 ± 49.3 pieces kg^{-1}	PE	Huang et al. (2020)
	15 Years of continuous mulching	308 ± 138.1 pieces kg^{-1}	PE	
	24 Years of continuous mulching	1075.6 ± 346.8 pieces kg^{-1}	PE	
China	Raw composts	200–420 items kg^{-1}	PP	Zhang et al. (2020)
	Semi-finished products	500–910 items kg^{-1}	PP	
	Fnished products	150–410 items kg^{-1}	PP	
China	Landfills, soil	3570 ± 688 n kg^{-1}	PVAL	Chai et al. (2020)
China	Mulching farmlands	571 pieces kg^{-1}	PE, PP, polyester, rayon, acrylic, PA	Zhou et al. (2020a)
	Non-mulching farmlands	263 pieces kg^{-1}	PE, PP, polyester, rayon, acrylic, PA	
China	Landfills, leachate	0.42–24.58 items L^{-1}	PP, PE	He et al. (2019)
China	Sewage sludge	1.60–56.4 × 10^3 particles kg^{-1}	Polyolefin, acrylic fibers, PE, PA	Li et al. (2018)
Chile	Sewage sludge	18–41 particles g^{-1}		Corradini et al. (2019)
Germany	Certified composts	22 particles kg^{-1}	Styrene-based polymers	Weithmann et al. (2018)
	Fresh digestate-fertilizer	146 particles kg^{-1}	Polyester, PE	
China	Influents	18–890 particles L^{-1}	PE, PP, PS	Wang et al. (2020b)
	Effluents	6–26 particles L^{-1}	PE, PP, PS	
Germany	Effluents	2.8 × 10^4 items m^{-3}		Schmidt et al. (2020)
France	Atmospheric fallout	29–280 particles m^{-2} day^{-1}		Dris et al. (2015)
China	Atmospheric fallout	175–313 particles m^{-2} day^{-1}	PE, PP, PS	Cai et al. (2017)
UK	Atmospheric fallout	575–1008 particles m^{-2} day^{-1}	Polyacrylonitrile	Wright et al. (2020)

(continued)

Table 9.1 (continued)

Location	Occurrence	MPs concentration	Commonly detected polymer	References
France	Atmospheric fallout	365 ± 69 particles m^{-2} day^{-1}	PS	Allen et al. (2019)

PVAL, polyvinyl alcohol; PE, polyethylene; PP, polypropylene; PA, polyamide; PS, polystyrene.

Microplastics in Compost and Organic Fertilizers

A majority of sewage sludge is recycled for land application in many countries (Nizzetto et al. 2016; Peccia and Westerhoff 2015). The reuse of sludge in agriculture can spread MPs into farm soils (Keller et al. 2020). Weithmann et al. (2018) found that the average MPs content in certified composts from an aerobic biowaste composting plant in Germany was 22 particles kg^{-1} dry weight. Moreover, there were 146 particles kg^{-1} dry weight in the fresh compost from an anaerobic biowaste digester. Most of the MPs found in the high-quality composts were styrene-based polymers, while those in the fresh digestate fertilizer were polyester, followed by PE. Zhang et al. (2020) detected that the abundance of MPs in the raw compost collected from three WWTPs in China averaged 353.3 ± 97.0 items kg^{-1} (range 200 to 420 items kg^{-1}), while that in the finished compost averaged 245.6 ± 84.1 items kg^{-1} (range 150 to 410 items kg^{-1}). On the contrary, MPs in the semi-finished products, averaging 707.8 ± 153.8 items kg^{-1} (range from 500 to 910 items kg^{-1}), were significantly more than in the finished composts. MPs flakes were dominant (>90%) in compost made from sewage sludge. Meanwhile, six types of MPs were identified, with PP accounting for the highest proportion (59%). These results indicated that the utilization of compost products could be a crucial source of MPs for the soil environment. The study also clearly demonstrated that the number of MPs in soils was closely correlated with the sludge-based compost application rates. Annual amendments with 30 and 15 t ha^{-1} of sludge compost resulted in total MPs abundances of 545.9 and 87.6 items kg^{-1} in soils, respectively, which were significantly more than without compost application (5.0 items kg^{-1}).

Microplastics in Atmospheric Precipitation

MPs can be carried long distances by the wind to remote regions. Atmospheric deposition can also be a source of MPs in soils (Bläsing and Amelung 2018). For example, MPs like fibers were detected in atmospheric fallout (29–280 particles m^{-2} day^{-1}) in the urban area of Greater Paris (Dris et al. 2015). Similarly, Cai et al. (2017) reported that the MPs concentration in atmospheric fallout from Dongguan city, an industrial city in southern China, ranged from 175 to 313 particles m^{-2} day^{-1}. PE, PP, and PS were the MP types detected in the samples, and fibers were the main shape

of the airborne MPs. Scanning electron microscopy (SEM) showed some signs of degradation on the MPs surfaces. Wright et al. (2020) found that the deposition rates of MPs in central London ranged from 575 to 1008 particles m^{-2} day^{-1}, while the concentration of MPs of similar size in a remote location was 20 times lower. Evidence is unequivocal that MPs can be transported through the atmosphere over a distance of up to 95 km and deposited to a remote area of the Pyrenees mountains in France (Allen et al. 2019). In this case, the average daily deposition rate was 365 ± 69 particles m^{-2} day^{-1}, with PS fragments as the dominant MPs in the samples.

Occurrence of Microplastics in Different Types of Soils

Plastic pollution in farmland has received increasing attention because it is closely related to food safety and human health. Plastic films are widely used in agriculture to control soil temperature, restrict weed growth, reduce moisture loss, and increase crop yield as well as (Wu et al. 2017; Zhang et al. 2017). Four million tons of mulch films were produced globally in 2016 and the annual growth rate of mulch film production is expected to reach 5.6% by 2030 (Xu et al. 2020). The consumption of agricultural plastic films in China was 2.60 million tons in 2015, of which 1.46 million tons were mulch films. This accounts for about 90% of the global consumption and covers an area of over 18.33 million hectares. Yet the recovery rate of mulch films is less than 60% (Luo et al. 2018). The increasing use of mulch films undoubtedly results in the exacerbation of MPs pollution in the soils of farmland (Qi et al. 2020).

Zhou et al. (2020a) investigated MPs in farmland soils on the coasts of Hangzhou Bay in eastern China. They revealed that mulched farmlands contained higher soil MPs levels than non-mulched farmlands, with an average 571 pieces kg^{-1} versus 263 pieces kg^{-1}, respectively. There were 10 polymer types with fragments as the dominant type. Moreover, Huang et al. (2020) demonstrated that sustaining plastic mulching results in the increase of MPs in China. MPs accumulated gradually in fields where plastic mulching was applied, with 80.3 ± 49.3, 308 ± 138.1, and 1075.6 ± 346.8 pieces kg^{-1} soil in locations after 5, 15, and 24 years of constant mulching. It was estimated that a hundred trillion mulching-based MPs could have been emitted into the soils, which created a severe threat to food safety and human health. Corradini et al. (2021) showed that the MPs concentration in croplands and pastures were 306 ± 360 and 184 ± 266 particles kg^{-1}, respectively, but there was no evidence of MPs in rangelands and natural grasslands in Chile's central valley. The most common type of MPs was fibers (68%), followed by films (23%), fragments (7%) and pellets (2%), across all land uses. Acrylates, polyurethane, and varnish were the most frequent plastic polymers detected in samples.

At Guiyu, in large flagrant e-waste disassembling zones, the MPs concentration significantly altered among distinct soils, ranging from 0 to 34,100 particles kg^{-1}, which suggested that the e-waste disassembling area could be considered as an MPs hotspot (Chai et al. 2020). To be specific, the average concentration of MPs in dilapidated e-waste dismantling areas and polluted agricultural soils were 13,900 ± 7260

and 12,300 ± 10,500 particles kg^{-1}, respectively. The soils around the garbage dump contained high concentrations of MPs (mean: 3570 ± 688 particles kg^{-1}). However, soils from fruit lands contained 36.7 ± 24.3 particles kg^{-1} of MPs. Totally six different shapes and ten colors of MPs were detected in the soils and most of them were secondary MPs (Fig. 9.2). The MPs primarily comprised of engineering plastics and modified plastics, while 88.6% had a size less than 1 mm, suggesting that the major source of MPs at Guiyu was mainly from e-wastes. The surface of MPs had clear signs of aging and degradation due to the primitive disassembling methods and long-term exposure to the soils. Additionally, the average Pb, Cd, Cr, Ba, Cu, Co, and As concentrations of MPs were 20.9, 0.7, 11.8, 308.8, 4.1, 1.3, and 3.1 $\mu g\ g^{-1}$, respectively, suggesting these MPs could be a source of metals in the soil environment.

Effects of Microplastics on Soil Properties and Plants

Soils are an integral part of terrestrial ecosystems because many processes critical to the functioning and service of ecosystems occur in the soils. But soils are increasingly faced with the threat of MPs pollution. Once MPs enter soils, they could alter fundamental soil properties and affect the growth performance of plants.

Effects of Microplastics on Soil Physicochemical Properties

MPs have a prominent impact on the physicochemical properties of soils. Liu et al. (2017) conducted experiments with 3 concentrations [0, 7, and 28% (w/w)] of MPs (PE plastic residue) added to loess soils to determine the effects on soil dissolved organic matter (DOM) and enzymatic activity. The addition of MPs increased soil microbial activity based on the monitoring of fluorescein diacetate hydrolase. The addition of MPs also significantly increased the concentrations of dissolved organic carbon, dissolved organic nitrogen, dissolved organic phosphorus, high-molecular-weight humic-like material, and fulvic acid. These results indicated that the accumulation of MPs may change soil microbial activity and alter the chemical properties of the soils. De Souza Machado et al. (2018b) added different concentrations (up to 2%) of four common MPs types (polyacrylic fibers, polyester fibers, PA beads, and PE fragments) to a loamy sandy soil during a five-week garden experiment. All tested MPs affected soil bulk density, with polyester fibers producing the most conspicuous changes in the proxies for the soil biophysical properties. Increasing polyester fiber concentrations significantly prompted the water-holding capacity of the soil, but decreased water-stable aggregates. Polyacrylic fibers also affected soil structure, resulting in a significant decline in water-stable aggregates. However, no significant changes in the soil bulk density of clayey soils from the field (one year) and pot (six wet-dry cycles) experiments were detected between the polyester fiber

Fig. 9.2 MPs found in the soils of Guiyu, a notable e-waste disassembling zone of China

(0.1 and 0.3%) and control treatments (Zhang et al. 2019). Furthermore, polyester fibers significantly increased the volume of pores >30 μm and reduced the volume of pores <30 μm, which also enhanced soil aggregation in the pot experiment. But the effects were less apparent in the field experiment. In another study, three types of MPs, biodegradable polylactic acid (PLA), conventional high-density PE (HDPE), and MPs clothing fibers, were added to a sandy clay loam soil in a mesocosm experiment (Boots et al. 2019). After 30 days of MPs exposure, the soil pH and water-stable soil aggregate profiles significantly differed between the MPs treatments. De Souza Machado et al. (2019) concluded that soil structure is affected by MPs, but the effects vary with the types and size of MPs.

Effects of Microplastics on Terrestrial Plants

Since MPs can change the physicochemical properties of soils, it is not surprising that they also affect the growth performance of plants. Qi et al. (2018) conducted the first study on the effects of MPs from mulch films on the growth of wheat (*Triticum aestivum*). They found that the addition of low-density PE (LDPE) and a type of starch-based plastics to sandy soils affected the growth of both above-ground and below-ground tissues of wheat plants. Interestingly, biodegradable plastics exhibited stronger negative effects than PE. The study by de Souza Machado et al. (2019) also focused on the effects of 6 types of MPs on the growth of spring onion (*Allium fistulosum*) in loamy sandy soils. The plant biomass, tissue elemental composition, root traits, and soil microbial activities were significantly changed after MPs exposure. However, MPs having a shape similar to soil particles had relatively less negative impacts on the plant. Polyester fibers and PA beads had the most significant effects on plant physiology. In another experiment, Boots et al. (2019) showed that the germination rate of perennial ryegrass (*Lolium perenne*) seeds was reduced by MPs clothing fibers or PLA. Meanwhile, PLA significantly reduced the shoot height. However, compared to the control, there were no significant differences in germination rate or shoot height when HDPE was added, indicating the toxicity of MPs varies with polymer types. Wang et al. (2020c) demonstrated that a high dose of biodegradable MPs (PLA) produces more phytotoxicity than non-biodegradable MPs (PE). The addition of 10% PLA reduced maize biomass and its leaf chlorophyll concentrations. MPs may also affect the interactions between soil microbial communities and plants. The type and dose of MPs were found to have significant effects on arbuscular mycorrhizal fungi (AMF) community structure and diversity. When combined with Cd, MPs produced more significant impacts on plant growth and the AMF community than PE. Lozano and Rillig (2020) assessed the effect of microfibers on plant productivity and structure in a community of grasses and herbs. The results showed that shoot and root mass increased with the addition of microfibers at the community level, probably due to decreased soil bulk density, prompted aeration, or increased root penetration in the soil. Microfibers also affected plant community structure, promoting the growth of the highly invasive species.

As mentioned above, plastics in the environment will eventually degrade into smaller-sized MPs, even NPs (<100 nm). Previous studies have revealed the influences of NPs on aquatic plants, but little data is indicating the internalization of NPs in land plants. Li et al. (2020) have opened the door with their experiments, analyzing the uptake of different NPs from treated wastewater by crop plants [wheat (*T. aestivum*) and lettuce (*Lactuca sativa*)] in different growth matrix (Rillig 2020). They demonstrated that nano-sized PS and polymethylmethacrylate (PMMA) beads could penetrate the stele of both species via "crack entry" at the locations of lateral root emergence. The crack-entry pathway and NP characteristics resulted in the efficient uptake of the plastic beads. The NPs were finally moved from the roots to the shoots by transpiration. Sun et al. (2020) demonstrated that positively and negatively charged NPs accumulated differently in *Arabidopsis thaliana* depending on their surface charge. The NPs aggregation and root exudates restricted the uptake of amino-modified PS NPs with the positively charged surface. Although only a few of the positively charged NPs were accumulated at the root tips, it caused a greater accumulation of reactive oxygen species, resulting in inferior plant growth and seedling development compared to negatively charged sulfonic-acid-modified NPs.

Conclusions and Future Research Needs

Study on MPs and NPs pollution in terrestrial ecosystems is still in its early stages. Still, a growing body of evidence suggests that MPs can generate a significant impact on terrestrial ecosystems. Researchers have jointly worked to establish various methods to characterize MPs and NPs, investigate the occurrence of MPs in different environments, and analyze the effects of MPs and NPs on soils and plants.

Further studies are required to develop and standardize the analysis procedure for MPs, especially for particle sizes smaller than 10 μm. Procedures for sample preparations and methods for MPs detection need to be further refined to generate reliable data. Extensive surveys are needed to understand the degree of MPs pollution in different types of terrestrial ecosystems. The degradation of plastics in different environments also needs to be tested. Biodegradable plastics such as PLA are increasingly applied and their fates in the environment remain largely unknown. Several studies have clearly illustrated the ecotoxicity of MPs, but more research is needed regarding MP accumulation in plants and the subsequent trophic transfer, which have important implications for food safety and risk for human health.

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References

Allen S, Allen D, Phoenix VR et al (2019) Atmospheric transport and deposition of microplastics in a remote mountain catchment. Nat Geosci 12:339–344. https://doi.org/10.1038/s41561-019-0335-5

Arthur C, Baker J, Bamford H (eds) (2009) Proceedings of the international research workshop on the occurrence, effects and fate of microplastic marine febris, Sept 9–11, 2008. NOAA Technical Memorandum NOS-OR&R-30

Avio CG, Gorbi S, Regoli F (2015) Experimental development of a new protocol for extraction and characterization of microplastics in fish tissues: first observations in commercial species from Adriatic Sea. Mar Environ Res 111:18–26. https://doi.org/10.1016/j.marenvres.2015.06.014

Barnes DKA, Galgani F, Thompson RC et al (2009) Accumulation and fragmentation of plastic debris in global environments. Philos Trans R Soc Lond B Biol Sci 364(1526):1985–1998. https://doi.org/10.1098/rstb.2008.0205

Bläsing M, Amelung W (2018) Plastics in soil: analytical methods and possible sources. Sci Total Environ 612:422–435. https://doi.org/10.1016/j.scitotenv.2017.08.086

Blair RM, Waldron S, Gauchotte-Lindsay C (2019) Average daily flow of microplastics through a tertiary wastewater treatment plant over a ten-month period. Water Res 163:114909. https://doi.org/10.1016/j.watres.2019.114909

Boots B, Russell CW, Green DS (2019) Effects of microplastics in soil ecosystems: above and below ground. Environ Sci Technol 53(19):11496–11506. https://doi.org/10.1021/acs.est.9b03304

Cabernard L, Roscher L, Lorenz C et al (2018) Comparison of Raman and Fourier transform infrared spectroscopy for the quantification of microplastics in the aquatic environment. Environ Sci Technol 52(22):13279–13288. https://doi.org/10.1021/acs.est.8b03438

Cai L, Wang J, Peng J et al (2017) Characteristic of microplastics in the atmospheric fallout from Dongguan city, China: preliminary research and first evidence. Environ Sci Pollut R 24:24928–24935. https://doi.org/10.1007/s11356-017-0116-x

Catarino AI, Thompson R, Sanderson W et al (2017) Development and optimization of a standard method for extraction of microplastics in mussels by enzyme digestion of soft tissues. Environ Toxicol Chem 36(4):947–951. https://doi.org/10.1002/etc.3608

Chai B, Wei Q, She Y et al (2020) Soil microplastic pollution in an e-waste dismantling zone of China. Waste Manag 118:291–301. https://doi.org/10.1016/j.wasman.2020.08.048

Cole M, Lindeque P, Halsband C et al (2011) Microplastics as contaminants in the marine environment: areview. Mar Pollut Bull 62(12):2588–2597. https://doi.org/10.1016/j.marpolbul.2011.09.025

Cole M, Webb H, Lindeque PK et al (2014) Isolation of microplastics in biota-rich seawater samples and marine organisms. Sci Rep 4:4528. https://doi.org/10.1038/srep04528

Corradini F, Casado F, Leiva V et al (2021) Microplastics occurrence and frequency in soils under different land uses on a regional scale. Sci Total Environ 752:141917. https://doi.org/10.1016/j.scitotenv.2020.141917

Corradini F, Meza P, Eguiluz R et al (2019) Evidence of microplastic accumulation in agricultural soils from sewage sludge disposal. Sci Total Environ 671:411–420. https://doi.org/10.1016/j.scitotenv.2019.03.368

da Costa JP, Paço A, Santos PSM (2019) Microplastics in soils: assessment, analytics and risks. Environ Chem 16:18–30. https://doi.org/10.1071/EN18150

David J, Steinmetz Z, Kučerík J et al (2018) Quantitative analysis of poly(ethylene terephthalate) microplastics in soil via thermogravimetry-mass spectrometry. Anal Chem 90(15):8793–8799. https://doi.org/10.1021/acs.analchem.8b00355

Dehaut A, Cassone AL, Frere L et al (2016) Microplastics in seafood: benchmark protocol for their extraction and characterization. Environ Pollut 215:223–233. https://doi.org/10.1016/j.envpol.2016.05.018

de Souza Machado AA, Kloas W, Zarfl C et al (2018a) Microplastics as an emerging threat to terrestrial ecosystems. Glob Chang Biol 24(4):1405–1416. https://doi.org/10.1111/gcb.14020

de Souza Machado AA, Lau CW, Kloas W et al (2019) Microplastics can change soil properties and affect plant performance. Environ Sci Technol 53(10):6044–6052. https://doi.org/10.1021/acs.est.9b01339

de Souza Machado AA, Lau CW, Till J et al (2018b) Impacts of microplastics on the soil biophysical environment. Environ Sci Technol 52(17):9656–9665. https://doi.org/10.1021/acs.est.8b02212

Dris R, Gasperi J, Rocher V et al (2015) Microplastic contamination in an urban area: a case study in Greater Paris. Environ Chem 12(5):592–599. https://doi.org/10.1071/En14167

Dümichen E, Eisentraut P, Bannick CG et al (2017) Fast identification of microplastics in complex environmental samples by a thermal degradation method. Chemosphere 174:572–584. https://doi.org/10.1016/j.chemosphere.2017.02.010

Fendall LS, Sewell MA (2009) Contributing to marine pollution by washing your face: microplastics in facial cleansers. Mar Pollut Bull 58(8):1225–1228. https://doi.org/10.1016/j.marpolbul.2009.04.025

Geyer R, Jambeck JR, Law KL (2017) Production, use, and fate of all plastics ever made. Sci Adv 3(7):e1700782. https://doi.org/10.1126/sciadv.1700782

He P, Chen L, Shao L et al (2019) Municipal solid waste (MSW) landfill: a source of microplastics?—Evidence of microplastics in landfill leachate. Water Res 159:38–45. https://doi.org/10.1016/j.watres.2019.04.060

He D, Luo Y, Lu S et al (2018) Microplastics in soils: analytical methods, pollution characteristics and ecological risks. TrAC Trends Anal Chem 109:163–172. https://doi.org/10.1016/j.trac.2018.10.006

Herrera A, Garrido-Amador P, Martínez I et al (2018) Novel methodology to isolate microplastics from vegetal-rich samples. Mar Pollut Bull 129(1):61–69. https://doi.org/10.1016/j.marpolbul.2018.02.015

Horton AA, Walton A, Spurgeon DJ et al (2017) Microplastics in freshwater and terrestrial environments: evaluating the current understanding to identify the knowledge gaps and future research priorities. Sci Total Environ 586:127–141. https://doi.org/10.1016/j.scitotenv.2017.01.190

Huang Y, Liu Q, Jia W et al (2020) Agricultural plastic mulching as a source of microplastics in the terrestrial environment. Environ Pollut 260:114096. https://doi.org/10.1016/j.envpol.2020.114096

Hurley RR, Lusher AL, Olsen M et al (2018) Validation of a method for extracting microplastics from complex, organic-rich, environmental matrices. Environ Sci Technol 52(13):7409–7417. https://doi.org/10.1021/acs.est.8b01517

Keller AS, Jimenez-Martinez J, Mitrano DM (2020) Transport of nano- and microplastic through unsaturated porous media from sewage sludge application. Environ Sci Technol 54(2):911–920. https://doi.org/10.1021/acs.est.9b06483

Li X, Chen L, Mei Q et al (2018) Microplastics in sewage sludge from the wastewater treatment plants in China. Water Res 142:75–85. https://doi.org/10.1016/j.watres.2018.05.034

Li L, Luo Y, Li R et al (2020) Effective uptake of submicrometre plastics by crop plants via a crack-entry mode. Nat Sustain. https://doi.org/10.1038/s41893-020-0567-9

Liu M, Lu S, Song Y et al (2018) Microplastic and mesoplastic pollution in farmland soils in suburbs of Shanghai, China. Environ Pollut 242(A):855–862. https://doi.org/10.1016/j.envpol.2018.07.051

Liu M, Song Y, Lu S et al (2019) A method for extracting soil microplastics through circulation of sodium bromide solutions. Sci Total Environ 691:341–347. https://doi.org/10.1016/j.scitotenv.2019.07.144

Liu H, Yang X, Li G et al (2017) Response of soil dissolved organic matter to microplastic addition in Chinese loess soil. Chemosphere 185:907–917. https://doi.org/10.1016/j.chemosphere.2017.07.064

Löder MGJ, Gerdts G (2015) Methodology used for the detection and identification of microplastics—a critical appraisal. In: Bergmann M, Gutow L, Klages M (eds) Marine anthropogenic litter. Springer, Cham, p 201–227 https://doi.org/10.1007/978-3-319-16510-3_8

Löder MGJ, Imhof HK, Ladehoff M et al (2017) Enzymatic purification of microplastics in environmental samples. Environ Sci Technol 51(24):14283–14292. https://doi.org/10.1021/acs.est.7b03055

Long Z, Pan Z, Wang W et al (2019) Microplastic abundance, characteristics, and removal in wastewater treatment plants in a coastal city of China. Water Res 155:255–265. https://doi.org/10.1016/j.watres.2019.02.028

Lozano YM, Rillig MC (2020) Effects of microplastic fibers and drought on plant communities. Environ Sci Technol 54(10):6166–6173. https://doi.org/10.1021/acs.est.0c01051

Luo Y, Zhou Q, Zhang H et al (2018) Pay attention to research on microplastic pollution in soil for prevention of ecological and food chain risks. Environ Pollut Control Strategy Microplastics 33(10):1021–1030. https://doi.org/10.16418/j.issn.1000-3045.2018.10.003

Mason SA, Garneau D, Sutton R et al (2016) Microplastic pollution is widely detected in US municipal wastewater treatment plant effluent. Environ Pollut 218:1045–1054. https://doi.org/10.1016/j.envpol.2016.08.056

Möller JN, Löder MGJ, Laforsch C (2020) Finding microplastics in soils: A review of analytical methods. Environ Sci Technol 54(4):2078–2090. https://doi.org/10.1021/acs.est.9b04618

Ng EL, Huerta Lwanga E, Eldridge SM et al (2018) An overview of microplastic and nanoplastic pollution in agroecosystems. Sci Total Environ 627:1377–1388. https://doi.org/10.1016/j.scitotenv.2018.01.341

Nizzetto L, Futter M, Langaas S (2016) Are agricultural soils dumps for microplastics of urban origin? Environ Sci Technol 50(20):10777–10779. https://doi.org/10.1021/acs.est.6b04140

Nuelle MT, Dekiff JH, Remy D et al (2014) A new analytical approach for monitoring microplastics in marine sediments. Environ Pollut 184:161–169. https://doi.org/10.1016/j.envpol.2013.07.027

Pansu M, Gautheyrou J (2006) Handbook of soil analysis: mineralogical, organic and inorganic methods. Springer, Berlin Heidelberg. https://doi.org/10.1007/978-3-540-31211-6

Peccia J, Westerhoff P (2015) We should expect more out of our sewage sludge. Environ Sci Technol 49(14):8271–8276. https://doi.org/10.1021/acs.est.5b01931

Qi R, Jones DL, Li Z et al (2020) Behavior of microplastics and plastic film residues in the soil environment: a critical review. Sci Total Environ 703:134722. https://doi.org/10.1016/j.scitotenv.2019.134722

Qi Y, Yang X, Pelaez AM et al (2018) Macro- and micro- plastics in soil-plant system: effects of plastic mulch film residues on wheat (*Triticum aestivum*) growth. Sci Total Environ 645:1048–1056. https://doi.org/10.1016/j.scitotenv.2018.07.229

Rillig MC (2020) Plastic and plants. Nat Sustain 3:887–888. https://doi.org/10.1038/s41893-020-0583-9

Rillig MC, Lehmann A, de Souza Machado AA et al (2019) Microplastic effects on plants. New Phytol 223(3):1066–1070. https://doi.org/10.1111/nph.15794

Rocha-Santos T, Duarte AC (2015) A critical overview of the analytical approaches to the occurrence, the fate and the behavior of microplastics in the environment. TrAC Trends Anal Chem 65:47–53. https://doi.org/10.1016/j.trac.2014.10.011

Scheurer M, Bigalke M (2018) Microplastics in Swiss floodplain soils. Environ Sci Technol 52(6):3591–3598. https://doi.org/10.1021/acs.est.7b06003

Schmidt C, Kumar R, Yang S et al (2020) Microplastic particle emission from wastewater treatment plant effluents into river networks in Germany: loads, spatial patterns of concentrations and potential toxicity. Sci Total Environ 737:139544. https://doi.org/10.1016/j.scitotenv.2020.139544

Shim WJ, Hong SH, Eo SE (2017) Identification methods in microplastic analysis: a review. Anal Methods 9:1384–1391. https://doi.org/10.1039/c6ay02558g

Silva AB, Bastos AS, Justino CIL et al (2018) Microplastics in the environment: challenges in analytical chemistry—a review. Anal Chim Acta 1017:1–19. https://doi.org/10.1016/j.aca.2018.02.043

Sujathan S, Kniggendorf A, Kumar A et al (2017) Heat and bleach: a cost-efficient method for extracting microplastics from return activated sludge. Arch Environ Contam Toxicol 73:641–648. https://doi.org/10.1007/s00244-017-0415-8

Sun X, Yuan X, Jia Y et al (2020) Differentially charged nanoplastics demonstrate distinct accumulation in *Arabidopsis thaliana*. Nat Nanotechnol 15(9):755–760. https://doi.org/10.1038/s41565-020-0707-4

Tagg AS, Harrison JP, Ju-Nam Y et al (2017) Fenton's reagent for the rapid and efficient isolation of microplastics from wastewater. Chem Commun 53:372–375. https://doi.org/10.1039/c6cc08798a

Talvitie J, Mikola A, Koistinen A et al (2017a) Solutions to microplastic pollution—removal of microplastics from wastewater effluent with advanced wastewater treatment technologies. Water Res 123:401–407. https://doi.org/10.1016/j.watres.2017.07.005

Talvitie J, Mikola A, Setälä O et al (2017b) How well is microlitter purified from wastewater?—A detailed study on the stepwise removal of microlitter in a tertiary level wastewater treatment plant. Water Res 109:164–172. https://doi.org/10.1016/j.watres.2016.11.046

Thompson RC, Olsen Y, Mitchell RP et al (2004) Lost at sea: Where is all the plastic? Science 304(5672):838. https://doi.org/10.1126/science.1094559

Waller CL, Griffiths HJ, Waluda CM et al (2017) Microplastics in the Antarctic marine system: an emerging area of research. Sci Total Environ 598:220–227. https://doi.org/10.1016/j.scitotenv.2017.03.283

Wang Q, Bai J, Ning B et al (2020a) Effects of bisphenol A and nanoscale and microscale polystyrene plastic exposure on particle uptake and toxicity in human Caco-2 cells. Chemosphere 254:126788. https://doi.org/10.1016/j.chemosphere.2020.126788

Wang T, Li B, Zou X et al (2019) Emission of primary microplastics in mainland China: invisible but not negligible. Water Res 162:214–224. https://doi.org/10.1016/j.watres.2019.06.042

Wang F, Wang B, Duan L et al (2020b) Occurrence and distribution of microplastics in domestic, industrial, agricultural and aquacultural wastewater sources: a case study in Changzhou, China. Water Res 182:115956. https://doi.org/10.1016/j.watres.2020.115956

Wang F, Zhang X, Zhang S et al (2020c) Interactions of microplastics and cadmium on plant growth and arbuscular mycorrhizal fungal communities in an agricultural soil. Chemosphere 254:126791. https://doi.org/10.1016/j.chemosphere.2020.126791

Weithmann N, Moller JN, Loder MGJ et al (2018) Organic fertilizer as a vehicle for the entry of microplastic into the environment. Sci Adv 4(4):eaap8060 https://doi.org/10.1126/sciadv.aap8060

Wright SL, Ulke J, Font A et al (2020) Atmospheric microplastic deposition in an urban environment and an evaluation of transport. Environ Int 136:105411. https://doi.org/10.1016/j.envint.2019.105411

Wu Y, Du T, Ding R et al (2017) An isotope method to quantify soil evaporation and evaluate water vapor movement under plastic film mulch. Agric Water Manag 184:59–66. https://doi.org/10.1016/j.agwat.2017.01.005

Xu C, Zhang B, Gu C et al (2020) Are we underestimating the sources of microplastic pollution in terrestrial environment? J Hazard Mater 400:123228. https://doi.org/10.1016/j.jhazmat.2020.123228

Zang H, Zhou J, Marshall MR et al (2020) Microplastics in the agroecosystem: Are they an emerging threat to the plant-soil system? Soil Biol Biochem 148:107926. https://doi.org/10.1016/j.soilbio.2020.107926

Zarfl C (2019) Promising techniques and open challenges for microplastic identification and quantification in environmental matrices. Anal Bioanal Chem 411:3743–3756. https://doi.org/10.1007/s00216-019-01763-9

Zhang F, Zhang W, Li M et al (2017) Is crop biomass and soil carbon storage sustainable with long-term application of full plastic film mulching under future climate change? Agric Syst 150:67–77. https://doi.org/10.1016/j.agsy.2016.10.011

Zhang G, Liu Y (2018) The distribution of microplastics in soil aggregate fractions in southwestern China. Sci Total Environ 642:12–20. https://doi.org/10.1016/j.scitotenv.2018.06.004

Zhang G, Zhang F, Li X (2019) Effects of polyester microfibers on soil physical properties: perception from a field and a pot experiment. Sci Total Environ 670:1–7. https://doi.org/10.1016/j.scitotenv.2019.03.149

Zhang L, Xie Y, Liu Y et al (2020) An overlooked entry pathway of microplastics into agricultural soils from application of sludge-based fertilizers. Environ Sci Technol 54(7):4248–4255. https://doi.org/10.1021/acs.est.9b07905

Zhou B, Wang J, Zhang H et al (2020a) Microplastics in agricultural soils on the coastal plain of Hangzhou Bay, east China: multiple sources other than plastic mulching film. J Hazard Mater 388:121814. https://doi.org/10.1016/j.jhazmat.2019.121814

Zhou Y, Wang J, Zou M et al (2020b) Microplastics in soils: a review of methods, occurrence, fate, transport, ecological and environmental risks. Sci Total Environ 748:141368. https://doi.org/10.1016/j.scitotenv.2020.141368

Zhou Q, Zhang H, Fu C et al (2018) The distribution and morphology of microplastics in coastal soils adjacent to the Bohai Sea and the Yellow Sea. Geoderma 322:201–208. https://doi.org/10.1016/j.geoderma.2018.02.015

Chapter 10
Advances in the Toxicological Studies of Atmospheric Particulate Matter

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Abstract Ambient airborne particular matter (PM) is a major environmental risk to human health in the world. Numerous epidemiological, clinical and experimental studies have demonstrated the association between high exposure levels of PM and an increase in various diseases, including asthma, chronic obstructive pulmonary disease (COPD), pulmonary fibrosis, lung cancer, neurodegenerative diseases, heart diseases, and diabetes. The proposed underlying biological and molecular mechanisms whereby PM causes adverse health effects include oxidative stress, inflammation and genotoxicity. This chapter provides an overview of the recent literature reporting new insights about the molecular mechanisms linking ambient PM exposure and health effects.

Introduction

Air pollution in urban and industrial areas is a challenge to developing and developed countries (Lee et al. 2020). Air pollution causes adverse health effects through exposure to airborne particulate matter (PM) (Prada et al. 2020). In the past several decades, numerous epidemiological studies have demonstrated that exposure to urban PM is associated with several adverse health effects (Simkhovich et al. 2008; Hoek et al. 2013; Kim et al. 2015). Short-term exposure to PM can cause exacerbation of bronchitis, asthma and changes in heart rate, whereas long-term exposure to

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high concentrations of PM increases the risk of respiratory diseases, arteriosclerosis, neurodegenerative diseases, type-2 diabetes and lung cancer (Rhinehart et al. 2020; Heusinkveld et al. 2016; Rajagopalan et al. 2020; Ye et al. 2020; Sun et al. 2013). Combined with the population-based epidemiological investigations, accumulating evidence in laboratory animals and controlled human exposures has also revealed a stronger correlation of adverse health effects with fine respirable particles than with other atmospheric gas pollutants (Valavanidis et al. 2013; Mukherjee and Agrawal 2018).

Particulate Air Pollution and Its Physicochemical Properties

Ambient airborne PM, which includes dust, dirt, soot, smoke, and liquid droplets emitted into the air, is small enough to be suspended in the atmosphere (Supharakonsakun et al. 2020). It is well acknowledged that PM originates from natural and anthropogenic sources (Hopke et al. 2020; Zhu et al. 2018). Airborne particles that come from natural sources, such as evaporated sea spray, windborne pollen, dust, and volcanic or other geothermal eruptions, tend to be coarse (Gao et al. 2017). In contrast, almost all fine particles are generated as a result of combustion processes from anthropogenic sources, including the burning of fossil fuels for steam generation, heating and household cooking, agricultural field burning, diesel-fueled engine combustion, and various industrial processes (Ren et al. 2016; Guo et al. 2014). Due to the advancement of urbanization, most people live in cities with quite a high population density (Han et al. 2018). Especially, domestic coal burning and traffic-related emissions as anthropogenic emission sources may make a substantial contribution to the PM content of urban air (Thorpe and Harrison 2008). Solid fuel burning, mobile emissions, dust and solid waste burning affect PM concentrations and composition (Secrest et al. 2017). Moreover, strong evidence shows the newly formed fine PM has a close link to the oxidation of volatile organic compounds which are usually accumulated at high concentrations in urban areas (Ehn et al. 2014; Zhou et al. 2016b). Distribution and concentration variations of PM in urban air over time and space are generally quite complex for interactions between local sources and meteorological conditions (Wang et al. 2020; Li et al. 2007; Fujitani et al. 2012).

Urban airborne PM represents a complex mixture of different-size particles (Kim et al. 2015). PM can be characterized by its physical attributes, influencing their transport and deposition (de la Torre et al. 2018). The physical attributes of airborne PM include mass concentration ($\mu g/m^3$) and size distribution (aerodynamic diameter). According to the aerodynamic diameter (AD), PM is divided into total suspended particulates (TSP, less than or equal to 100 μm), coarse particulate matter (PM_{10}), fine particulate matter ($PM_{2.5}$), submicron particulate matter (PM_1) and ultrafine particles ($PM_{0.1}$) (van Berlo et al. 2012). Airborne PM has been highlighted as a crucial pollutant. Presently, PM_{10} and $PM_{2.5}$ are used to reflect the daily air pollution level in the whole world.

Previous studies have reported that airborne PM can interact with different inorganic and organic substances in the air to form organic or inorganic chemical compounds (Daellenbach et al. 2020; Huang et al. 2014a). The smaller particles ($PM_{2.5}$, PM_1, $PM_{0.1}$) contain the secondarily formed aerosols and combustion particles, as well as recondensed organic and metal vapors (Kuwayama et al. 2013; Englert 2004; Clarke et al. 2004). The carbonaceous component of fine particles contains both elemental carbon (graphite and soot) and nonvolatile organic carbon (hydrocarbons emitted in combustion exhaust, and secondary organic compounds formed by photochemistry) (Pöschl 2005; Tao et al. 2021; Koçak et al. 2021). Additionally, atmospheric reactions of sulfates and nitrogen oxides produce nitric acid (HNO_3) vapors that may accumulate in fine and coarse forms. Generally, the acid component of particulate matter is contained in fine particles, although some coarse acid droplets are also present in fog (Cisneros et al. 2010; Wang et al. 2021b, b; Wang and Li, 2021). In addition, the most common combination of coarse particles mainly consists of insoluble crust-derived minerals, sea salt, and the material of biological origin (Wang et al. 2021b; Zhou et al. 2016a,). By contrast, the fine and ultrafine particles are mainly carbonaceous, which aggregate with metals and organic species adsorbed on their surface cavities (Rui et al. 2016; Deng et al. 2013b; Guan et al. 2016a, Xu et al. 2020). The smaller particles can adsorb more toxic substances such as heavy metals and polycyclic aromatic hydrocarbons due to their larger specific surface area (Valavanidis et al. 2008). Therefore, the chemical composition of PM varies greatly and depends on many factors, including combustion sources, climate, season, and type of urban or industrial pollution (Solomon and Sioutas 2008; Lighty et al. 2000).

PM, as a complex mixture of particles, is an important carrier of organic and inorganic chemicals, such as polycyclic aromatic hydrocarbons (PAHs), quinones, elemental and organic carbon (mainly from combustion processes and vehicular exhaust particles), reactive gases (ozone, peroxides, aldehydes), inorganic components (sulfates, nitrates, ammonium, chloride and trace metals), and biological components (endotoxins, bacteria, viruses, spores, pollens, animal and plant debris) (Han et al. 2015, 2016; Zhu et al. 2016). Especially, heavy metals (Cd, Cr, Cu, Ni, Pb, and Zn), mainly derived from traffic emissions or coal combustion, have a high distribution in fine and ultrafine metallic PM (Goix et al. 2014; Zhao et al. 2011; Sidi et al. 2012; Sun et al. 2016). For example, $PM_{2.5}$ has been shown to contain organic, inorganic and biological components, such as aromatic hydrocarbons, heavy metal particles and inorganic elements (Shi et al. 2015). The chemical composition (or elemental composition), particle structure and surface charge are intrinsic properties of PM, which drive the interactions with biomolecules in the human body after exposure to PM (Harrison 2020). Therefore, the combined toxic effects of PM with various chemical compositions should be considered in environmental toxicological research.

Exposure Routes of Particulate Matter

Exposure to airborne PM is one of the most significant environmental risks people face. The respiratory system is the major route of entry for airborne PM (Thompson 2018). In addition, ingestion and dermal absorption are recognized routes to PM exposure (Behroozy 2013). However, these routes of PM exposure will not be reviewed further.

Fine and ultrafine PM with high pulmonary deposition efficiency can reach deep inside the lung through the nose and bronchioles (Cheng 2003). It is well acknowledged that the lungs, in particular pulmonary epithelial cells, represent a primary biological target for injury arising from inhalation exposure to PM (Ulrich et al. 2002; Barkauskas et al. 2017). The deposition of PM in different parts of the human respiratory system depends on particle size, shape, density, and individual breathing patterns (mouth or nose breathing) (Behera et al. 2015; Manojkumar et al. 2019; Martins et al. 2015; Madureira et al. 2020). Figure 10.1 shows the fractional deposition of inhaled PM in nasopharyngeal, tracheobronchial, and alveolar regions of the human respiratory tract during nose breathing based on International Commission on Radiological Protection (Sturm 2007; Haddrell et al. 2015). Usually, PM_{10} is deposited in the nose, pharynx, larynx, trachea, and main bronchus (van Berlo et al. 2012), while $PM_{2.5}$ and PM_1 can enter the bronchioles and alveoli (van Berlo et al. 2012). It has been demonstrated that PM_1, $PM_{0.1–1}$, and their soluble components can enter the blood circulation through the air-blood barrier and then interact with other tissues and organs to cause damage to the body (Brook 2008; Tong et al. 2010).

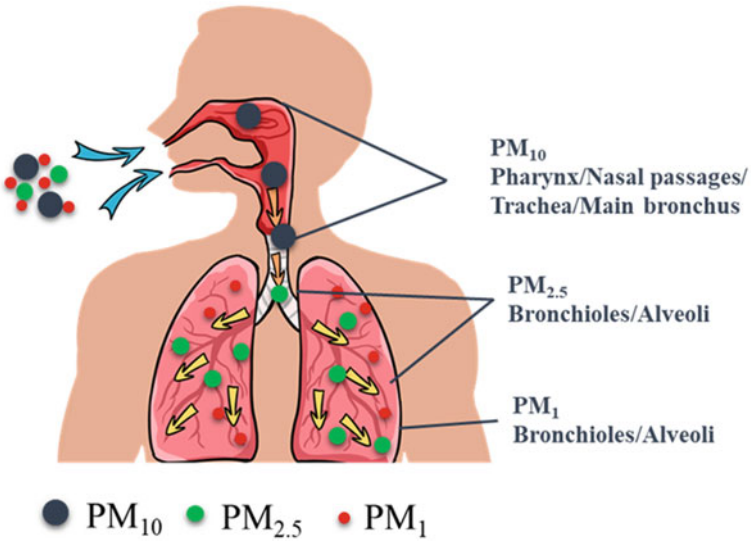

Fig. 10.1 The deposition of different fractions of PM in different parts of the respiratory system, based on the International Commission on Radiological Protection

Oxidative stress and inflammation induced by fine PM play an important role in its toxicity (Araujo and Nel 2009).

In order to determine the exact deposition dose of PM in the airway, a multiple-path-particle-deposition model (MPPD) and computational fluid dynamics (CFD) model have been developed. These models have been applied for estimating PM dose in the lower respiratory tract based on particle size-dependent deposition (Kelly et al. 2001; Cassee et al. 2002; Anjilvel and Asgharian 1995; Longest and Holbrook 2012). The modeled results can be used to extrapolate toxicological effects from animal tests in the risk estimation process (Anjilvel and Asgharian 1995; Longest and Holbrook 2012). However, the elucidation of the mechanisms for clearance, absorption or penetration of PM after migration into the lung alveolar region is still challenging for humans and animals (Semmler et al. 2004).

The adverse effects on the human organism are influenced by the chemical composition of PM, the duration of exposure, and individual susceptibility. While most of the smaller PM reaches the human lungs, the retention rate is highest for the fine PM (Manigrasso et al. 2017). Moreover, incomplete combustion products and toxic metals may be carried deep into the lungs with the fine PM, which significantly contributes to adverse health impacts (Xiao et al. 2020; Schultz et al. 2017). High deposition and low clearance of fine PM may lead to its accumulation in the alveoli, enhancing the interaction with the pulmonary cells and other biological components in the lung (Zhou et al. 2019). It has been documented that inhaled ultrafine PM with a high deposition efficiency can penetrate the alveolar epithelium into the bloodstream and increase the possibility of toxic effects to the body (Kermanizadeh et al. 2015).

In the lung, soluble fractions of PM can dissolve in the epithelial lining fluid and surfactants or biomolecules which may adsorb to the surface of insoluble PM fractions (Thompson 2018). The harmful components of PM can be released into the pulmonary surfactant and attached to the pulmonary epithelial cells (Ulrich et al. 2002; Müller et al. 1998). Finally, exposure of endothelial cells in the airways and alveoli to the chemical components of PM results in a series of cytotoxic effects, such as oxidative damage, inflammation, and even cell death (Duarte et al. 2012; Ulrich et al. 2002).

Health Effects of Particulate Matter

In the past decades, the adverse health effects of airborne PM have been extensively investigated in epidemiological, clinical and toxicological studies (Kim et al. 2015; Almetwally et al. 2020). Short-term and long-term exposures to $PM_{2.5}$ have been associated with mortality and morbidity caused by cardiovascular and chronic cardiopulmonary diseases, including lung cancer (Donaldson and Seaton 2012; Pope et al. 2002). Early, Pope and his colleagues obtained data from an ongoing prospective mortality study, collecting information about half a million people in 151 U.S. metropolitan areas during 1982–1989. They found that death rates in the most polluted areas with fine PM were 17% higher than in the least polluted areas due to

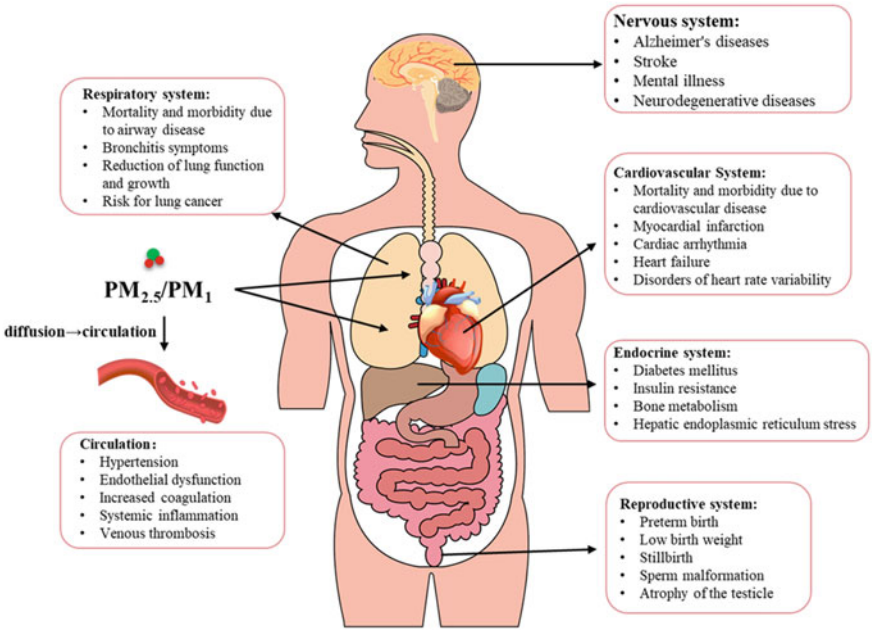

Fig. 10.2 The impact of air pollution on human health

31% higher rate of death from heart and lung diseases (Pope et al. 1995). Recent epidemiological studies in Taiwan, Sweden, Demark, and the U.S. have revealed the adverse effects of $PM_{2.5}$ on the central nervous system (CNS) (Jung et al. 2015; Ritz et al. 2016; Oudin et al. 2016) and reproductive system (Mahalingaiah et al. 2016, 2018; Jurewicz et al. 2018). Experimental evidence has also shown direct effects of PM on the lung receptors and cardiovascular system and/or indirect effects through PM-mediated pulmonary oxidative stress and inflammatory responses (Brook et al. 2004). The potential pathophysiological effects of PM exposure are summarized in Fig. 10.2.

Exposure to Particulate Matter and Respiratory System

Accumulating evidence has demonstrated a strong association between air pollution and respiratory diseases, including chronic obstructive pulmonary disease (COPD), asthma, bronchitis symptoms, decreased lung function, and lung cancer (Guan et al. 2016b ; Almetwally et al. 2020; Faustini et al. 2013; Mannucci et al. 2015). A nationwide time-series study indicated a significant short-term association between exposure to PM and increased hospital admissions for pneumonia in Chinese adults (Tian et al. 2019). In particular, the elderly and females were relatively more sensitive to the

outdoor air pollutants (PM_{10}, SO_2, and NO_2), which caused an increase in emergency room admissions for respiratory diseases in Beijing, China (Pan et al. 2007). In addition, short-term ambient PM exposure has been associated with asthma symptoms, especially in asthmatic children and adults with allergic sensitization, suggesting that PM exacerbates the severity of allergic asthma (Mann et al. 2010). Based on the evidence regarding the association of $PM_{2.5}$ and PM_{10} to lung cancer risk, PM in outdoor air has been designated as a Group I carcinogen by the International Agency for Research on Cancer (IARC) (Hamra et al. 2014).

Exposure to Particulate Matter and Cardiovascular System

Both acute and chronic exposure to PM air pollution is associated with increased cardiovascular diseases, such as ischemic heart disease, heart failure and thrombotic stroke (Hamanaka and Mutlu 2018). A recent study in China attributed 40.3% of deaths to stroke and 26.8% of deaths to ischemic heart disease due to $PM_{2.5}$ exposure (Song et al. 2017). In addition, a study about the effects of PM exposure on cardiorespiratory health in low-income and middle-income countries showed that a 10 $\mu g/m^3$ increase in same-day PM_{10} exposure was associated with a 0.27% increase in cardiovascular mortality (Newell et al. 2017). It was found that PM in all sizes disarranged the coagulative balance and short- and prolonged-time exposure to PM caused a dramatic hypercoagulable state (Signorelli et al. 2017). Furthermore, PM, especially $PM_{2.5,}$ was associated with increased atrial fibrillation (AF) onset within hours following exposure in patients with cardioverter-defibrillators (Link et al. 2013).

Exposure to Particulate Matter and Nervous System

Several studies have reported an association between airborne $PM_{2.5}$ exposure and neurodevelopmental and neurodegenerative diseases (Costa et al. 2020, 2017; Buoli et al. 2018). Long-term exposure to traffic-related PM impaired cognitive function in the elderly, indicating that PM may contribute to the pathogenesis of Alzheimer's disease (AD) (Ranft et al. 2009). High concentrations of PM can lead to cognitive decline, olfactory bulb dysfunction, hearing impairment, and depression in children, adults and elderly people (Suades-González et al. 2015; Guxens and Sunyer 2012). Compared with children living in the areas with clear air, young urbanites in Mexico City with high air pollution levels exhibited systemic, brain and intrathecal inflammation, low cerebrospinal fluid Aβ42, breakdown of the blood–brain barrier, short-term memory deficits, and Alzheimer's and Parkinson's disease hallmarks (Calderón-Garcidueñas et al. 2015, 2016). A recent experimental study has reported that the extract of diesel exhaust particles (DEPs) caused behavioral deficits and a significant decrease in neurons in zebrafish, while DEPs induced progressive and nonselective

loss of neurons alongside an increase in aggregation-prone neuronal protein (Barnhill et al. 2020). In addition, DEPs selectively damaged dopaminergic (DA) neurons through the phagocytic activation of microglial nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX2) and consequent oxidative stress (Block et al. 2004). Microglia, the resident innate immune cells in the brain, have been reported to be a crucial factor responsible for cellular damage caused by air pollution (Brown and Neher 2010). Activated microglia can release inflammatory cytokines, chemokines and other inflammatory factors, leading to neurotoxicity and even neurodegenerative diseases (Yang et al. 2018b). Inhaled components of urban PM can activate microglia through both direct and indirect pathways (Jayaraj et al. 2017). Among them, the microglial integrin receptor Mac1 and its downstream effector NOX2 play a critical role in mediating reactive microgliosis-generated chronic neuroinflammation and progressive neurodegeneration (Chen et al. 2016).

Exposure to Particulate Matter and Endocrine System

Epidemiological investigations and animal studies have established an association between long-term exposure to PM and the risk of obesity and diabetes mellitus. Activation of the immune system, endoplasmic reticulum stress, hepatic lipid deposition, and reduced gluconeogenesis are believed to be involved in the pathophysiology of air pollution-mediated diabetes (Rao et al. 2015). Also, the available evidence supports a prospective association between exposure to the main air pollutants $PM_{2.5}$ and NO_2 and an increased risk for type 2 diabetes (Balti et al. 2014). A national multisite-case-crossover analysis conducted in 121 cities in the U.S. revealed a significant association between short-term exposure to $PM_{2.5}$ and increased hospitalization risk for diabetes (1.14% increase) (Zanobetti et al. 2014). However, no significant association was observed between the levels of glycated hemoglobin (HbA1c) and PM_{10} in children and adolescents with type 1 diabetes in Germany (Lanzinger et al. 2018; Fleisch et al. 2014). Still, other studies have shown that exposure to high concentrations of PM and other traffic-related pollutants may contribute to abnormal glycemia with impaired glucose tolerance (IGT) in pregnancy (Lanzinger et al. 2018; Fleisch et al. 2014) and changes in heart rate variability in individuals with diabetes or IGT (Hampel et al. 2012). Another study showed that long-term exposure to ambient $PM_{2.5}$ induced impaired glucose tolerance, insulin resistance, inflammation, and mitochondrial alteration in adipose tissue (Xu et al. 2011). Therefore, further studies on the association between airborne PM and the endocrine system in diabetes mellitus are needed to elucidate the underlying molecular mechanisms.

Exposure to Particulate Matter and Reproductive System

In recent years, researchers have focused on the effects of $PM_{2.5}$ exposure on human reproductive health. Studies have shown that long-term exposure to $PM_{2.5}$ can increase the risk of low birth weight, preterm birth, and stillbirth (Li et al. 2019, Yuan et al. 2019). Especially, exposure to $PM_{2.5}$ during pregnancy is associated with the risk of low birth weight in the context of a very high pollution level of $PM_{2.5}$ (Wu et al. 2018). A recent meta-analysis of exposure to PM and adverse birth outcomes indicated that birth weight was negatively associated with a 10 $\mu g/m^3$ increase in PM_{10} and $PM_{2.5}$ exposure during the entire pregnancy. In addition, a significantly increased risk of preterm birth per 10 $\mu g/m^3$ increase in PM_{10} and $PM_{2.5}$ during pregnancy was also observed (Lamichhane et al. 2015). Moreover, several Chinese national cohort studies have demonstrated that an increase of 10 $\mu g/m^3$ in the concentration of atmospheric pollutants such as PM_{10}, $PM_{2.5}$, and NO_2 over the entire pregnancy is significantly associated with increased risk of preterm birth (Wang et al. 2018a; Li et al. 2018; Wang et al. 2018b). Furthermore, accumulating evidence indicates that prenatal exposure to ambient air pollution is associated with an elevated stillbirth risk. Every 10 $\mu g/m^3$ increase of $PM_{2.5}$ in each the entire pregnancy was associated with an increased stillbirth rate (Zang et al. 2019; Smith et al. 2020; Yang et al. 2018a). Consistent with the epidemiological and clinical observations, a series of experimental studies have also reported that oxidative stress, DNA methylation, alterations in the mitochondrial DNA (mtDNA) content, and endocrine disruption play an important role in $PM_{2.5}$-induced adverse effects in pregnant women and fetuses (Liu et al. 2017; Yi et al. 2017). Transcriptomic analysis has identified that the differentially expressed genes induced by $PM_{2.5}$ exposure are mainly enriched in the pathways of ovarian steroidogenesis, reactive oxygen species (ROS) generation, and oxidative phosphorylation (Zhou et al. 2020). Several animal studies have shown that various $PM_{2.5}$ components can reach male rodents' reproductive organs or tissues, such as testis, epididymis and spermatogenic tubules (Liu et al. 2018, Yang et al. 2019). Moreover, $PM_{2.5}$ exposure reduces sperm quality, including sperm malformation, concentration, vitality and morphology (Li et al. 2019). Exposure to $PM_{2.5}$ can disturb the hormone levels in vivo, which is a potential threat to male fertility (Radwan et al. 2016). Taken together, these findings suggest that $PM_{2.5}$ can exert various pathological effects on the reproductive system and embryo development (Wang et al. 2021a) .

Potential Toxicity Mechanisms of Particulate Matter

Toxicological studies conducted in vitro and in vivo to date have collectively implicated that PM-induced ROS impairs the cellular physiological/biochemical processes by the mechanisms of inducing oxidative stress and inflammation as well as genotoxicity (Radwan et al. 2016). Consequently, the normal physiological functions and/or

fates of target cells are altered and the tissues and organs are damaged (Radwan et al. 2016; Cho et al. 2018).

Oxidative Stress

Oxidative stress is caused by an imbalance between ROS production and the activity of antioxidant substances (Limón-Pacheco and Gonsebatt 2009). ROS play an important role as secondary messengers in various intracellular signaling cascades, but they are also involved in pathological processes associated with excessive ROS generation (Lu et al. 2018). The major types of ROS are superoxide anions ($O_2^{-}\bullet$), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), singlet oxygen, hydroperoxy radicals (HO_2), alkoxy radicals (RO•), peroxy radicals (ROO•) and ozone (O_3) (Zuo et al. 2019). It has been demonstrated that various organic chemicals, metals, and environmentally persistent free radicals (EPFRs) absorbed on $PM_{2.5}$ can produce or increase intracellular ROS (Torres-Ramos et al. 2011, Huang et al. 2014b, Gehling and Dellinger 2013). Deng et al. (2014) found that $PM_{2.5}$-triggered oxidative stress-activated multiple cell death pathways in human lung epithelial A549 cells, including apoptosis pathway as evidenced by increased pro-apoptotic protein Bax expression levels and decreased in anti-apoptotic protein Bcl-2 expression. In $PM_{2.5}$-treated human bronchial epithelial cells (BEAS-2B), inhibition of AMP-activated protein kinase (AMPK) activity plays an important role in decreasing cell viability and increasing intracellular ROS and p-nuclear factor kappa B (NF-κB) levels (Dornhof et al. 2017). Wei et al. (2016) found that $PM_{2.5}$ exposure triggered ROS generation in human umbilical vein cells (EA.hy926) in a time- and dose-dependent manner. Moreover, $PM_{2.5}$-induced oxidative stress significantly increased intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1(VCAM-1) expression in EA.hy926 cells through extracellular signal regulatory kinase (ERK)/ protein kinase B (AKT)/NF-κB-dependent signaling pathway (Rui et al. 2016). The redox effects of metal ions of $PM_{2.5}$ on ROS generation and microglia activation were investigated by Chen et al. (2020) who found that $PM_{2.5}$ induced oxidative stress and microglial activation, which was accompanied by increased glutaminase-containing extracellular vesicle release in the olfactory bulb. Moreover, long-term exposure to airborne $PM_{2.5}$ significantly induced hepatic insulin resistance by Nrf2/Jun N-terminal kinase (JNK)-mediated signaling pathway (Xu et al. 2017) and profoundly exacerbated ovarian oxidative stress and inflammation in female C57BL/6 J mice through the NF-κB/IL-6 signaling pathway (Zhou et al. 2020). Therefore, PM-induced oxidative stress has been recognized as a key molecular mechanism of PM-mediated toxicity (Deng et al. 2013b; Wei et al. 2021; Gehling and Dellinger 2013).

The enzymatic antioxidant defense is one of the major molecular mechanisms to eliminate ROS production (Kouassi et al. 2010). The major antioxidant enzymes, including superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and heme oxygenase-1 (HO-1) (Liu and Meng 2005) are induced to restore cellular redox homeostasis in the presence of $PM_{2.5}$-induced oxidative stress (Li et al. 2008). Many

studies have demonstrated that nuclear factor erythroid-2-related factor 2 (Nrf2), a transcription factor, is essential for protection against chemically induced oxidative stress to restore cellular redox balance (Deng et al. 2013a, b; Chen et al. 2018). The molecular mechanisms of Nrf2-mediated protection can be attributed to the induction and enhanced expression of antioxidant and detoxification genes (Saha et al. 2020). During oxidative stress, Nrf2 is activated following its detachment from Kelch-like ECH-associated protein (Keap1) and then translocated to the nucleus where it binds to the antioxidant response element (ARE) in the promoter region of target genes, such as HO-1, NADPH: quinone oxidoreductase 1 (NQO-1), SOD and glutamate-cysteine ligase catalytic subunit (GCLC), leading to their transcriptional induction (Ma 2013; Bellezza et al. 2018; Tonelli et al. 2018). Recently, several studies have demonstrated that Nrf2 is activated in vivo and in vitro after exposure to DEPs and PM (Lawal 2017; Rao et al. 2018; Harmon et al. 2018; Chen et al. 2018). These results indicate that activation of Nrf2/ARE-dependent transcription is a key adaptive response in cellular defense against $PM_{2.5}$-induced oxidative stress (Deng et al. 2013a).

Immune Response and Inflammation

The adverse health effects of PM are closely related to immune system damage mediated by the inflammatory response (Heo et al. 2015; Feng et al. 2016). Fine PM can penetrate the lower respiratory tract and alveoli and directly stimulate alveolar macrophages (Feng et al. 2016; Montiel-Davalos et al. 2010). It is well known that alveolar macrophages are the first line of defense in the lung and are essential in clearing ambient PM from the lung surface and stimulating epithelial cells to produce pro-inflammatory cytokines and chemokines (Ishii et al. 2005). In vitro, exposure to PM has been shown to induce polarization of M1 macrophages and cause oxidative damage, death and lysis of macrophages (Montiel-Davalos et al. 2010; Mitkus et al. 2013; Borthwick et al. 2016). Research further confirmed that organic components and metals (Mn, Ni, Mo and V) in PM extract from Los Angeles Basin triggered ROS generation and reduced the phagocytosis and survival ability of alveolar macrophages (Heo et al. 2015). Moreover, $PM_{2.5}$ exposure significantly increased the expression of tumor necrosis factor α (TNFα), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-1β (IL-1β), granulocyte–macrophage colony-stimulating factor (GM-CSF) in a dose-dependent manner (Feng et al. 2016; Zhao et al. 2016). In vivo, acute and chronic $PM_{2.5}$ exposure is involved in local and systemic inflammation. It was found that $PM_{2.5}$ significantly increased lung inflammation in a dose-dependent manner in healthy mice (Wang et al. 2019; Wang et al. 2018c). The cell numbers, protein and pro-inflammatory mediator (TNFα and IL-6) levels in bronchoalveolar lavage fluid (BALF) were markedly increased. Meanwhile, histological analysis and immunohistochemical staining of lung sections further revealed that $PM_{2.5}$ exposure caused lung injury and infiltration of macrophages and neutrophils (Wang et al. 2019; Wang et al. 2018c). Also, $PM_{2.5}$ exposure can increase the inflammatory response in the brain (Cserbik et al. 2020), liver (Chen et al. 2021), heart (Yue et al. 2019),

kidneys (Zare Sakhvidi et al. 2020), ovaries (Zhou et al. 2020) and testes (Yang et al. 2019) as evidenced by increased expression of pro-inflammatory genes, NF-κB pathway activation and infiltration of inflammatory cells (Chen et al. 2018; Xu et al. 2013; Xie et al. 2013; Ying et al. 2014).

In addition, $PM_{2.5}$ exposure may cause the alteration of the immune system, which is linked to adverse health effects, including asthma (Guarnieri and Balmes 2014). It was found that $PM_{2.5}$ reduced the numbers of toll-like receptor 2 or 4 (TLR4 or TLR2) positive cells and increased the release of Th2-related cytokines IL-4, IL-5, IL-10 and IL-13, in both BALF and blood of mice, driving a Th2-biased immune response by an inflammasome-associated mechanism (Ogino et al. 2014; Zhao et al. 2012). Furthermore, nucleotide-binding domain, leucine-rich repeat-containing domain receptors (NLRs) and inflammatory bodies are considered to be involved in the inflammatory response induced by PM (Lai et al. 2017; Zheng et al. 2018). Further, $PM_{2.5}$ can activate NOD-, LRR- and pyrin domain-containing 3 (NLRP3) inflammasome through cathepsin B release, ROS production, and potassium efflux, causing lung inflammation and pulmonary fibrosis (Lai et al. 2017; Zheng et al. 2018). The P38-p53-ERK1/2 signaling pathway has also been identified to regulate alveolar type II cells exposed to $PM_{2.5}$ (Niu et al. 2013; Soberanes et al. 2009). Therefore, various signaling pathways may be involved in systemic inflammatory and immune responses induced by $PM_{2.5}$.

Genotoxicity and Mutagenicity

Oxidative stress is associated with excessive levels of ROS that attack biological macromolecules (e.g., lipids, proteins, and DNA) inside cells, causing oxidative damage, which leads to DNA breakage, induction of apoptosis, and ultimately, carcinogenesis (Brigelius-Flohe 2009; Niki 2016). It has been shown that aqueous suspensions of PM and water-soluble constituents from particles generate strand breaks in DNA, which are mainly driven by ROS production due to the action of transition metals (Dellinger et al. 2001; Sharma et al. 2007; Xu and Zhang 2004). Several studies have demonstrated that air pollution particles generate oxidatively damaged DNA by promoting a milieu of oxidative stress and inflammation (Møller et al. 2014; Vattanasit et al. 2014; Corsini et al. 2013). The chemical composition of PM is a decisive factor for its genotoxicity (Chuang et al. 2011; Bo et al. 2019; Yang et al. 2017; Sese et al. 2018). Some studies have demonstrated that the extractable organic compounds with mutagenic and cytotoxic properties contribute to various mechanisms of toxicity. For example, PAHs in PM are considered the main carcinogenic compounds associated with lung cancer (Chuang et al. 2011; Bo et al. 2019; Yang et al. 2017; Sese et al. 2018). In addition, the water-soluble fraction containing transition metals with redox potential also plays an important role in the initiation of oxidative DNA damage and membrane lipid peroxidation (Valavanidis et al. 2008). Extractable organic matter of $PM_{2.5}$ from a highway site with high traffic intensity generates high levels of DNA strand breaks in A549 cells (Bonetta et al. 2009). Thus,

these findings are consistent with the knowledge that associations between chemical composition and particle toxicity tend to be stronger for the fine and ultrafine PM size fractions.

Oxidatively damaged DNA, strand breaks, and cytogenetic markers are often used to assess PM-induced DNA damage (Valavanidis et al. 2008; Møller et al. 2014). The level of oxidative damage in DNA bases is an important general indicator of intracellular oxidative stress, reflecting a specific mechanism of carcinogenesis (Møller et al. 2014). Meanwhile, ROS can oxidize guanine in DNA and RNA to form 8-hydroxyguanine associated with gene damage and carcinogenesis (Floyd 1990; Feig et al. 1994). The levels of the oxidized base 8-oxo-7,8-dihydro-guanine (8-oxoGua) or the 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) are considered as important biomarkers of PM-induced DNA oxidative damage in animal organs (Møller et al. 2010), and consistently increased in the mononuclear blood cells and urine of subjects exposed to ambient airborne PM (Demetriou et al. 2012). It has been demonstrated that human exposure to each 10 $\mu g/m^3$ $PM_{2.5}$ could result in an 11% increase in the levels of 8-oxodGuo in lymphocyte DNA (Sørensen et al. 2003).

Current research has uncovered that $PM_{2.5}$ mass, its organic and/or elemental constituents, or the duration of exposure are associated with increased levels of urinary or serum 8-oxodGuo in humans (Huang et al. 2012; Kim et al. 2004; Wei et al. 2016; Tan et al. 2017). An earlier study has reported that children exposed to high levels of a complex mixture of urban airborne PM in Mexico City presented higher levels of 8-oxodGuo in nasal biopsies from the posterior inferior turbinate compared to those of controls in a low-polluted coastal town (Calderón-Garcidueñas et al. 1999). Regarding cancer risk, there is an association between urinary 8-oxodGuo excretion and lung cancer risk in humans (Loft et al. 2012) and in colon adenoma and carcinoma patients (Obtulowicz et al. 2010). These observations are consistent with an earlier report demonstrating that DEPs exposure increases the levels of 8-oxodGuo levels in the lung tumors of mice (Ichinose et al. 1997).

Epigenetic Modification

In addition to the genotoxic effect of PM, another important consequence of the exposure is the induction of epigenetic changes. Accumulating evidence has revealed that PM exposure can alter the epigenome, leading to dysregulation of gene expression (de Oliveira et al. 2018; Sun et al. 2018; Kupsco et al. 2020; Xu et al. 2019). Based on strong evidence from the epidemiological, clinical and experimental studies, PM-induced epigenetic changes are associated with increased disease susceptibility and progression (Jirtle and Skinner 2007). It has been shown that the global DNA methylation changes associated with PM exposure contribute to PM-induced cardiovascular, pulmonary and neurodegenerative diseases as well as cancer development (Wang et al. 2012; Bellavia et al. 2015; Baccarelli 2009; Feil and Fraga 2012; De Prins et al. 2013; Bakulski and Fallin 2014).

DNA methylation and gene expression variations after $PM_{2.5}$ exposure are considered to be directly involved in its toxicity and pathological processes. Studies comparing the PM-induced epigenetic changes in DNA methylation and hydroxymethylation profiles in tissues of animal models, human tissues and human cells would allow a better understanding of the applicability of such detailed information obtained in an animal model (de Oliveira et al. 2018). Long interspersed nucleotide element (LINE)-1, a transposable element in the human genome, has been used as a surrogate assay for quantifying a total 5mC in the majority of the studies on associations between ambient PM exposure and global DNA methylation alterations (Ding et al. 2016). A previous study has identified that DNA methylation of LINE is decreased in blood samples after acute exposure to traffic PM (Baccarelli 2009). Similarly, exposure to $PM_{2.5}$ and PM_{10} was associated with changes in DNA methylation in rat lung as evidenced by decreased methylation of LINE1 and inducible nitric oxide synthase (*iNOS*) promoter, and increased promoter methylation of adenomatous polyposis coli (*APC*) (Ding et al. 2016). On the other hand, global blood DNA methylation in humans was associated with a hypomethylation effect of PM exposure (Baccarelli 2009; Guo et al. 2014; Wei et al. 2016; Chen et al. 2016). Moreover, DNA methylomic change occurred when bronchial epithelial BEAS-2B cells were exposed to either low (1 $\mu g/cm^2$) or high (30 $\mu g/cm^2$) concentrations of $PM_{2.5}$ for 24 h. Cells exposed to $PM_{2.5}$ at a low concentration demonstrated a comparable, but more attenuated change in gene expression compared to cells exposed to $PM_{2.5}$ at a high concentration. DNA methylomic analysis further revealed that nearly half of the differentially expressed genes were found to have DNA methylation changes, with just a slightly greater trend toward overall hypomethylation across the genome. Interestingly, single and repeated exposure to $PM_{2.5}$ (1 $\mu g/cm^2$) resulted in widespread transcriptomic and DNA methylomic changes. Compared to the single exposure, repeated exposure to $PM_{2.5}$ caused a more notable degree of hypomethylation across the genome (Huang et al. 2021). A recent study reported that acute exposure to PM at high concentrations for only 24 h resulted in reduced global DNA methylation and increased global DNA hydroxymethylation levels in the lungs of mice (Li et al. 2019). Notably, these alterations of DNA methylation and DNA hydroxymethylation in lungs were all reversed after the air purification for 120 h. In addition, the effects of oxidative stress and DNA hydroxymethylation in neuronal pathology of $PM_{2.5}$ were investigated by Wei et al. (2016). They found that $PM_{2.5}$ and its organic extracts increased global DNA hydroxymethylation and gene-specific DNA hydroxymethylation of neuronal genes and subsequently interfered with their mRNA expression in human neuroblastoma cells (SH-SY5Y). Meanwhile, metal-rich PM_1 exposure triggered the methylation of mitochondrial transfer RNA phenylalanine (MT-TF) and 12S ribosomal RNA (MT-RNR1) DNA (Byun et al. 2013).

The assessment of the total levels of 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) in DNA can be used to evaluate the effect of PM on the epigenome (Bakulski and Fallin 2014). Sanchez-Guerra et al. (2015) found that ambient PM_{10} exposure significantly increased the levels of 5hmC in human blood samples over time, but not the levels of 5mC. However, significantly lower levels of 5-hmC were observed in human bronchial epithelial cells (HBECs) exposed to DEPs

at 5 mg/cm^2 for 24 h (Somineni et al. 2016). Additionally, increased human exposure to $PM_{2.5}$ or PM_{10} has been associated with a decrease of 5hmC levels in buccal cells (De Nys et al. 2018). $PM_{2.5}$ exposure leads to an abnormal mRNA 5mC gain and loss in the fibrotic lung tissues of mice (Han et al. 2020). Several genes (*lcn2*, *mmp9*, *chi3l1*, *adipoq*, *atp5j2*, *atp5l, atpif1*, *ndufb6*, *fgr*, *slc11a1* and *tyrobp*) related to oxidative stress response, inflammatory responses and immune system processes acted as 5mC upregulation factors.

RNA methylation, another epigenetic mechanism, is highly sensitive to airborne environmental exposure, including air pollution (Li et al. 2019; Deng et al. 2018; Kupsco et al. 2020). N6-methyladenosine (m6A) is the most abundant product of RNA methylation, with roles in modulating mRNA transcript processing and regulation (Niu et al. 2013). A recent study showed that exposure to ambient black carbon (BC), but not to $PM_{2.5}$ or PM_{10}, significantly increased the levels of global m6A in RNA in human blood (Kupsco et al. 2020). Conversely, $PM_{2.5}$ (62 μg/ml) exposure decreased the level of global m6A methylation in A549 lung epithelial cells, however, expression of m6A writers (METTL3 and WTAP), erasers (FTO and ALKBH5) and readers (HNRPC) was significantly increased (Cayir et al. 2019). These experimental results indicate variable m6A responses to environmental stressors, suggesting that the dynamic and reversible chemical m6A modification of RNA may serve as a novel epigenetic marker of profound biological disturbance (Niu et al. 2013).

Histone modifications associated with PM exposure have been studied in epigenetic regulation of basic life processes in mammals (Vrijens et al. 2020; Wu et al. 2019; Li et al. 2020; Zhang et al. 2019; Cui et al. 2021). Histone modifications regulate gene expression by influencing chromatin structure that can change gene expression status (Zheng et al. 2017), allowing transcription factors to enter the promoter site (Gilmour et al. 2003). The data obtained from a study that involved Beijing truck drivers and their exposure to air pollution showed that global histone H3 modifications were associated with traffic-derived PM_{10} exposures, but not with $PM_{2.5}$ or elemental components (Zheng et al. 2017). Circulating total histone H3 levels, trimethylated H3 lysine 4 and trimethylated H3 lysine 36 levels in maternal cord blood were increased after gestational $PM_{2.5}$ exposure, which suggested that circulating histones may be a risk factor in the development of air pollution-associated disease later in life (Vrijens et al. 2020). In addition, some recent studies in mice have implicated that $PM_{2.5}$ exposure significantly decreases the levels of GATA binding protein 4 (GATA4) and acetylated histone 3 lysine 9 (H3K9ac) in GATA4 promoter region in hearts, accompanied by downregulation of histone acetyltransferase (HAT)-p300 and upregulation of histone deacetylase-SIRT3, suggesting that maternal exposure to $PM_{2.5}$ may cause cardiac injury in children through histone modifications regulating the transcription factor GATA4 (Wu et al. 2019; Li et al. 2020). It is important to note that DNA methyltransferase (DNMT), histone acetyltransferase (HAT), and histone deacetylase (HDAC) contribute to the epigenetic changes induced by PM or its components (Wang et al. 2012). Notably, various organic and inorganic components in PM can directly or indirectly alter histone acetylation and transgene silencing. Especially, nickel and chromium significantly reduced the acetylation of histones H2A, H2B, H3 and H4 (Ke et al. 2006). Moreover,

$PM_{2.5}$ exposure significantly increased the protein levels of transcription activators p300, histone acetyltransferases CBP, H3K9ac and the mRNA levels of GATA4 myocyte enhancer factor 2C (Mef2c) in the hearts of female mice (Wu et al. 2019). These results indicate that $PM_{2.5}$-induced histone acetylation modification may play an important role in the programming of cardiac hypertrophy.

Conclusions and Perspectives

To sum up, ambient airborne PM pollution is associated with an increased risk of adverse health effects worldwide. Inhaled fine PM in the airway can enter the blood circulation through the blood-air barrier and reach other tissues and organs, resulting in damage to the respiratory, cardiovascular, endocrine, reproductive and central nervous system. Moreover, in vitro cell and in vivo animal studies have provided fundamental insights into the mechanisms linking PM exposure and health effects. Among them, oxidative stress, inflammation, genotoxicity and epigenotoxicity have been considered as important molecular mechanisms of PM-induced adverse effects in disease progression. However, the knowledge on the toxicity mechanisms of PM is not comprehensive, and many problems still need to be addressed and solved.

Regarding the health effect studies of PM, it is necessary to focus on the temporal and spatial distribution properties and physicochemical characteristics of PM and analyze the toxicity of PM at different stages, locations, and exposure levels as well as the effects of PM with varying physicochemical properties. It remains to be evaluated whether PM only acts as a carrier or interacts with the toxic substances carried by it, resulting in damage to human health. In addition, the interactions between different components of PM and their combined effects with other air pollutants should be investigated. Also, it would be important to assess the biological effects of lifetime exposures to outdoor and indoor environmental pollutants: the level and extent of exposure, as well as the characteristics of PM, all play a significant role in the resulting health effects and should be thoroughly investigated.

Currently, it is difficult to reach a conclusion about the toxic effects of the chemical components of PM in different regions and seasons. Moreover, identifying the "safe" threshold concentration of fine PM, below which no health effects occur, is considered ambiguous. A recent study has demonstrated that even small changes in the allowed limit of annual mean $PM_{2.5}$ concentrations could significantly lower mortality rates (Giannadaki et al. 2016). In the future, the risk assessment of long-term exposure to airborne $PM_{2.5}$ is required, especially for people living in relatively populous areas. Current research has uncovered epigenetic changes associated with PM-derived effects on human health. Epigenetic changes can be used as potential biomarkers and therapeutic targets for disease induced by PM. To better address the knowledge gaps, various innovative technologies and experimental and analytical methods are needed to explore the interactions between PM and biosystems. Furthermore, a systematic and in-depth study of the pathological mechanisms of PM and other air pollutants is not only conducive to the prevention and treatment

of relevant patients but also beneficial for improving the life quality of the general public.

References

Almetwally AA, Bin-Jumah M, Allam AA (2020) Ambient air pollution and its influence on human health and welfare: an overview. Environ Sci Pollut Res Int 27:24815–24830

Anjilvel S, Asgharian B (1995) A multiple-path model of particle deposition in the rat lung. Fundam Appl Toxicol 28:41–50

Araujo JA, Nel AE (2009) Particulate matter and atherosclerosis: role of particle size, composition and oxidative stress. Part Fibre Toxicol 6:24

Baccarelli A (2009) Breathe deeply into your genes!: genetic variants and air pollution effects. Am J Respir Crit Care Med 179(6):431–432

Balti EV, Echouffo-Tcheugui JB, Yako YY et al (2014) Air pollution and risk of type 2 diabetes mellitus: a systematic review and meta-analysis. Diabetes Res Clin Pract 106:161–172

Bakulski KM, Fallin MD (2014) Epigenetic epidemiology: Promises for public health research. Environ Mol Mutagen 55(3):171–183

Barkauskas CE, Chung MI, Fioret B et al (2017) Lung organoids: current uses and future promise. Development 144:986–997

Barnhill LM, Khuansuwan S, Juarez D et al (2020) Diesel exhaust extract exposure induces neuronal toxicity by disrupting autophagy. Toxicol Sci 176:193–202

Behera SN, Betha R, Huang X et al (2015) Characterization and estimation of human airway deposition of size-resolved particulate-bound trace elements during a recent haze episode in Southeast Asia. Environ Sci Pollut Res Int 22:4265–4280

Behroozy A (2013) On dermal exposure assessment. Int J Occup Environ Med 4:113–127

Bellavia A, Urch B, Speck M et al (2015) DNA hypomethylation, ambient particulate matter, and increased blood pressure: findings from controlled human exposure experiments. J Am Heart Assoc 4(10):e001981

Bellezza I, Giambanco I, Minelli A et al (2018) Nrf2-Keap1 signaling in oxidative and reductive stress. Biochim Biophys Acta Mol Cell Res 1865:721–733

Block ML, Wu X, Pei Z et al (2004) Nanometer size diesel exhaust particles are selectively toxic to dopaminergic neurons: the role of microglia, phagocytosis, and NADPH oxidase. Faseb J 18:1618–1620

Bo Y, Guo C, Lin C et al (2019) Dynamic changes in long-term exposure to ambient particulate matter and incidence of hypertension in adults. Hypertension 74:669–677

Bonetta S, Gianotti V, Bonetta S et al (2009) DNA damage in A549 cells exposed to different extracts of PM2.5 from industrial, urban and highway sites. Chemosphere 77:1030–1034

Borthwick LA, Barron L, Hart KM et al (2016) Macrophages are critical to the maintenance of IL-13-dependent lung inflammation and fibrosis. Mucosal Immunol 9:38–55

Brigelius-Flohe R (2009) Commentary: oxidative stress reconsidered. Genes Nutr 4:161–163

Brook RD (2008) Cardiovascular effects of air pollution. Clin Sci (lond) 115:175–187

Brook RD, Franklin B, Cascio W et al (2004) Air pollution and cardiovascular disease: a statement for healthcare professionals from the expert panel on population and prevention science of the American heart association. Circulation 109:2655–2671

Brown GC, Neher JJ (2010) Inflammatory neurodegeneration and mechanisms of microglial killing of neurons. Mol Neurobiol 41:242–247

Buoli M, Grassi S, Caldiroli A et al (2018) Is there a link between air pollution and mental disorders? Environ Int 118:154–168

Byun HM, Panni T, Motta V et al (2013) Effects of airborne pollutants on mitochondrial DNA methylation. Part Fibre Toxicol 10:18

Calderón-Garcidueñas L, Reynoso-Robles R, Vargas-Martínez J et al (2016) Prefrontal white matter pathology in air pollution exposed Mexico City young urbanites and their potential impact on neurovascular unit dysfunction and the development of Alzheimer's disease. Environ Res 146:404–417

Calderón-Garcidueñas L, Vojdani A, Blaurock-Busch E et al (2015) Air pollution and children: Neural and tight junction antibodies and combustion metals, the role of barrier breakdown and brain immunity in neurodegeneration. J Alzheimers Dis 43:1039–1058

Calderón-Garcidueñas L, Wen-Wang L, Zhang YJ et al (1999) 8-hydroxy-2'-deoxyguanosine, a major mutagenic oxidative DNA lesion, and DNA strand breaks in nasal respiratory epithelium of children exposed to urban pollution. Environ Health Perspect 107(6):469–474

Cassee FR, Muijser H, Duistermaat E et al (2002) Particle size-dependent total mass deposition in lungs determines inhalation toxicity of cadmium chloride aerosols in rats. Application of a multiple path dosimetry model. Arch Toxicol 76:277–286

Cayir A, Barrow TM, Guo L et al (2019) Exposure to environmental toxicants reduces global N6-methyladenosine RNA methylation and alters expression of RNA methylation modulator genes. Environ Res 175:228–234

Chen X, Guo J, Huang Y et al (2020) Urban airborne PM2.5-activated microglia mediate neurotoxicity through glutaminase-containing extracellular vesicles in olfactory bulb. Environ Pollut 264:114716

Chen X, Liu S, Zhang W et al (2018) Nrf2 deficiency exacerbates PM2.5-induced olfactory bulb injury. Biochem Biophys Res Commun 505:1154–1160

Chen SH, Oyarzabal EA, Hong JS (2016) Critical role of the Mac1/NOX2 pathway in mediating reactive microgliosis-generated chronic neuroinflammation and progressive neurodegeneration. Curr Opin Pharmacol 26:54–60

Chen J, Wu L, Yang G et al (2021) The influence of PM2.5 exposure on non-alcoholic fatty liver disease. Life Sci 270:119135

Cheng YS (2003) Aerosol deposition in the extrathoracic region. Aerosol Sci Technol 37:659–671

Cho CC, Hsieh WY, Tsai CH et al (2018) In vitro and in vivo experimental studies of PM2.5 on disease progression. Int J Environ Res Public Health 15:1380

Chuang K-J, Yan Y-H, Chiu S-Y et al (2011) Long-term air pollution exposure and risk factors for cardiovascular diseases among the elderly in Taiwan. Occup Environ Med 68:64–68

Cisneros R, Bytnerowicz A, Schweizer D et al (2010) Ozone, nitric acid, and ammonia air pollution is unhealthy for people and ecosystems in southern Sierra Nevada, California. Environ Pollut 158:3261–3271

Clarke AG, Robertson LA, Hamilton RS et al (2004) A lagrangian model of the evolution of the particulate size distribution of vehicular emissions. Sci Total Environ 334–335:197–206

Corsini E, Budello S, Marabini L et al (2013) Comparison of wood smoke PM2.5 obtained from the combustion of FIR and beech pellets on inflammation and DNA damage in A549 and THP-1 human cell lines. Arch Toxicol 87:2187–2199

Costa LG, Cole TB, Coburn J et al (2017) Neurotoxicity of traffic-related air pollution. Neurotoxicology 59:133–139

Costa LG, Cole TB, Dao K et al (2020) Effects of air pollution on the nervous system and its possible role in neurodevelopmental and neurodegenerative disorders. Pharmacol Ther 210:107523

Cserbik D, Chen JC, McConnell R et al (2020) Fine particulate matter exposure during childhood relates to hemispheric-specific differences in brain structure. Environ Int 143:105933

Cui H, Xie N, Banerjee S et al (2021) Lung myofibroblasts promote macrophage profibrotic activity through lactate-induced histone lactylation. Am J Respir Cell Mol Biol 64:115–125

Daellenbach KR, Uzu G, Jiang J et al (2020) Sources of particulate-matter air pollution and its oxidative potential in Europe. Nature 587:414–419

de la Torre A, Barbas B, Sanz P et al (2018) Traditional and novel halogenated flame retardants in urban ambient air: gas-particle partitioning, size distribution and health implications. Sci Total Environ 630:154–163

Dellinger B, Pryor WA, Cueto R et al (2001) Role of free radicals in the toxicity of airborne fine particulate matter. Chem Res Toxicol 14:1371–1377
Demetriou CA, Raaschou-Nielsen O, Loft S et al (2012) Biomarkers of ambient air pollution and lung cancer: a systematic review. Occup Environ Med 69(9):619–627
Deng X, Rui W, Zhang F et al (2013a) PM2.5 induces Nrf2-mediated defense mechanisms against oxidative stress by activating PIK3/AKT signaling pathway in human lung alveolar epithelial A549 cells. Cell Biol Toxicol 29:143–157
Deng X, Zhang F, Rui W et al (2013b) PM2.5-induced oxidative stress triggers autophagy in human lung epithelial A549 cells. Toxicol in Vitro 27:1762–1770
Deng X, Zhang F, Wang L et al (2014) Airborne fine particulate matter induces multiple cell death pathways in human lung epithelial cells. Apoptosis 19:1099–1112
De Nys S, Duca RC, Nawrot T, et al (2018) Temporal variability of global DNA methylation and hydroxymethylation in buccal cells of healthy adults: association with air pollution. Environ Int 111:301–308
De Oliveira AAF, de Oliveira TF, Dias MF et al (2018) Genotoxic and epigenotoxic effects in mice exposed to concentrated ambient fine particulate matter (PM2.5) from São Paulo city, Brazil. Part Fibre Toxicol 15(1):40
De Prins S, Koppen G, Jacobs G et al (2013) Influence of ambient air pollution on global DNA methylation in healthy adults: a seasonal follow-up. Environ Int 59:418–424
Ding R, Jin Y, Liu X et al (2016) Characteristics of DNA methylation changes induced by traffic-related air pollution. Mutat Res Genet Toxicol Environ Mutagen 796:46–53
Donaldson K, Seaton A (2012) A short history of the toxicology of inhaled particles. Part Fibre Toxicol 9:13
Dornhof R, Maschowski C, Osipova A et al (2017) Stress fibers, autophagy and necrosis by persistent exposure to PM2.5 from biomass combustion. PLoS One 12:e0180291
Duarte FV, Teodoro JS, Rolo AP et al (2012) Exposure to dibenzofuran triggers autophagy in lung cells. Toxicol Lett 209:35–42
Ehn M, Thornton JA, Kleist E et al (2014) A large source of low-volatility secondary organic aerosol. Nature 506:476–479
Englert N (2004) Fine particles and human health—a review of epidemiological studies. Toxicol Lett 149:235–242
Faustini A, Stafoggia M, Colais P et al (2013) Air pollution and multiple acute respiratory outcomes. Eur Respir J 42:304–313
Feig DI, Reid TM, Loeb LA (1994) Reactive oxygen species in tumorigenesis. Cancer Res 54:1890s–1894s
Feil R, Fraga MF (2012) Epigenetics and the environment: emerging patterns and implications. Nat Rev Genet 13(2):97–109
Feng S, Gao D, Liao F et al (2016) The health effects of ambient PM2.5 and potential mechanisms. Ecotoxicol Environ Saf 128:67–74
Fleisch AF, Gold DR, Rifas-Shiman SL et al (2014) Air pollution exposure and abnormal glucose tolerance during pregnancy: the project Viva cohort. Environ Health Perspect 122:378–383
Floyd RA (1990) The role of 8-hydroxyguanine in carcinogenesis. Carcinogenesis 11:1447–1450
Fujitani Y, Kumar P, Tamura K et al (2012) Seasonal differences of the atmospheric particle size distribution in a metropolitan area in Japan. Sci Total Environ 437:339–347
Gao J, Woodward A, Vardoulakis S et al (2017) Haze, public health and mitigation measures in China: A review of the current evidence for further policy response. Sci Total Environ 578:148–157
Gehling W, Dellinger B (2013) Environmentally persistent free radicals and their lifetimes in PM2.5. Environ Sci Technol 47:8172–8178
Giannadaki D, Lelieveld J, Pozzer A (2016) Implementing the US air quality standard for PM2.5 worldwide can prevent millions of premature deaths per year. Environ Health 15:88

Gilmour PS, Rahman I, Donaldson K et al (2003) Histone acetylation regulates epithelial IL-8 release mediated by oxidative stress from environmental particles. Am J Physiol Lung Cell Mol Physiol 284:533–540

Goix S, Lévêque T, Xiong TT et al (2014) Environmental and health impacts of fine and ultrafine metallic particles: assessment of threat scores. Environ Res 133:185–194

Guan L, Rui W, Bai R et al (2016a) Effects of size-fractionated particulate matter on cellular oxidant radical generation in human bronchial epithelial BEAS-2B cells. Int J Environ Res Public Health 13:483

Guan WJ, Zheng XY, Chung KF et al (2016b) Impact of air pollution on the burden of chronic respiratory diseases in China: time for urgent action. Lancet 388:1939–1951

Guarnieri M, Balmes JR (2014) Outdoor air pollution and asthma. Lancet 383:1581–1592

Guo S, Hu M, Zamora ML et al (2014) Elucidating severe urban haze formation in China. Proc Natl Acad Sci U S A 111:17373–17378

Guxens M, Sunyer J (2012) A review of epidemiological studies on neuropsychological effects of air pollution. Swiss Med Wkly 141:w13322

Haddrell AE, Davies JF, Reid JP (2015) Dynamics of particle size on inhalation of environmental aerosol and impact on deposition fraction. Environ Sci Technol 49:14512–14521

Hamanaka RB, Mutlu GM (2018) Particulate matter air pollution: effects on the cardiovascular system. Front Endocrinol 9:680–680

Hampel R, Breitner S, Schneider A et al (2012) Acute air pollution effects on heart rate variability are modified by SNPs involved in cardiac rhythm in individuals with diabetes or impaired glucose tolerance. Environ Res 112:177–185

Hamra GB, Guha N, Cohen A et al (2014) Outdoor particulate matter exposure and lung cancer: a systematic review and meta-analysis. Environ Health Perspect 122:906–911

Han X, Liu H, Zhang Z et al (2020) Epitranscriptomic 5-methylcytosine profile in PM-induced mouse pulmonary fibrosis. Genomics Proteomics Bioinform 18:41–51

Han T, Qiao L, Zhou M et al (2015) Chemical and optical properties of aerosols and their interrelationship in winter in the megacity Shanghai of China. J Environ Sci (China) 27:59–69

Han B, Zhang R, Yang W et al (2016) Heavy haze episodes in Beijing during January 2013: inorganic ion chemistry and source analysis using highly time-resolved measurements from an urban site. Sci Total Environ 544:319–329

Han L, Zhou W, Li W et al (2018) Urbanization strategy and environmental changes: an insight with relationship between population change and fine particulate pollution. Sci Total Environ 642:789–799

Harmon AC, Hebert VY, Cormier SA et al (2018) Particulate matter containing environmentally persistent free radicals induces AhR-dependent cytokine and reactive oxygen species production in human bronchial epithelial cells. PLoS One 13:e0205412

Harrison RM (2020) Airborne particulate matter. Philos Trans A Math Phys Eng Sci 378:20190319

Heo J, Antkiewicz DS, Shafer MM et al (2015) Assessing the role of chemical components in cellular responses to atmospheric particle matter (PM) through chemical fractionation of PM extracts. Anal Bioanal Chem 407:5953–5963

Heusinkveld HJ, Wahle T, Campbell A et al (2016) Neurodegenerative and neurological disorders by small inhaled particles. Neurotoxicology 56:94–106

Hoek G, Krishnan RM, Beelen R et al (2013) Long-term air pollution exposure and cardio-respiratory mortality: a review. Environ Health 12:43

Hopke PK, Dai Q, Li L et al (2020) Global review of recent source apportionments for airborne particulate matter. Sci Total Environ 740:14009

Huang HB, Lai CH, Chen GW et al (2012) Traffic-related air pollution and DNA damage: a longitudinal study in Taiwanese traffic conductors. PLoS One 7(5):e37412

Huang RJ, Zhang Y, Bozzetti C et al (2014a) High secondary aerosol contribution to particulate pollution during haze events in China. Nature 514:218–222

Huang Q, Zhang J, Peng S et al (2014b) Effects of water soluble PM2.5 extracts exposure on human lung epithelial cells (A549): a proteomic study. J Appl Toxicol 34:675–687

Huang SK, Tripathi P, Koneva LA et al (2021) Effect of concentration and duration of particulate matter exposure on the transcriptome and DNA methylome of bronchial epithelial cells. Environ Epigenet 7(1):dvaa022

Ichinose T, Yajima Y, Nagashima M et al (1997) Lung carcinogenesis and formation of 8-hydroxydeoxyguanosine in mice by diesel exhaust particles. Carcinogenesis 18(1):185–192

Ishii H, Hayashi S, Hogg JC et al (2005) Alveolar macrophage-epithelial cell interaction following exposure to atmospheric particles induces the release of mediators involved in monocyte mobilization and recruitment. Respir Res 6:87

Jayaraj RL, Rodriguez EA, Wang Y et al (2017) Outdoor ambient air pollution and neurodegenerative diseases: the neuroinflammation hypothesis. Curr Environ Health Rep 4:166–179

Jirtle RL, Skinner MK (2007) Environmental epigenomics and disease susceptibility. Nat Rev Genet 8(4):253–262

Jung CR, Lin YT, Hwang BF (2015) Ozone, particulate matter, and newly diagnosed Alzheimer's disease: a population-based cohort study in Taiwan. J Alzheimers Dis 44:573–584

Jurewicz J, Dziewirska E, Radwan M et al (2018) Air pollution from natural and anthropic sources and male fertility. Reprod Biol Endocrinol 16:109

Ke Q, Davidson T, Chen H et al (2006) Alterations of histone modifications and transgene silencing by nickel chloride. Carcinogenesis 27:1481–1488

Kelly JT, Bobbitt CM, Asgharian B (2001) In vivo measurement of fine and coarse aerosol deposition in the nasal airways of female long-evans rats. Toxicol Sci 64:253–258

Kermanizadeh A, Balharry D, Wallin H et al (2015) Nanomaterial translocation-the biokinetics, tissue accumulation, toxicity and fate of materials in secondary organs—a review. Crit Rev Toxicol 45:837–872

Kim JY, Mukherjee S, Ngo LC et al (2004) Urinary 8-hydroxy-2'-deoxyguanosine as a biomarker of oxidative DNA damage in workers exposed to fine particulates. Environ Health Perspect 112(6):666–671

Kim KH, Kabir E, Kabir S (2015) A review on the human health impact of airborne particulate matter. Environ Int 74:136–143

Koçak E, Kılavuz SA, Öztürk F et al (2021) Characterization and source apportionment of carbonaceous aerosols in fine particles at urban and suburban atmospheres of Ankara, Turkey. Environ Sci Pollut Res Int 28:25701–25715

Kouassi KS, Billet S, Garcon G et al (2010) Oxidative damage induced in A549 cells by physically and chemically characterized air particulate matter (PM2.5) collected in Abidjan, Cote D'ivoire. J Appl Toxicol 30:310–320

Kupsco A, Gonzalez G, Baker BH et al (2020) Associations of smoking and air pollution with peripheral blood RNA N6-methyladenosine in the Beijing truck driver air pollution study. Environ Int 144:106021

Kuwayama T, Ruehl CR, Kleeman MJ (2013) Daily trends and source apportionment of ultrafine particulate mass (PM0.1) over an annual cycle in a typical California city. Environ Sci Technol 47:13957–13966

Lai C-H, Huang H-B, Chang Y-C et al (2017) Exposure to fine particulate matter causes oxidative and methylated DNA damage in young adults: a longitudinal study. Sci Total Environ 598:289–296

Lamichhane DK, Leem JH, Lee JY et al (2015) A meta-analysis of exposure to particulate matter and adverse birth outcomes. Environ Health Toxicol 30:e2015011

Lanzinger S, Rosenbauer J, Sugiri D et al (2018) Impact of long-term air pollution exposure on metabolic control in children and adolescents with type 1 diabetes: results from the DPV registry. Diabetologia 61:1354–1361

Lawal AO (2017) Air particulate matter induced oxidative stress and inflammation in cardiovascular disease and atherosclerosis: the role of Nrf2 and AhR-mediated pathways. Toxicol Lett 270:88–95

Lee KK, Bing R, Kiang J et al (2020) Adverse health effects associated with household air pollution: a systematic review, meta-analysis, and burden estimation study. Lancet Glob Health 8:e1427–e1434

Li N, Xia T, Nel AE (2008) The role of oxidative stress in ambient particulate matter-induced lung diseases and its implications in the toxicity of engineered nanoparticles. Free Radic Biol Med 44:1689–1699

Li R, Zhao Y, Shi J et al (2020) Effects of PM2.5 exposure in utero on heart injury, histone acetylation and GATA4 expression in offspring mice.Chemosphere 2020;256:127–133

Li Q, Wang YY, Guo Y et al (2018) Effect of airborne particulate matter of 2.5 μm or less on preterm birth: a national birth cohort study in China. Environ Int 121:1128–1136

Li XL, Wang JS, Tu XD et al (2007) Vertical variations of particle number concentration and size distribution in a street canyon in Shanghai, China. Sci Total Environ 378:306–316

Li Z, Tang Y, Song X et al (2019) Impact of ambient PM2.5 on adverse birth outcome and potential molecular mechanism. Ecotoxicol Environ Saf 169:248–254

Lighty JS, Veranth JM, Sarofim AF (2000) Combustion aerosols: factors governing their size and composition and implications to human health. J Air Waste Manag Assoc 50:1565–1618

Limón-Pacheco J, Gonsebatt ME (2009) The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. Mutat Res 674:137–147

Link MS, Luttmann-Gibson H, Schwartz J et al (2013) Acute exposure to air pollution triggers atrial fibrillation. J Am Coll Cardiol 62:816–825

Liu X, Meng Z (2005) Effects of airborne fine particulate matter on antioxidant capacity and lipid peroxidation in multiple organs of rats. Inhal Toxicol 17:467–473

Liu J, Ren L, Wei J et al (2018) Fine particle matter disrupts the blood-testis barrier by activating TGF-β3/p38 MAPK pathway and decreasing testosterone secretion in rat. Environ Toxicol 33:711–719

Liu W, Zhang M, Feng J et al (2017) The influence of quercetin on maternal Immunity, oxidative stress, and inflammation in mice with exposure of fine particulate matter during gestation. Int J Environ Res Public Health 14:592

Loft S, Svoboda P, Kawai K et al (2012) Association between 8-oxo-7,8-dihydroguanine excretion and risk of lung cancer in a prospective study. Free Radic Biol Med 52(1):167–172

Longest PW, Holbrook LT (2012) In silico models of aerosol delivery to the respiratory tract—development and applications. Adv Drug Deliv Rev 64:296–311

Lu J, Wang Z, Cao J et al (2018) A novel and compact review on the role of oxidative stress in female reproduction. Reprod Biol Endocrinol 16:80

Ma Q (2013) Role of nrf2 in oxidative stress and toxicity. Annu Rev Pharmacol Toxicol 53:401–426

Madureira J, Slezakova K, Silva AI et al (2020) Assessment of indoor air exposure at residential homes: inhalation dose and lung deposition of PM10, PM2.5 and ultrafine particles among newborn children and their mothers. Sci Total Environ 717:137293

Mahalingaiah S, Hart JE, Laden F et al (2016) Adult air pollution exposure and risk of infertility in the Nurses' Health Study II. Hum Reprod 31:638–647

Mahalingaiah S, Missmer SE, Cheng JJ et al (2018) Perimenarchal air pollution exposure and menstrual disorders. Hum Reprod 33:512–519

Manigrasso M, Natale C, Vitali M et al (2017) Pedestrians in traffic environments: ultrafine particle respiratory doses. Int J Environ Res Public Health 14:288

Mann JK, Balmes JR, Bruckner TA et al (2010) Short-term effects of air pollution on wheeze in asthmatic children in Fresno, California. Environ Health Perspect 118:1497–1502

Mannucci PM, Harari S, Martinelli I et al (2015) Effects on health of air pollution: a narrative review. Intern Emerg Med 10:657–662

Manojkumar N, Srimuruganandam B, Shiva Nagendra SM (2019) Application of multiple-path particle dosimetry model for quantifying age specified deposition of particulate matter in human airway. Ecotoxicol Environ Saf 168:241–248

Martins V, Cruz Minguillón M, Moreno T et al (2015) Deposition of aerosol particles from a subway microenvironment in the human respiratory tract. J Aerosol Sci 90:103–113

Mitkus RJ, Powell JL, Zeisler R et al (2013) Comparative physicochemical and biological characterization of NIST Interim Reference Material PM2.5 and SRM 1648 in human A549 and mouse RAW264.7 cells. Toxicol in Vitro 27:2289–2298

Møller P, Danielsen PH, Karottki DG et al (2014) Oxidative stress and inflammation generated DNA damage by exposure to air pollution particles. Mutat Res Rev Mutat Res 762:133–166
Møller P, Løhr M, Folkmann JK et al (2010) Aging and oxidatively damaged nuclear DNA in animal organs. Free Radic Biol Med 48:1275–1285
Montiel-Davalos A, Ibarra-Sanchez Mde J, Ventura-Gallegos JL et al (2010) Oxidative stress and apoptosis are induced in human endothelial cells exposed to urban particulate matter. Toxicol in Vitro 24:135–141
Mukherjee A, Agrawal M (2018) A global perspective of fine particulate matter pollution and its health effects. Rev Environ Contam Toxicol 244:5–51
Müller B, Seifart C, Barth PJ (1998) Effect of air pollutants on the pulmonary surfactant system. Eur J Clin Invest 28:762–777
Newell K, Kartsonaki C, Lam KBH et al (2017) Cardiorespiratory health effects of particulate ambient air pollution exposure in low-income and middle-income countries: a systematic review and meta-analysis. Lancet Planet Health 1:e368–e380
Niki E (2016) Oxidative stress and antioxidants: distress or eustress? Arch Biochem Biophys 595:19–24
Niu J, Liberda EN, Qu S et al (2013) The role of metal components in the cardiovascular effects of PM2.5. PLoS One 8:e83782
Obtulowicz T, Winczura A, Speina E et al (2010) Aberrant repair of etheno-DNA adducts in leukocytes and colon tissue of colon cancer patients. Free Radic Biol Med 49(6):1064–1071
Ogino K, Zhang R, Takahashi H et al (2014) Allergic airway inflammation by nasal inoculation of particulate matter (PM2.5) in NC/Nga mice. PLoS One 9:e92710
Oudin A, Forsberg B, Adolfsson AN et al (2016) Traffic-Related air pollution and dementia incidence in northern Sweden: a longitudinal study. Environ Health Perspect 124:306–312
Pan X, Yue W, He K et al (2007) Health benefit evaluation of the energy use scenarios in Beijing, China. Sci Total Environ 374:242–251
Pope CA 3rd, Burnett RT, Thun MJ et al (2002) Lung cancer, cardiopulmonary mortality, and long-term exposure to fine particulate air pollution. JAMA 287:1132–1141
Pope CA 3rd, Thun MJ, Namboodiri MM et al (1995) Particulate air pollution as a predictor of mortality in a prospective study of U.S. adults. Am J Respir Crit Care Med 151:669–674
Pöschl U (2005) Atmospheric aerosols: composition, transformation, climate and health effects. Angew Chem Int Ed Engl 44:7520–7540
Prada D, López G, Solleiro-Villavicencio H et al (2020) Molecular and cellular mechanisms linking air pollution and bone damage. Environ Res 185:109465
Radwan M, Jurewicz J, Polańska K et al (2016) Exposure to ambient air pollution—Does it affect semen quality and the level of reproductive hormones? Ann Hum Biol 43:50–56
Rajagopalan S, Park B, Palanivel R et al (2020) Metabolic effects of air pollution exposure and reversibility. J Clin Invest 130:6034–6040
Ranft U, Schikowski T, Sugiri D et al (2009) Long-term exposure to traffic-related particulate matter impairs cognitive function in the elderly. Environ Res 109:1004–1011
Rao X, Montresor-Lopez J, Puett R et al (2015) Ambient air pollution: an emerging risk factor for diabetes mellitus. Curr Diab Rep 15:33
Rao X, Zhong J, Brook RD et al (2018) Effect of particulate matter air pollution on cardiovascular oxidative stress pathways. Antioxid Redox Signal 28:797–818
Ren J, Li B, Yu D et al (2016) Approaches to prevent the patients with chronic airway diseases from exacerbation in the haze weather. J Thorac Dis 8:E1–E7
Rhinehart ZJ, Kinnee E, Essien UR et al (2020) Association of fine particulate matter and risk of stroke in patients with atrial fibrillation. JAMA Netw Open 3:e2011760
Ritz B, Lee PC, Hansen J et al (2016) Traffic-related air pollution and Parkinson's disease in Denmark: a case-control study. Environ Health Perspect 124:351–356
Rui W, Guan L, Zhang F et al (2016) PM2.5-induced oxidative stress increases adhesion molecules expression in human endothelial cells through the ERK/AKT/NF-κB-dependent pathway. J Appl Toxicol 36:48–59

Saha S, Buttari B, Panieri E et al (2020) An overview of Nrf2 signaling pathway and its role in inflammation. Molecules 25:5474
Sanchez-Guerra M, Zheng Y, Osorio-Yanez C et al (2015) Effects of particulate matter exposure on blood 5-hydroxymethylation: Results from the Beijing truck driver air pollution study. Epigenetics 10(7):633–642
Schultz ES, Litonjua AA, Melén E (2017) Effects of long-term exposure to traffic-related air pollution on lung function in children. Curr Allergy Asthma Rep 17:41
Secrest MH, Schauer JJ, Carter EM et al (2017) Particulate matter chemical component concentrations and sources in settings of household solid fuel use. Indoor Air 27:1052–1066
Semmler M, Seitz J, Erbe F et al (2004) Long-term clearance kinetics of inhaled ultrafine insoluble iridium particles from the rat lung, including transient translocation into secondary organs. Inhal Toxicol 16:453–459
Sese L, Nunes H, Cottin V et al (2018) Role of atmospheric pollution on the natural history of idiopathic pulmonary fibrosis. Thorax 73:145–150
Sharma AK, Jensen KA, Rank J et al (2007) Genotoxicity, inflammation and physico-chemical properties of fine particle samples from an incineration energy plant and urban air. Mutat Res 633:95–111
Shi Y, Ji Y, Sun H et al (2015) Nanoscale characterization of PM2.5 airborne pollutants reveals high adhesiveness and aggregation capability of soot particles. Sci Rep 5:11232
Sidi D, Keming M, Le B (2012) Distribution and heavy metal character of foliar dust on roadside tree *Sophora japonica* of urban area in Beijing. Acta Ecol Sin 32:5095–5102
Signorelli SS, Ferrante M, Gaudio A et al (2017) Deep vein thrombosis related to environment (Review). Mol Med Rep 15:3445–3448
Simkhovich BZ, Kleinman MT, Kloner RA (2008) Air pollution and cardiovascular injury epidemiology, toxicology, and mechanisms. J Am Coll Cardiol 52:719–726
Smith RB, Beevers SD, Gulliver J et al (2020) Impacts of air pollution and noise on risk of preterm birth and stillbirth in London. Environ Int 134:105290
Soberanes S, Urich D, Baker CM et al (2009) Mitochondrial complex III-generated oxidants activate ASK1 and JNK to induce alveolar epithelial cell death following exposure to particulate matter air pollution. J Biol Chem 284:2176–2186
Solomon PA, Sioutas C (2008) Continuous and semicontinuous monitoring techniques for particulate matter mass and chemical components: a synthesis of findings from EPA's Particulate Matter Supersites Program and related studies. J Air Waste Manag Assoc 58:164–195
Song C, He J, Wu L et al (2017) Health burden attributable to ambient PM2.5 in China. Environ Pollut 223:575–586
Sørensen M, Autrup H, Hertel O et al (2003) Personal exposure to PM2.5 and biomarkers of DNA damage. Cancer Epidemiol Biomarkers Prev 12(3):191–196
Sturm R (2007) A computer model for the clearance of insoluble particles from the tracheobronchial tree of the human lung. Comput Biol Med 37:680–690
Suades-González E, Gascon M, Guxens M et al (2015) Air pollution and neuropsychological development: a review of the latest evidence. Endocrinology 156:3473–3482
Sun B, Shi Y, Yang X et al (2018) DNA methylation: a critical epigenetic mechanism underlying the detrimental effects of airborne particulate matter. Ecotoxicol Environ Saf 161:173–183
Sun Z, Mukherjee B, Brook RD et al (2013) Air-pollution and cardiometabolic diseases (AIRCMD): a prospective study investigating the impact of air pollution exposure and propensity for type II diabetes. Sci Total Environ 448:72–78
Sun L, Wu Q, Liao K et al (2016) Contribution of heavy metals to toxicity of coal combustion related fine particulate matter (PM2.5) in *Caenorhabditis elegans* with wild-type or susceptible genetic background. Chemosphere 144:2392–2400
Supharakonsakun Y, Areepong Y, Sukparungsee S (2020) The performance of a modified EWMA control chart for monitoring autocorrelated PM2.5 and carbon monoxide air pollution data. PeerJ 8:e10467

Tan C, Lu S, Wang Y, Zhu Y et al (2017) Long-term exposure to high air pollution induces cumulative DNA damages in traffic policemen. Sci Total Environ 593–594:330–336
Tao J, Zhang Z, Zhang L et al (2021) Quantifying the relative importance of major tracers for fine particles released from biofuel combustion in households in the rural North China Plain. Environ Pollut 268:115764
Thompson JE (2018) Airborne particulate matter: human exposure and health effects. J Occup Environ Med 60:392–423
Thorpe A, Harrison RM (2008) Sources and properties of non-exhaust particulate matter from road traffic: a review. Sci Total Environ 400:270–282
Tian Y, Liu H, Wu Y et al (2019) Ambient particulate matter pollution and adult hospital admissions for pneumonia in urban China: a national time series analysis for 2014 through 2017. PLoS Med 16:e1003010
Tonelli C, Chio IIC, Tuveson DA (2018) Transcriptional regulation by Nrf2. Antioxid Redox Signal 29:1727–1745
Tong H, Cheng WY, Samet JM et al (2010) Differential cardiopulmonary effects of size-fractionated ambient particulate matter in mice. Cardiovasc Toxicol 10:259–267
Torres-Ramos YD, Montoya-Estrada A, Guzman-Grenfell AM et al (2011) Urban PM2.5 induces ROS generation and RBC damage in COPD patients. Front Biosci (elite Ed) 3:808–817
Ulrich MM, Alink GM, Kumarathasan P et al (2002) Health effects and time course of particulate matter on the cardiopulmonary system in rats with lung inflammation. J Toxicol Environ Health A 65:1571–1595
Valavanidis A, Fiotakis K, Vlachogianni T (2008) Airborne particulate matter and human health: toxicological assessment and importance of size and composition of particles for oxidative damage and carcinogenic mechanisms. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 26:339–362
Valavanidis A, Vlachogianni T, Fiotakis K et al (2013) Pulmonary oxidative stress, inflammation and cancer: respirable particulate matter, fibrous dusts and ozone as major causes of lung carcinogenesis through reactive oxygen species mechanisms. Int J Environ Res Public Health 10:3886–3907
van Berlo D, Hullmann M, Schins RP (2012) Toxicology of ambient particulate matter. In: Luch A (ed) Molecular, clinical and environmental toxicology, volume 3, molecular toxicology series. Experientia Supplementum, Birkhäuser Basel, p 165–217
Vattanasit U, Navasumrit P, Khadka MB et al (2014) Oxidative DNA damage and inflammatory responses in cultured human cells and in humans exposed to traffic-related particles. Int J Hyg Environ Health 217:23–33
Vrijens K, Trippas AJ, Lefebvre W et al (2020) Association of prenatal exposure to ambient air pollution with circulating histone levels in maternal cord blood. JAMA Netw Open 3(5):e205156
Wang Q, Benmarhnia T, Zhang H et al (2018a) Identifying windows of susceptibility for maternal exposure to ambient air pollution and preterm birth. Environ Int 121:317–324
Wang W, Ding X, Turap Y et al (2020) Distribution, sources, risks, and vitro DNA oxidative damage of PM2.5-bound atmospheric polycyclic aromatic hydrocarbons in Urumqi, NW China. Sci Total Environ 739:139518
Wang T, Garcia JG, Zhang W (2012) Epigenetic regulation in particulate matter-mediated cardiopulmonary toxicities: a systems biology perspective. Curr Pharmacogenomics Person Med 10:314–321
Wang Z, Hu W, Niu H et al (2021a) Variations in physicochemical properties of airborne particles during a heavy haze-to-dust episode in Beijing. Sci Total Environ 762:143081
Wang S, Li H (2021) NO_3-initiated gas-phase formation of nitrated phenolic compounds in polluted atmosphere. Environ Sci Technol 55:2899–2907
Wang YY, Li Q, Guo Y et al (2018b) Association of long-term exposure to airborne particulate matter of 1 μm or less with preterm birth in China. JAMA Pediatr 172:e174872
Wang L, Luo D, Liu X et al (2021b) Effects of PM2.5 exposure on reproductive system and its mechanisms. Chemosphere 264:128436

Wang H, Shen X, Liu J et al (2019) The effect of exposure time and concentration of airborne PM2.5 on lung injury in mice: a transcriptome analysis. Redox Biol 26:101264
Wang H, Shen X, Tian G et al (2018c) AMPKα2 deficiency exacerbates long-term PM2.5 exposure-induced lung injury and cardiac dysfunction. Free Radic Biol Med 121:202–214
Wei H, Liang F, Meng G et al (2016) Redox/methylation mediated abnormal DNA methylation as regulators of ambient fine particulate matter-induced neurodevelopment related impairment in human neuronal cells. Sci Rep 6:33402
Wei H, Yuan W, Yu H et al (2021) Cytotoxicity induced by fine particulate matter PM2.5 via mitochondria-mediated apoptosis pathway in rat alveolar macrophages. Environ Sci Pollut Res Int 28:25819–25829
Wu H, Jiang B, Geng X et al (2018) Exposure to fine particulate matter during pregnancy and risk of term low birth weight in Jinan, China, 2014–2016. Int J Hyg Environ Health 221:183–190
Wu X, Pan B, Liu L et al (2019) In utero exposure to PM2.5 during gestation caused adult cardiac hypertrophy through histone acetylation modification. J Cell Biochem 120:4375–4384
Xiao X, Yao T, Du S et al (2020) Age differences in the pulmonary and vascular pathophysiologic processes after long-term real-time exposure to particulate matter in rats. Chemosphere 261:127710
Xie Y, Zhang X, Tian Z et al (2013) Preexposure to PM2.5 exacerbates acute viral myocarditis associated with Th17 cell. Int J Cardiol 168:3837–3845
Xu MX, Ge CX, Qin YT et al (2019) Prolonged PM2.5 exposure elevates risk of oxidative stress-driven nonalcoholic fatty liver disease by triggering increase of dyslipidemia. Free Radic Biol Med 130:542–556
Xu X, Jiang SY, Wang TY et al (2013) Inflammatory response to fine particulate air pollution exposure: neutrophil versus monocyte. PLoS One 8:e71414
Xu W, Kuang Y, Liang L et al (2020) Dust-dominated coarse particles as a medium for rapid secondary organic and inorganic aerosol formation in highly polluted air. Environ Sci Technol 54:15710–15721
Xu X, Liu C, Xu Z et al (2011) Long-term exposure to ambient fine particulate pollution induces insulin resistance and mitochondrial alteration in adipose tissue. Toxicol Sci 124:88–98
Xu DQ, Zhang WL (2004) Monitoring of pollution of air fine particles (PM2.5) and study on their genetic toxicity. Biomed Environ Sci 17:452–458
Xu J, Zhang W, Lu Z et al (2017) Airborne PM2.5-induced hepatic insulin resistance by Nrf2/JNK-mediated signaling pathway. Int J Environ Res Public Health 14:787
Yang Y, Boza-Serrano A, Dunning CJR et al (2018a) Inflammation leads to distinct populations of extracellular vesicles from microglia. J NEuroinflammation 15:168
Yang S, Tan Y, Mei H et al (2018b) Ambient air pollution the risk of stillbirth: a prospective birth cohort study in Wuhan, China. Int J Hyg Environ Health 221:502–509
Yang D, Yang X, Deng F et al (2017) Ambient air pollution and biomarkers of health effect. Adv Exp Med Biol 1017:59–102
Yang Y, Yang T, Liu S et al (2019) Concentrated ambient PM2.5 exposure affects mice sperm quality and testosterone biosynthesis. PeerJ 7:e8109
Ye B, Zhong C, Li Q et al (2020) The association of ambient fine particulate matter exposure during pregnancy with blood glucose levels and gestational diabetes mellitus risk: a prospective cohort study in Wuhan, China. Am J Epidemiol 189:1306–1315
Yi L, Wei C, Fan W (2017) Fine-particulate matter (PM2.5), a risk factor for rat gestational diabetes with altered blood glucose and pancreatic GLUT2 expression. Gynecol Endocrinol 33:611–616
Ying Z, Xu X, Bai Y et al (2014) Long-term exposure to concentrated ambient PM2.5 increases mouse blood pressure through abnormal activation of the sympathetic nervous system: a role for hypothalamic inflammation. Environ Health Perspect 122:79–86
Yuan L, Zhang Y, Gao Y et al (2019) Maternal fine particulate matter (PM2.5) exposure and adverse birth outcomes: an updated systematic review based on cohort studies. Environ Sci Pollut Res Int 26:13963–13983

Yue W, Tong L, Liu X et al (2019) Short term PM2.5 exposure caused a robust lung inflammation, vascular remodeling, and exacerbated transition from left ventricular failure to right ventricular hypertrophy. Redox Biol 22:101161

Zang H, Cheng H, Song W et al (2019) Ambient air pollution and the risk of stillbirth: a population-based prospective birth cohort study in the coastal area of China. Environ Sci Pollut Res Int 26:6717–6724

Zanobetti A, Dominici F, Wang Y et al (2014) A national case-crossover analysis of the short-term effect of PM2.5 on hospitalizations and mortality in subjects with diabetes and neurological disorders. Environ Health 13:38

Zare Sakhvidi MJ, Lequy E, Goldberg M et al (2020) Air pollution exposure and bladder, kidney and urinary tract cancer risk: a systematic review. Environ Pollut 267:115328

Zhang D, Tang Z, Huang H et al (2019) Metabolic regulation of gene expression by histone lactylation. Nature 574:575–580

Zhao Q, Chen H, Yang T et al (2016) Direct effects of airborne PM2.5 exposure on macrophage polarizations. Biochim Biophys Acta 1860:2835–2843

Zhao H, Li X, Wang X (2011) Heavy metal contents of road-deposited sediment along the urban-rural gradient around Beijing and its potential contribution to runoff pollution. Environ Sci Technol 45:7120–7127

Zhao C, Liao J, Chu W et al (2012) Involvement of TLR2 and TLR4 and Th1/Th2 shift in inflammatory responses induced by fine ambient particulate matter in mice. Inhal Toxicol 24:918–927

Zheng R, Tao L, Jian H et al (2018) NLRP3 inflammasome activation and lung fibrosis caused by airborne fine particulate matter. Ecotoxicol Environ Saf 163:612–619

Zheng Y, Sanchez-Guerra M, Zhang Z et al (2017) Traffic-derived particulate matter exposure and histone H3 modification: A repeated measures study. Environ Res 153:112–119

Zhou X, Cao Z, Ma Y et al (2016a) Concentrations, correlations and chemical species of PM2.5/PM10 based on published data in China: potential implications for the revised particulate standard. Chemosphere 144:518–526

Zhou S, Davy PK, Wang X et al (2016b) High time-resolved elemental components in fine and coarse particles in the Pearl River Delta Region of southern China: dynamic variations and effects of meteorology. Sci Total Environ 572:634–648

Zhou T, Hu Y, Wang Y et al (2019) Fine particulate matter PM2.5 aggravates apoptosis of cigarette-inflamed bronchial epithelium in vivo and vitro. Environ Pollut 248:1–9

Zhou S, Xi Y, Chen Y et al (2020) Ovarian dysfunction induced by chronic whole-body PM2.5 exposure. Small 16:e2000845

Zhu CS, Cao JJ, Tsai CJ et al (2016) The rural carbonaceous aerosols in coarse, fine, and ultrafine particles during haze pollution in northwestern China. Environ Sci Pollut Res Int 23:4569–4575

Zhu Y, Huang L, Li J et al (2018) Sources of particulate matter in China: insights from source apportionment studies published in 1987–2017. Environ Int 115:343–357

Zuo L, Prather ER, Stetskiv M et al (2019) Inflammaging and oxidative stress in human diseases: from molecular mechanisms to novel treatments. Int J Mol Sci 20:4472

Chapter 11
Mechanisms of Action of Emerging Contaminants: Pharmaceuticals and Personal Care Products (PPCP)

Wei Shi and Haoyue Tan

Abstract Pharmaceuticals and personal care products (PPCPs) are emerging contaminants present in the environment. The general population is inevitably exposed to PPCPs in daily life. The PPCP family contains two types of chemicals: pharmaceuticals and personal care products. Pharmaceuticals are used primarily to prevent or treat human and animal diseases, whereas personal care products are used to improve the quality of daily life and include products such as toothpaste, shampoo, lotions, cosmetics, and hair colors. Due to the structural similarity to biologically active compounds, PPCPs have raised public concerns regarding their possible effects on human health and the environment. Over the past two decades, many studies have found that PPCPs are endocrine-disrupting chemicals (EDCs) with profound adverse effects on the endocrine system. Therefore, in this chapter, we discuss several typical PPCPs, such as bisphenol A and its analogues, triclosan, triclocarban, and phthalates, their adverse endocrine-disrupting activities and three typical endocrine system-related modes of action (MOAs) through interaction with estrogen, androgen, and thyroid receptors.

Introduction

Pharmaceuticals and personal care products (PPCPs) are emerging contaminants, which include two major chemical classes (i) pharmaceuticals and (ii) personal care products (PCPs). Pharmaceuticals are used primarily to prevent or treat human and animal diseases, whereas PCPs are used to improve the quality of daily life and include products such as toothpaste, shampoo, lotions, cosmetics, and hair colors (Boxall et al. 2012). Different from other contaminants, the general population is inevitably exposed to PPCPs in daily life. Although many PPCPs (such as phthalates) can be quickly metabolized in the human body, PPCPs have been detected in the

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natural environment across the world (Alejandro et al. 2009; Hirsch et al. 1999; Kolpin et al. 2002). Additionally, pharmaceuticals, as well as several PCPs, are biologically active compounds that are designed to interact with a target (such as a specific receptor, enzyme, or biological process) in humans and animals to deliver the therapeutic effect. If these targets are present in organisms in the natural environment, exposure to some PPCPs might elicit harmful effects in those organisms. Therefore, as pseudo-persistent contaminants (Monteiro and Boxall 2009), PPCPs have raised public concerns regarding their possible effects on human health and the environment. Over the past two decades, a substantial number of studies have been conducted to determine the occurrence, fate, effects, and risks of PPCPs in the environment.

Many studies have found that PPCPs are biologically active in experimental models, and epidemiological studies have linked PPCP exposure to several chronic human diseases, such as cancer and infertility (Binder et al. 2018). Studies have shown that many PPCPs are endocrine-disrupting chemicals (EDCs) with profound health effects (Ma et al. 2019; Prins et al. 2019; Rochester 2013; Wazir and Mokbel 2019), and in vitro (Vandenberg 2014; Villar-Pazos et al. 2017) and in vivo (Jenkins et al. 2011; Leung et al. 2017) experiments support this causal relationship between PPCP exposure and the pathogenesis of diseases caused by endocrine system disruption. Therefore, in this chapter, we introduce several typical PPCPs and their adverse effects, focusing on their mechanisms of action, mainly endocrine system-related effects, which is the main mode of action (MOA) of PPCPs.

Personal Care Products

PCPs include numerous chemical compounds from different classes, such as phenols and phthalates. Many studies have been conducted to identify the negative effects of PCPs on human health and it has been shown that certain components of PCPs are associated with severe diseases, mainly by disrupting the endocrine system. In the next sections, the toxicological studies of several typical components of PCPs that have been related to health effects via endocrine disruption are discussed.

Bisphenol A and Its Analogues

Bisphenol A (BPA) and its analogues are produced in high volumes all over the world and are widely used as components of polycarbonate plastics and epoxy resins in many consumer products, such as toys, food and beverage packaging (Rubin 2011). It is worth noting that such uses were banned in France in 2015. Several studies have shown that BPA and its analogues cause developmental, reproductive, and neurodevelopmental toxicity in children and adults, mostly via endocrine disruption. Since the estrogenic activity of BPA was reported in 1993 (Krishnan et al. 1993), a considerable amount of research about endocrine-disrupting activities of BPA and

its analogues has been conducted. BPA disrupts hormonal homeostasis in the human body, for which molecular mechanisms and adverse outcomes have been established and show a plausible causal link. The main molecular mechanisms of BPA that may be related to adverse outcomes in human health are summarized below.

Endocrine-Related Mechanisms

Competitive binding to estrogen receptors (ERs) and agonistic and/or antagonistic properties at the cellular level are the most important activities of BPA (Welshons et al. 2006). BPA can exert a mix of agonist and antagonist activities on ERα, and an agonist activity on ERβ (Chen et al. 2020; Kurosawa et al. 2002). Estrogen-related receptors (ERRs), including ERRα, ERRβ, ERRγ, together with ERs compose the estrogen signaling pathway. BPA was found to bind strongly to ERRγ in both in vitro (Matsushima et al. 2007; Takayanagi et al. 2006; Takeda et al. 2009) and in vivo (Tohmé et al. 2014) assays, potentially generating disrupting activities in the estrogen signaling pathway. Additionally, BPA can bind to estrogen membrane receptors such as G protein-coupled receptor 30 (GPR30) (Thomas and Dong 2006) and form the ligand-receptor complexes to disrupt the estrogen signaling pathway (Alonso-Magdalena et al. 2012; Watson et al. 2007). This kind of nongenomic estrogenic action instead of activating/repressing traditional estrogen-responsive genes can produce rapid responses at very low BPA concentrations in the range of 10 fM to 10 nM (Wetherill et al. 2007; Zsarnovszky et al. 2005).

BPA can also exert antagonistic activity on androgen receptor (AR) (Chen et al. 2019; Molina-Molina et al. 2013; Wetherill et al. 2007), although the binding potency to AR is relatively weaker than to ERs (Bonefeld-Jørgensen et al. 2007; Roy et al. 2004). Two analogues of BPA, 4,4'-sulfonylbis(2-methylphenol) (dBPS) and 4,4'-thiodiphenol (THIO) were identified as androgen antagonists in vitro assays (Kolšek et al. 2015). Aside from competitive binding, several studies have found that BPA can also inhibit nuclear translocation of the AR and interfere with its function via multiple mechanisms (Teng et al. 2013).

The thyroid hormone receptor (THR) is another nuclear receptor (NR) that has been extensively studied because THRs are crucial in brain development. Low levels of thyroid hormone (TH) are associated with impaired neurodevelopment in the offspring (Ghassabian and Trasande 2018). BPA may bind to both THRα/β subtypes directly, acting as an antagonist (Delfosse et al. 2014; Moriyama et al. 2002; Shigeyuki et al. 2005; Zoeller et al. 2005), suppressing the transcriptional activities and gene expression of THRs (Gentilcore et al. 2013; Sheng et al. 2012), and inducing adverse neuroendocrine regulations (Chen et al. 2014; Gore 2010; Panagiotidou et al. 2014). Some studies also found that the antagonistic activities of BPA towards THR subtypes are different. Namely, BPA acts as an antagonist on THRβ, which mediates the negative feedback effect of TH on the pituitary gland. However, BPA is less effective at antagonizing TH on THRα, which responds to elevated thyroxine (T4) levels (Zoeller et al. 2005). Halogenated BPAs can also

inhibit the transcriptional activities of THRs directly (Delfosse et al. 2014). Moreover, extensive experimental research documents that BPA can affect THR-mediated signaling pathways indirectly. BPA did not only alter the transcriptional activities involved in TH synthesis but also changed the activities of thyroid peroxidase in rat thyroid-related in vitro assays (Wu et al. 2016). BPA as a non-competitive inhibitor inhibited sodium/iodide symporter (NIS)-mediated iodide uptake in a concentration-dependent manner (Wu et al. 2016). BPA can also repress TH sulfotransferase activity (Butt and Stapleton 2013). Many in vivo experiments were performed to elucidate the mechanisms of negative effects of BPA on THRs. Exposure of pregnant rats to BPA caused an increase in total T4 in serum, whereas serum thyroid-stimulating hormone (TSH) levels did not change significantly (Zoeller et al. 2005). BPA up-regulated the expression of the TH-responsive gene RC3/neurogranin (Zoeller et al. 2005).

It has been found that, in addition to estrogen, androgen and thyroid receptors, BPA can interact with many NRs, such as glucocorticoid receptor (GR) (Chen et al. 2020) and pregnane X receptor (PXR) (Delfosse et al. 2014). Analogues of BPA, bisphenol Z (BPZ) and bis[4-(2-hydroxyethoxy)phenyl] sulfone (BHEPS) were identified as anti-glucocorticoids, and bisphenol F (BPF) was identified as a glucocorticoid-like ligand in reporter gene assays (Kolšek et al. 2015). BPA can also bind to many other receptors besides NR and result in non-transcriptional mechanisms (Diamanti-Kandarakis et al. 2009), such as sex hormone-binding globulin (SHBG) and aryl hydrocarbon receptor (AhR). For SHBG, BPA acts as a competitive binder to displace the endogenous hormone ligands from the SHBG ligand-binding domain and further increases the imbalance in the ratio of androgen to estrogen (Déchaud et al. 1999; Takeuchi and Tsutsumi 2002). For AhR, BPA acts as an antagonist to decrease the activity of AhR in vitro and as an agonist to upregulate the mRNA expression of AhR in the brain in vivo (Bonefeld-Jørgensen et al. 2007; Nishizawa et al. 2005). Additionally, BPA can also reduce aromatase activity in vitro (Bonefeld-Jørgensen et al. 2007) and the synthesis of testosterone and estradiol in vivo (Akingbemi et al. 2004).

Endocrine-Related Adverse Outcomes of BPA

The growth and development of male genital organs are mainly determined by endogenous androgens. As mentioned above, many molecular- and cellular-level studies have found that BPA can be an antagonist to disrupt AR-mediated signaling pathways. Many in vivo studies on rats elucidated the plausible causal relationship between AR and developmental toxicity. Rats exposed to BPA exhibited a delay in puberty onset, as well as testicular damage and spermatogenesis, which was affected in most treated rats (Tan et al. 2003). It is worth noting that the concentrations of testosterone in plasma did not change significantly in polecats (Nieminen et al. 2002) after exposure to BPA. The results suggest that BPA impacts on the AR and the consequent adverse outcomes may be species-specific.

NRs are also crucial for the development of the nervous system, which has been identified as another target for BPA. However, the adverse outcome of BPA exposure

on the human brain is a relatively new issue and there are only a few relevant studies. In vivo animal studies and epidemiological investigations have underlined the potential relationship between the exposure to BPA, possible receptors and neurobehavioral outcomes in childhood and adults. For instance, the gene encoding aromatase AroB is an estrogen receptor target gene that is expressed in the developing brain. AroB can convert testosterone to estradiol (E2) to regulate numerous developmental processes of the brain. Researchers have found that BPA can induce drastic overexpression of AroB in the brain and that induction is largely through ERs (Chung et al. 2011). Claire et al. (2017) found that BPA was positively associated with relationship problems and hyperactivity-inattention. Perinatal exposure to BPA can affect the corticosterone-regulated actions, resulting in the sex-differential neuroendocrine stress and behavior in rodents (Panagiotidou et al. 2014; Poimenova et al. 2010; Prasanth et al. 2010). Results show that children prenatally exposed to BPA are most likely to be negatively impacted, especially regarding the neurobehavioral function (Evans et al. 2014; Hong et al. 2013; Joe et al. 2011; Kim et al. 2013; Maserejian et al. 2012a, 2012b; Miodovnik et al. 2011; Perera et al. 2012; Philippat et al. 2017; Roen et al. 2015). It is worth noting that the adverse outcomes in the brain and behavior are sex-dependent. In other words, boys and girls may show different and even opposite behaviors after exposure to BPA. For example, after being exposed to BPA during gestation, boys had increased emotional reactivity and aggressive behavior, whereas girls had decreased anxiety/depression and were less aggressive (Perera et al. 2012; Roen et al. 2015).

Early-life exposures to BPA may also have a long-term adverse effect on respiratory health. Recent evidence suggests that prenatal or early postnatal exposure to BPA may be deleterious to the developing immune system, resulting in abnormal respiratory impairments (Kwak et al. 2009). In animal studies (mice), prenatal exposure to BPA has been associated with increased allergic sensitization and bronchial inflammation (Nakajima et al. 2012). In human studies, prenatal exposure to BPA was associated with increased odds of wheeze and asthma in children (Donohue et al. 2013; Gascon et al. 2015a; Ku et al. 2015; Spanier et al. 2012; Spanier et al. 2014). Additionally, an in utero exposure study showed that many phenols, including BPA, tended to be associated with altered respiratory health (Vernet et al. 2017).

Triclosan

Triclosan (TCS), a chlorophenol, is a broad-spectrum antibacterial agent which is widely used in personal care products such as antibacterial soaps and toothpaste (Fang et al. 2010; Witorsch and Thomas 2010). In many studies, TCS has been found in human breast milk (Adolfsson-Erici et al. 2002; Allmyr et al. 2006; Toms et al. 2011), blood (Allmyr et al. 2006, 2008), urine (Heffernan et al. 2015; Philippat et al. 2013; Provencher et al. 2014; Yin et al. 2016), and amniotic fluid (Philippat et al. 2013). In 2016, the U.S. Food and Drug Administration (FDA) banned the antimicrobial TCS for use in soaps (Weatherly and Gosse 2017), although it is still

widely used in toothpaste and mouthwash. Therefore, FDA has nominated TCS to the National Toxicology Program (NTP) for toxicological evaluations and found that it is associated with endocrine-disrupting effects (Fang et al. 2010).

Endocrine-Related Mechanisms

In ER-responsive bioassays based on mammalian cells, TCS exhibited concentration-dependent antagonist activity (Ahn et al. 2008). However, after exposure to TCS, the ER-dependent growth of ovarian cancer cells induced cell proliferation and migration, suggesting that TCS is a xenoestrogen and has ER-related endocrine-disrupting activity (Kim et al. 2014). In another in vitro study with zebrafish cell lines, TCS showed no estrogenic activity (Serra et al. 2018), implying that the species differences play a role in responses to ER-mediated activity. Additionally, experiments with rats showed that TCS greatly bioaccumulated in the placenta of pregnant rats, up-regulated the transcriptional expression levels of E2 and inhibited the production of circulating E2, posing the potential negative effects on rats and their offspring (Feng et al. 2016).

TCS is predicted to be an AR antagonist as a competitive binder (inhibitory concentration at 50% or IC_{50}: 10 μM) by several in silico methods (Kenda et al. 2020). The binding affinity assays confirmed that TCS is a competitive binder for AR (Gee et al. 2008). Many AR-responsive bioassays showed the anti-androgenic effects of TCS with the IC_{50} in the range of 0.7–6.1 μM (Ahn et al. 2008; Chen et al. 2007; Di Paolo et al. 2016; Gee et al. 2008; Kolšek et al. 2015). However, in fish (stickleback) AR transactivation assays, the human anti-androgen TCS had no effect (Lange et al. 2015). This phenomenon indicates the species differences in responses to antiandrogens. Conversely, TCS showed AR agonistic activity in the MDA-kb2 cell line (Christen et al. 2010). TCS also enhanced the dihydrotestosterone (DHT)-dependent activation of AR, and the responsive gene expression reached 180% (Christen et al. 2010). This stimulatory action of the exogenous substrate on the gene expression instead of acting through competition with the receptor's primary binding site may represent a novel mode of action of the endocrine activity (Chen et al. 2008).

Considering the controversial results regarding the TCS-mediated AR disruption, many animal experiments were conducted to elucidate the probable MOA of TCS on AR. However, different in vivo studies with male rats have indicated that TCS exposure can cause contradictory AR disrupting activities. In one study, TCS showed anti-androgenic effects by decreasing testicular weight (Kumar et al. 2009), however, in another study, TCS showed no AR disrupting activity in Hershberger assays which suggested no change in the weight of the accessory sex organs (Farmer et al. 2018). Moreover, it has been demonstrated that TCS can act as an EDC of AR indirectly. TCS decreased the synthesis of DHT (natural androgen) in testicular tissue which resulted in the reduction of sperm production in male rats (Kumar et al. 2009). The decrease of steroidogenesis may be mediated by the decreased synthesis of serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH), thus involving the

hypothalamus-pituitary-gonadal (HPG) axis and can result in an abnormal reproductive system. Furthermore, prostate cancer is a global health concern and is mainly caused by the overexpression of the AR-mediated signaling pathways. TCS was shown to act as a xenoandrogen in LNCaP human prostate cancer cells via AR signaling pathway by mimicking the action of DHT, increasing the induction of cell proliferation and migration (Kim et al. 2015), which suggested the risk of TCS to prostate cancer progression.

Although previous in vitro assays based on zebrafish liver cell line showed that TCS had no activity on THRs (Zhou et al. 2017), the chemical structural similarity to THs, triiodothyronine (T3) and T4 suggests that TCS may have thyroid disrupting properties and may further alter thyroid homeostasis. In fact, many studies claim that TCS can be a direct antagonist for THRs with moderate potency (IC_{50}: 3.61 μM) (Kenda et al. 2020). It is generally accepted that TCS disturbs the thyroid system in different experimental organisms by acting through several pathways in addition to binding to receptors, including altering TH synthesis, metabolism, and transport. Studies that have investigated the effects of TCS on THRs using in vitro and kinetics experiments show that TCS may inhibit iodine uptake in a non-competitive manner and inhibit the activity of thyroid peroxidase, resulting in the decrease of TH synthesis (Wu et al. 2016). However, no significant changes were detected in the expression of genes involved in TH synthesis (Wu et al. 2016). Many in vivo studies have demonstrated the negative effects of TCS on thyroid homeostasis. After treating with TCS, rats exhibited hypothyroidism which was mainly contributed to the decreased activity of thyroid peroxidase in thyroid cells (Zhang et al. 2018). In TCS-treated rats and mice, a significant reduction of T4 serum levels was observed (Crofton et al. 2007; Fang et al. 2015; Farmer et al. 2018; Paul et al. 2010), which implied that TCS may alter circulating concentrations of T4. Perinatal exposure of rats to TCS resulted in decreased circulating T4 concentrations via up-regulation of hepatic catabolism and elimination of T4 (Paul et al. 2012).

AhR is a transcriptional factor that activates gene expression in a ligand-dependent manner. In the AhR-responsive bioassays, TCS exhibited weak agonistic activity (Ahn et al. 2008). Additionally, TCS (0.1–10 μM) significantly enhanced the interaction with ryanodine (RyR1) and stimulated Ca^{2+} mobilization (Ahn et al. 2008). Agonist activities of TCS on both receptors in the brain may contribute to the alteration of neurodevelopment and neuroplasticity function (Juliette et al. 2004).

Endocrine-Related Adverse Outcomes of Triclosan

There is no available data regarding a large population study that would have established a relationship between the developmental neurotoxicity of children and the possible endocrine-disrupting properties of TCS.

Triclocarban (TCC)

Due to the suspected EDC activity, triclocarban (TCC), a TCS analogue, is not recognized as safe for long-term daily use. In 2016, the U.S. FDA also banned the TCC from use in soap (Weatherly and Gosse 2017), but it is now still allowed in some personal care products and as a disinfectant in the health care industry.

Endocrine-Related Molecular Mechanisms of TCC

Similar to TCS, TCC has the ability to amplify the transcriptional activity of endogenous ligands. For example, TCC has been found to enhance estrogenic activity following cellular co-exposure with endogenous estrogens (E2), and enhance the hormone-dependent induction of ER-dependent gene expression (Ahn et al. 2008; Tarnow et al. 2013; Yueh et al. 2012). But TCC shows little or moderate agonist activity in reporter gene assays (Kenda et al. 2020), implying the weak competitive binding potency. Moreover, TCC did not induce the proliferation of E2-dependent MCF-7 cells (Tarnow et al. 2013), confirming that TCC cannot induce estrogenic activity by itself. These in vitro results suggest that TCC is a potential indirect ER agonist that interacts with the ligand-binding domains of ER. Many in vivo tests have been done to further study the endocrine-disrupting activity of TCC on ER. It has been demonstrated that TCC interacts with ERα and leads to the induction of CYP1B1 in ovaries of female mice to further induce promoter activity in vivo (Enright et al. 2017). Also some in vitro assays show that TCC can interact with ERα and promote the induction of human CYP2B6 (Yueh et al. 2012). In aquatic animals, TCC can interfere with ER-relevant signaling pathways and up-regulate estrogen-sensitive vitellogenin (vtg) transcripts, as was demonstrated using male and female fathead minnows (Zenobio et al. 2014). Co-exposure to exogenous estrogen and TCC drastically enhanced the transcriptional expression of AroB in zebrafish embryos (Chung et al. 2011), implying that the amplification of estrogenic activity also happens in vivo. However, single exposure to TCC could only weakly stimulate the expression of AroB (Chung et al. 2011). As mentioned above, aromatase AroB can convert androgens to estrogens in the brain. The negative effects of TCC on AroB by disrupting ER may potentially elevate the level of endogenous estrogens in the developing brain and cause abnormal behavior or reproduction. Interestingly, in the same study, TCC didn't increase AroB transcription mediated by another hormonally active pollutant, BPA, but suppressed it instead (Chung et al. 2011). The study highlighted the potentially unforeseeable effects of chemical mixtures.

Many in vitro assays measuring AR activity showed that TCC and its analogues enhanced AR-responsive gene expression, but exhibited little or no agonistic activity (Ahn et al. 2008; Blake et al. 2010; Chen et al. 2008; Christen et al. 2010; Tarnow et al. 2013). This enhancement of androgenic activity indicated a novel MOA similar to the previously discussed estrogenic activity. For in-depth analysis, the OECD-validated AR-EcoScreen cell line, which is more sensitive to androgens, was used

to identify the MOA of TCC agonist AR with and without DHT (Kenda et al. 2020). Results showed that TCC had no binding affinity for isolated AR, while it induced AR-mediated transcription, which implied that complex interactions underlie TCC's androgenic activity. Additionally, the AR agonistic effects of DHT-combined TCC were blocked by anti-androgen flutamide, which further confirmed the AR-mediated mechanism of action (Christen et al. 2010). It should be noted that although TCC amplified the action of DHT and testosterone in reporter gene assays, TCC itself did not change the endogenous gene expression (Tarnow et al. 2013). The testosterone-induced gene expression amplification effect of TCC was also apparent in in vivo assays, where rats were co-exposed to testosterone and TCC and as a result, castrated rats exhibited significantly increased sex accessory organs (Chen et al. 2008). Furthermore, TCC induced hyperplasia of intact male reproductive organs significantly and potentiated androgen effects in prostate cancer cells via AR-dependent actions (Duleba et al. 2011). However, TCC was found to downregulate the gene expression of AR in male fathead minnows (Zenobio et al. 2014), suggesting species-specific MOA. Still, although the studies using different species indicate differences in AR-disrupting activity for TCC, animal experiments emphasize the existence of potential developmental toxicity of TCS both in humans and wildlife.

In addition to androgenic and estrogenic activities, TCC can potentially affect THRs to disrupt the endocrine system. TCC (1 μM) has been found to show antagonist activities on TR in reporter gene assays (Kenda et al. 2020). Furthermore, TCC (1–5 μM) has been found to have antagonistic activity on GR in reporter gene assays (Kenda et al. 2020; Kolšek et al. 2015), while no agonist activity was found (Yueh et al. 2012). However, TCC did not bind to isolated GR, which indicated that antagonistic activity of GR was not induced through the competitive binding to the ligand-binding domain of GR. Additionally, TCC was shown to promote the activity of constitutive androstane receptor (CAR) in the reporter screening assay and induce the up-regulation of CAR-dependent UDP-glucuronosyltransferase (UGT1A) gene expression in mice liver (Yueh et al. 2012). Exposure of CAR, one of the nuclear xenobiotic receptors, to TCC may have the potential to alter normal xenobiotic metabolism. TCC was shown to disrupt the regulon of the AhR and further co-stimulate the expression of AhR-dependent genes CYP1A1 and CYP1B1 by lowering the transcriptional threshold for both genes in the presence of estrogens (Tarnow et al. 2013). Results show that TCC may be involved in crosstalk between ER and AhR.

Phthalates

Phthalates are high-volume chemicals, extensively used as plasticizers, present in many plastic products such as toys and food packaging (Hauser and Calafat 2005; Koniecki et al. 2011). Among them, di-2-ethylhexyl phthalate (DEHP), diisononyl phthalate (DiNP), butylbenzyl phthalate (BBzP), dibutyl phthalates (DBPs), and diethyl phthalate (DEP) are the highest-produced phthalates (Miodovnik et al. 2014). The widely used phthalates have been frequently detected in the environment and

have become highly concerning organic pollutants (Gao and Wen 2016). One of the reasons for serious health concerns is the fact that phthalates can cross the placental barrier and transfer to the fetus during pregnancy (Ejaredar et al. 2015), potentially inducing adverse outcomes to offspring. Recent studies have established a significant correlation between phthalates and reproduction abnormalities. According to the Registration, Evaluation, Authorization & Restriction of Chemicals (REACH), regulation of the European Union (EU), several phthalates linked to reproductive effects should be prohibited from application in consumer products. As a result, the Socio-Economic Analysis Committee of the European Chemicals Agency voted in 2017 to limit the use of BBzP, DEHP, DBP, and diisobutyl phthalate (DiBP) according to REACH (C&EN 2017a). Additionally, five phthalates, di-n-hexyl phthalate, di-n-pentyl phthalate, dicyclohexyl phthalate, DiBP, and DiNP, are considered to be harmful to male reproductive development. In 2017, the U.S. Consumer Product Safety Commission (CPSC) decided to ban these five substances from toys and items such as teething rings and pacifiers (C&EN 2017b). Furthermore, the U.S. Congress has urged the reduction of phthalates in medical equipment (C&EN 2021). CPSC has concluded that, aside from male reproductive development, phthalates can impair brain development. Therefore, in this section, we summarize endocrine-disrupting activities of typical phthalates and the related MOAs associated with reproductive and/or developmental toxicity in humans.

Endocrine-Related Molecular Mechanisms of Action of Phthalates

According to in vitro tests, four phthalates (DEP, DBP, BBP, DEHP and DiNP) showed no estrogenic effect on ERα(Czernych et al. 2017) but strong anti-estrogenic activity, with IC_{50} of 8.66 μM, 3.61 μM and 0.065 μM for BBP, DEHP and DINP, respectively, was demonstrated (Czernych et al. 2017). Similar to ERα, the four phthalates (DEP, DBP, BBP, DEHP and DiNP) show no significant effect on AR (Czernych et al. 2017), while strong anti-androgenic activity, with IC_{50} of 5.30 μM, 2.87 μM and 0.068 μM for BBP, DEHP, and DINP, respectively, was established (Czernych et al. 2017).

Endocrine-Related Adverse Outcomes of Phthalates

Hormone-disrupting activities were shown to be the most significant effects of phthalates due to the potential of causing different adverse outcomes. Specifically, the potential of phthalates to induce developmental and reproductive toxicity is one of the most concerning issues. A report of the National Toxicology Program of the U.S. Department of Health and Human Services reviewed the reproductive and developmental effects of seven phthalates, DiNP (Kavlock et al. 2002a), di-isodecyl phthalate (DiDP) (Kavlock et al. 2002b), di-n-octyl phthalate (DnOP) (Kavlock et al. 2002c), DEHP (Kavlock et al. 2002d), di-n-butyl phthalate (DnBP) (Kavlock et al. 2002e), di-n-hexyl phthalate (DnHP) (Kavlock et al. 2002f), and butyl benzyl phthalate (BBP)

(Kavlock et al. 2002g). The expert group collected as many animal experiments as available. The results showed that the assessed phthalates are endocrine-disrupting compounds, which can seriously impact the development of animals. For example, in rat and mouse experiments, upon oral administration of DBP to pregnant animals, the fetal loss was the most significant effect, while increased fetal external, skeletal, and visceral abnormalities were secondary (Kavlock et al. 2002e). Additionally, the adverse effects of offspring included sexual differences. Female sexual development was not affected, while the abnormalities in male sexual development included hypospadias, epididymis and seminal vesicle hypoplasia and missing or undescended testes. Since the development of male sexual organs such as testes and seminal vesicles are controlled by androgen, the anti-androgen activity of DBP may most strongly affect prenatal males, compared to juveniles or adults. Also DEHP was shown to induce anti-androgen effects and lead to male genital tract malformation in rats (Christiansen et al. 2010; Moore et al. 2001). The effects of DEHP on the sexual behavior of male rodents were also studied by assessing the anti-androgenic activity and the results demonstrated that DEHP was considerably more toxic to the male reproductive system than DBP (Andrade et al. 2006a; Dalsenter et al. 2006; Moore et al. 2001).

Several phthalates with endocrine-disrupting activities have also been associated with children's neurodevelopment and behavior. Data from epidemiological studies indicate that exposure to phthalates can lead to neurodevelopmental disorders in children and increase the risk of autism, attention deficit and other diseases. The adverse effects of phthalates such as DEHP, DBPs, DEP and BBzP on children have been systematically reviewed (Ejaredar et al. 2015; Zhang et al. 2019). The results showed that prenatal exposure to these phthalates would affect children's behavior, attention, visual-spatial abilities, and social responsiveness. Additionally, gender-specific adverse outcomes of neurodevelopment in children have been reported. Prenatal exposure to phthalate metabolites, mono butyl phthalate (MBP) and mono-benzyl phthalate (MBzP), was positively correlated with impaired behavior, interpersonal relationships and emotional symptoms in male infants (Philippat et al. 2017). Moreover, the concentrations of metabolites such as DBP (Engel et al. 2010; Factor-Litvak et al. 2014; Hyland et al. 2019), DEHP (Lien et al. 2015), DiBP (Kobrosly et al. 2014) and BBzP (Factor-Litvak et al. 2014) have also been positively correlated with the problematic behavior of boys. Although the results of neurobehavioral studies are not always consistent (Braun et al. 2014; Gascon et al. 2015b; Hyland et al. 2019; Shin et al. 2018), the differences do not affect the conclusion that phthalates and/or their metabolites may cause impaired neurodevelopment. In vivo experiments have been conducted to elucidate the relationship between phthalate exposure and neurodevelopmental toxicity, and the results are generally consistent with the observations from epidemiological studies (Gore et al. 2019), indicating sex-specific outcomes. From in vitro tests, it is known that many phthalates have anti-androgenic activity at the cellular level. Many in vivo tests also show that phthalates inhibit fetal testosterone production by disrupting the organization and function of the HPG axis which results in sex-specific neurobehavioral outcomes (Engel et al. 2021; Gore et al. 2019; Kougias et al. 2018). However, researchers have found that DEHP exposure disrupted

the courtship behavior of adult male mice by inducing neural AR downregulation in the nucleus and upstream chemosensory regions instead of causing traditional anti-androgenic effects on HPG (Dombret et al. 2017). The study demonstrated that the disruption of sexual behavior by DEHP can also occur via the effects on neural mechanisms (such as neural AR expression). In addition to androgens (testosterone), estrogens play an important role in brain development (Diotel et al. 2018). Phthalates have been shown to interfere also with the estrogen signaling pathway by suppressing the activity of aromatase, which can modulate estrogen synthesis and result in neurotoxicity (Andrade et al. 2006b; Diotel et al. 2018). Disruption of THR pathway is another potential mechanism to explain the results of the epidemiological and in vivo studies (Miodovnik et al. 2014) because THs are essential for brain development. Lastly, there are many potential non-endocrine mechanisms that have been associated with phthalate exposure and could impair the neurodevelopment of children, such as disruption of ion homeostasis, peroxisome proliferator-activated receptors activation, and lipid metabolism (Engel et al. 2021). However, the relevant mechanisms are still unclear.

There are also concerns over the long-term adverse effects of phthalates on respiratory health. Experimental evidence suggests that phthalates such as DEHP, DiNP, DiDP, and BBzP or their monoester metabolites can penetrate the placental barrier and may have allergenic properties and induce respiratory arrest (Bornehag and Nanberg 2010; Kwak et al. 2009; Whyatt et al. 2014). Only a few epidemiological studies have shown that metabolites of certain high-molecular-weight phthalates, including BBzP, MBzP, DiNP, and DEHP can cause increased rates of asthma, wheezing and respiratory infection (Gascon et al. 2015a; Smit et al. 2015; Whyatt et al. 2014). However, no similar phenomenon was observed for the metabolites of low-molecular-weight phthalates (Gascon et al. 2015a).

Pharmaceuticals

Most pharmaceuticals are designed to interact with a target (such as a specific receptor, enzyme, or biological process) in humans and animals to deliver the therapeutic effect.

Endocrine-Disrupting Activity

An increasing number of pharmaceuticals, including human and veterinary drugs, are being detected in the environment (Pope et al. 2009; Williams 2005). In theory, if pharmaceuticals are delivered to the environment, they will interact with wildlife. Organisms that possess these specific targets of the drugs are likely to be affected by the environmental pharmaceuticals and their elicited adverse effects (Ankley et al. 2007). Specifically, most medicines, designed to affect specific biological pathways,

tend to induce endocrine-related bioactivities to organisms at environmentally relevant concentrations. For example, one of the widely recognized consequences of such environmental pharmaceutical actions is the feminization of male fish (World Health Organization 2002). The feminization occurs due to exogenous chemicals acting as endogenic estrogens that activate ERs. The chemicals that induce feminization include synthetic drugs such as 17α-ethinylestradiol (EE2) (Gross-Sorokin et al. 2006). EE2 is a synthetic steroid widely used in contraceptives and it is not surprising that a compound that has a steroidal-like chemical structure can affect fish populations. The phenomenon illustrates the general issue with pharmaceuticals: many drugs are designed to target receptors with unique structures, but they can affect non-target species because of interactions with their receptors with similar properties. For example, 17β-trenbolone is an androgenic drug designed to be used in livestock. However, steroidal-like structures of these chemicals are highly similar to endogenous androgens of fish, resulting in an impact on the reproductive endocrine system of fish in the water (Durhan et al. 2006). These examples illustrate that some pharmaceuticals which target biological pathways that are conserved in most species pose risks associated with endocrine disruption.

Faced with this issue, many studies have been conducted to support the development of regulatory strategies for assessing drugs that potentially have endocrine-disrupting activity. Pharmaceutical industries have also conducted epidemiological studies to explore the outcomes of pharmaceutical exposures on workers involved in the manufacture of pharmaceuticals (Binks 2003; Heron and Pickering 2003; Sussman et al. 2016). Results showed that many drugs, especially steroids, have the capacity to cause cumulative damage. These pharmaceuticals include cytotoxic anticancer drugs and antibiotics which can mimic natural hormones in the human body. Only by accessing the wealth of data from animal tests and clinical trials, it is possible to fully evaluate the impacts of endocrine-disrupting activity of pharmaceuticals in the environment and quantify the extent of the risk they pose to environmental and human health (Brooks 2010; Huggett et al. 2003).

Antibiotic Resistance

Antibiotics are designed to be an effective weapon against pathogenic microorganisms, but the emergence of antibiotic resistance poses a great threat to life. Therefore, antibiotic resistance has become a global health concern (Brown and Wright 2016). In 1998, the World Health Organization (WHO) identified the emergence of antimicrobial resistance as one of the serious concerns of health policies in the future. In 2012, experts have identified the top 20 key outstanding issues regarding the effects of PPCPs on human and ecological health, and “antibiotic resistance” as one of the key issues (Boxall et al. 2012). In 2016, to tackle the global rising threat of drug-resistant bacteria, the U.S. Department of Health & Human Services, the U.K.’s Antimicrobial Resistance (AMR) Centre, Boston University and others formed a global non-profit partnership dedicated to accelerating antibacterial research: “Combating

Antibiotic-Resistant Bacteria Biopharmaceutical Accelerator" (CARB-X) (C&EN 2016, 2018). Many studies have found that antibacterial drugs in the environment may potentially select for antimicrobial resistance genes (Byrne-Bailey et al. 2008; Gaze et al. 2011; Knapp et al. 2010). There are three essential pathways by which bacteria can increase their resistance (Petchiappan and Chatterji 2017). In brief, (i) decreased influx or increased efflux of antibiotics. Bacteria can reduce the entry of antibiotics into the cell and activate the export of antibiotics from the cell, lowering the effectiveness of antibiotics. (ii) Modification of antibiotics. If antibiotics enter the cell, the resistant bacteria can synthesize enzymes to degrade them or modify their chemical structure so that they cannot bind to their target. (iii) Modification of the antibiotic target. Bacteria can modify, protect, or mutate their cellular targets to disrupt the binding process of antibiotics with targets. In addition to these pathways, bacteria have acquired multiple mechanisms to survive but the exact pathways are still unclear. Finally, new antibiotics targeting auxiliary pathways are necessary to be designed. Many studies have found that metabolism-related and stress-associated pathways may be ideal approaches in this regard (Petchiappan and Chatterji 2017), but there is still a long way to go to construct effective antibiotics using these new pathways as targets.

Summary

PPCPs have changed the way we live and they are ubiquitous in the environment. Biological activities of most pharmaceuticals and several components of personal care products are consciously designed, however, these biological activities of PPCPs lead to unexpected challenges to humans and wildlife, some of which are well investigated, while others are poorly understood. Endocrine disruption is one of the typical toxicity mechanisms for both pharmaceuticals and components of personal care products, while antibiotic resistance is a concerning issue with antibiotic drugs. This chapter summarized the findings of these major mechanisms of toxicity associated with PPCPs. Results show that PPCPs have profound effects on humans and wildlife, which cannot be adequately characterized because of the diversity of chemical structure and diversity of biological activity. Currently, a large body of experimental and epidemiological information is available on PPCPs in the environment. Only by fully harnessing the available toxicity information about PPCPs the effects and risks of PPCPs can be elucidated.

References

Adolfsson-Erici M, Pettersson M, Parkkonen J et al (2002) Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden. Chemosphere 46:1485–1489

Ahn KC, Zhao B, Chen J et al (2008) In vitro biologic activities of the antimicrobials triclocarban, its analogs, and triclosan in bioassay screens: receptor-based bioassay screens. Environ Health Perspect 116:1203–1210

Akingbemi BT, Sottas CM, Koulova AI et al (2004) Inhibition of testicular steroidogenesis by the xenoestrogen bisphenol A is associated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzyme gene expression in rat leydig cells. Endocrinology 145:592–603

Alejandro JR, Richard AB, Sascha U et al (2009) Occurrence of pharmaceuticals and personal care products in fish: Results of a national pilot study in the united states. Environ Toxicol Chem 28:2587–2597

Allmyr M, Adolfsson-EM MMS et al (2006) Triclosan in plasma and milk from Swedish nursing mothers and their exposure via personal care products. Sci Total Environ 372:87–93

Allmyr M, Harden F, Toms LML et al (2008) The influence of age and gender on triclosan concentrations in Australian human blood serum. Sci Total Environ 393:162–167

Alonso-Magdalena P, Ropero AB, Soriano S et al (2012) Bisphenol-A acts as a potent estrogen via non-classical estrog Alonso-Magdalena en triggered pathways. Mol Cell Endocrinol 355:201–207

Andrade AJM, Grande SW, Talsness CE et al (2006a) A dose response study following in utero and lactational exposure to di-(2-ethylhexyl) phthalate (DEHP): Reproductive effects on adult male offspring rats. Toxicology 228:85–97

Andrade AJM, Grande SW, Talsness CE et al (2006b) A dose–response study following in utero and lactational exposure to di-(2-ethylhexyl)-phthalate (DEHP): Non-monotonic dose–response and low dose effects on rat brain aromatase activity. Toxicology 227:185–192

Ankley GT, Brooks BW, Huggett DB et al (2007) Repeating history: pharmaceuticals in the environment. Environ Sci Technol 41:8211–8217

Binder AM, Corvalan C, Pereira A et al (2018) Prepubertal and pubertal endocrine-disrupting chemical exposure and breast density among chilean adolescents. Cancer Epidemiol Biomarkers Prev 27:1491–1499

Binks SP (2003) Occupational toxicology and the control of exposure to pharmaceutical agents at work. Occup Med 53:363–370

Blake LS, Martinović D, Gray J et al (2010) Characterization of the androgen-sensitive MDA-kb2 cell line for assessing complex environmental mixtures. Environ Toxicol Chem 29:1367–1376

Bonefeld-Jørgensen EC, Long M, Hofmeister MV, Vinggaard AM (2007) Endocrine-disrupting potential of bisphenol A, bisphenol A dimethacrylate, 4-n-nonylphenol, and 4-n-octylphenol in vitro: new data and a brief review. Environ Health Perspect 115:69–76

Bornehag CG, Nanberg E (2010) Phthalate exposure and asthma in children. Int J Androl 33:333–345

Boxall ABA, Rudd MA, Brooks BW et al (2012) Pharmaceuticals and personal care products in the environment: what are the big questions? Environ Health Perspect 120:1221–1229

Braun JM, Kalkbrenner AE, Just AC et al (2014) Gestational exposure to endocrine-disrupting chemicals and reciprocal social, repetitive, and stereotypic behaviors in 4- and 5-year-old children: the HOME study. Environ Health Perspect 122:513–520

Brooks B (2010) Leveraging mammalian pharmaceutical toxicology and pharmacology data to predict chronic fish responses to pharmaceuticals. Toxicol Lett 193:69–78

Brown ED, Wright GD (2016) Antibacterial drug discovery in the resistance era. Nature 529:336–343

Butt CM, Stapleton HM (2013) Inhibition of thyroid hormone sulfotransferase activity by brominated flame retardants and halogenated phenolics. Chem Res Toxicol 26:1692–1702

Byrne-Bailey KG, Gaze WH, Kay P et al (2008) Prevalence of sulfonamide resistance genes in bacterial isolates from manured agricultural soils and pig slurry in the United Kingdom. Antimicrob Agents Chemother 53:696–702

C&EN Global Enterprise (2016) Partnership Targets Antibiotic Resistance. 94:15

C&EN Global Enterprise (2017a) European Union Further Restricts Four Phthalates. 95:15

C&EN Global Enterprise (2017b) U.S. to restrict five phthalates in kids' products. 95:13

C&EN Global Enterprise (2018) $50 million more to fight antibiotic resistance. 96:11

C&EN Global Enterprise (2021) Congress Urges Reduction of Phthalates in Medical Equipment. 99:17
Chen F, Zhou L, Bai Y et al (2014) Sex differences in the adult HPA axis and affective behaviors are altered by perinatal exposure to a low dose of bisphenol A. Brain Res 1571:12–24
Chen J, Ahn KC, Gee NA et al (2007) Antiandrogenic properties of parabens and other phenolic containing small molecules in personal care products. Toxicol Appl Pharmacol 221:278–284
Chen J, Ahn KC, Gee NA et al (2008) Triclocarban enhances testosterone action: a new type of endocrine disruptor? Endocrinology 149:1173–1179
Chen Q, Wang X, Tan H et al (2019) Molecular initiating events of bisphenols on androgen receptor-mediated pathways provide guidelines for in silico screening and design of substitute compounds. Environ Sci Technol Lett 6:205–210
Chen Q, Zhou C, Shi W et al (2020) Mechanistic in silico modeling of bisphenols to predict estrogen and glucocorticoid disrupting potentials. Sci Total Environ 728:138854
Christen V, Crettaz P, Oberli-S A et al (2010) Some flame retardants and the antimicrobials triclosan and triclocarban enhance the androgenic activity in vitro. Chemosphere 81:1245–1252
Christiansen S, Boberg J, Axelstad M et al (2010) Low-dose perinatal exposure to di(2-ethylhexyl) phthalate induces anti-androgenic effects in male rats. Reprod Toxicol 30:313–321
Chung E, Genco MC, Megrelis L et al (2011) Effects of bisphenol A and triclocarban on brain-specific expression of aromatase in early zebrafish embryos. Proc Natl Acad Sci USA 108:17732–17737
Claire, Philippat Dorothy, Nakiwala Antonia M, Calafat Jérémie, Botton Maria, De Agostini Barbara, Heude Rémy, Slama (2017) Prenatal exposure to nonpersistent endocrine disruptors and behavior in boys at 3 and 5 Years. Environmental Health Perspectives 125(9):097014 https://doi.org/10.1289/EHP1314
Crofton KM, Paul KB, DeVito MJ et al (2007) Short-term in vivo exposure to the water contaminant triclosan: Evidence for disruption of thyroxine. Environ Toxicol Pharmacol 24:194–197
Czernych R, Chraniuk M, Zagożdżon P et al (2017) Characterization of estrogenic and androgenic activity of phthalates by the XenoScreen YES/YAS in vitro assay. Environ Toxicol Pharmacol 53:95–104
Déchaud H, Ravard C, Claustrat F et al (1999) Xenoestrogen interaction with human sex hormone-binding globulin (hSHBG). Steroids 64:328–334
Dalsenter PR, Santana GM, Grande SW et al (2006) Phthalate affect the reproductive function and sexual behavior of male Wistar rats. Hum Exp Toxicol 25:297–303
Delfosse V, Grimaldi M, Maire le A et al (2014) Nuclear receptor profiling of bisphenol-A and its halogenated analogues. Vitam Horm 94:229–251
Diamanti-Kandarakis E, Bourguignon JP, Giudice LC et al (2009) Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocr Rev 30:293–342
Diotel N, Charlier TD, Lefebvre d'Hellencourt C et al (2018) Steroid transport, local synthesis, and signaling within the brain: roles in neurogenesis, neuroprotection, and sexual behaviors. Front Neurosci 12:84
Di Paolo C, Kirchner K, Balk FGP et al (2016) Downscaling procedures reduce chemical use in androgen receptor reporter gene assay. Sci Total Environ 571:826–833
Dombret C, Capela D, Poissenot K et al (2017) Neural mechanisms underlying the disruption of male courtship behavior by adult exposure to di(2-ethylhexyl) phthalate in mice. Environ Health Perspect 125:097001
Donohue KM, Miller RL, Perzanowski MS et al (2013) Prenatal and postnatal bisphenol A exposure and asthma development among inner-city children. J Allergy Clin Immunol 131:736–742
Duleba AJ, Ahmed MI, Sun M et al (2011) Effects of triclocarban on intact immature male rat: augmentation of androgen action. Reprod Sci 18:119–127
Durhan EJ, Lambright CS, Makynen EA et al (2006) Identification of metabolites of trenbolone acetate in androgenic runoff from a beef feedlot. Environ Health Perspect 114:65–68
Ejaredar M, Nyanza EC, Ten Eycke K et al (2015) Phthalate exposure and childrens neurodevelopment: A systematic review. Environ Res 142:51–60

Engel SM, Miodovnik A, Canfield RL et al (2010) Prenatal phthalate exposure is associated with childhood behavior and executive functioning. Environ Health Perspect 118:565–571
Engel SM, Patisaul HB, Brody C et al (2021) Neurotoxicity of ortho-phthalates: recommendations for critical policy reforms to protect brain development in children. Am J Public Health 111:687–695
Enright HA, Falso MJS, Malfatti MA et al (2017) Maternal exposure to an environmentally relevant dose of triclocarban results in perinatal exposure and potential alterations in offspring development in the mouse model. PLoS ONE 12:17
Evans SF, Kobrosly RW, Barrett ES et al (2014) Prenatal bisphenol A exposure and maternally reported behavior in boys and girls. Neurotoxicology 45:91–99
Factor-Litvak P, Insel B, Calafat AM et al (2014) Persistent associations between maternal prenatal exposure to phthalates on child IQ at age 7 years. PLoS One 9:e114003
Fang JL, Stingley RL, Beland FA et al (2010) Occurrence, efficacy, metabolism, and toxicity of triclosan. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 28:147–171
Fang JL, Vanlandingham M, Juliar BE et al (2015) Dose-response assessment of the dermal toxicity of triclosan in B6C3F1 mice. Toxicol Res 4:867–877
Farmer WT, Louis GW, Buckalew AR et al (2018) Evaluation of triclosan in the Hershberger and H295R steroidogenesis assays. Toxicol Lett 291:194–199
Feng Y, Zhang P, Zhang Z et al (2016) Endocrine disrupting effects of triclosan on the placenta in pregnant rats. PLoS One 11:e0154758
Gao DW, Wen ZD (2016) Phthalate esters in the environment: A critical review of their occurrence, biodegradation, and removal during wastewater treatment processes. Sci Total Environ 541:986–1001
Gascon M, Casas M, Morales E et al (2015a) Prenatal exposure to bisphenol A and phthalates and childhood respiratory tract infections and allergy. J Allergy Clin Immunol 135:370–378
Gascon M, Valvi D, Forns J et al (2015b) Prenatal exposure to phthalates and neuropsychological development during childhood. Int J Hyg Environ Health 218:550–558
Gaze WH, Zhang L, Abdouslam NA et al (2011) Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment. ISME J 5:1253–1261
Gee RH, Charles A, Taylor N et al (2008) Oestrogenic and androgenic activity of triclosan in breast cancer cells. J Appl Toxicol 28:78–91
Gentilcore D, Porreca I, Rizzo F et al (2013) Bisphenol A interferes with thyroid specific gene expression. Toxicology 304:21–31
Ghassabian A, Trasande L (2018) Disruption in thyroid signaling pathway: a mechanism for the effect of endocrine-disrupting chemicals on child neurodevelopment. Front Endocrinol 9:204
Gore A (2010) Neuroendocrine targets of endocrine disruptors. Hormones 9:16–27
Gore AC, Krishnan K, Reilly MP (2019) Endocrine-disrupting chemicals: effects on neuroendocrine systems and the neurobiology of social behavior. Horm Behav 111:7–22
Gross-Sorokin MY, Roast SD, Brighty GC (2006) Assessment of feminization of male fish in English rivers by the Environment Agency of England and Wales. Environ Health Perspect 114:147–151
Hauser R, Calafat AM (2005) Phthalates and human health. Occup Environ Med 62:806–818
Heffernan AL, Baduel C, Toms LML et al (2015) Use of pooled samples to assess human exposure to parabens, benzophenone-3 and triclosan in Queensland, Australia. Environ Int 85:77–83
Heron RJ, Pickering FC (2003) Health effects of exposure to active pharmaceutical ingredients (APIs). Occup Med 53:357–362
Hirsch R, Ternes T, Haberer K et al (1999) Occurrence of antibiotics in the aquatic environment. Sci Total Environ 225:109–118
Hong SB, Hong YC, Kim JW et al (2013) Bisphenol A in relation to behavior and learning of school-age children. J Child Psychol Psychiatry 54:890–899
Huggett DB, Cook JC, Ericson JF et al (2003) A theoretical model for utilizing mammalian pharmacology and safety data to prioritize potential impacts of human pharmaceuticals to fish. Aquat Toxicol 9:1789–1799

Hyland C, Mora AM, Kogut K et al (2019) Prenatal exposure to phthalates and neurodevelopment in the CHAMACOS cohort. Environ Health Perspect 127:107010

Joe MB, Amy EK, Antonia MC et al (2011) Impact of early-life bisphenol A exposure on behavior and executive function in children. Pediatrics 128:873–882

Jenkins S, Wang J, Eltoum I et al (2011) Chronic oral exposure to bisphenol A results in a nonmonotonic dose response in mammary carcinogenesis and metastasis in MMTV-erbB2 mice. Environ Health Perspect 119:1604–1609

Juliette G, Wong PW, Pessah IN (2004) Non-coplanar 2,2′,3,5′,6-pentachlorobiphenyl (PCB 95) amplifies ionotropic glutamate receptor signaling in embryonic cerebellar granule neurons by a mechanism involving ryanodine receptors. Toxicol Sci 77:72–82

Kavlock R, Boekelheide K, Chapin R et al (2002a) NTP center for the evaluation of risks to human reproduction: phthalates expert panel report on the reproductive and developmental toxicity of di-isononyl phthalate. Reprod Toxicol 16:679–708

Kavlock R, Boekelheide K, Chapin R et al (2002b) NTP center for the evaluation of risks to human reproduction: phthalates expert panel report on the reproductive and developmental toxicity of di-isodecyl phthalate. Reprod Toxicol 16:655–678

Kavlock R, Boekelheide K, Chapin R et al (2002c) NTP center for the evaluation of risks to human reproduction: phthalates expert panel report on the reproductive and developmental toxicity of di-n-octyl phthalate. Reprod Toxicol 16:721–734

Kavlock R, Boekelheide K, Chapin R et al (2002d) NTP center for the evaluation of risks to human reproduction: phthalates expert panel report on the reproductive and developmental toxicity of di(2-ethylhexyl) phthalate. Reprod Toxicol 16:529–653

Kavlock R, Boekelheide K, Chapin R et al (2002e) NTP center for the rvaluation of risks to human reproduction: phthalates exper $50 million more to fight antibiotic resistance.t panel report on the reproductive and developmental toxicity of di-n-butyl phthalate. Reprod Toxicol 16:489–527

Kavlock R, Boekelheide K, Chapin R et al (2002f) NTP center for the evaluation of risks to human reproduction: phthalates expert panel report on the reproductive and developmental toxicity of di-n-hexyl phthalate. Reprod Toxicol 16:709–719

Kavlock R, Boekelheide K, Chapin R et al (2002g) NTP center for the evaluation of risks to human reproduction: phthalates expert panel report on the reproductive and developmental toxicity of butyl benzyl phthalate. Reprod Toxicol 16:453–487

Kenda M, Kuželički NK, Iida M et al (2020) Triclocarban, triclosan, bromochlorophene, chlorophene, and climbazole effects on nuclear receptors: an in silico and in vitro study. Environ Health Perspect 128:107005

Kim GH, Robert BG, Katherine K et al (2013) Prenatal and early childhood bisphenol A concentrations and behavior in school-aged children. Environ Res 126:43–50

Kim JY, Yi BR, Go RE et al (2014) Methoxychlor and triclosan stimulates ovarian cancer growth by regulating cell cycle- and apoptosis-related genes via an estrogen receptor-dependent pathway. Environ Toxicol Pharmacol 37:1264–1274

Kim SH, Hwang KA, Shim SM et al (2015) Growth and migration of LNCaP prostate cancer cells are promoted by triclosan and benzophenone-1 via an androgen receptor signaling pathway. Environ Toxicol Pharmacol 39:568–576

Knapp CW, Dolfing J, Ehlert PAI et al (2010) Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. Environ Sci Technol 44:580–587

Kobrosly RW, Evans S, Miodovnik A et al (2014) Prenatal phthalate exposures and neurobehavioral development scores in boys and girls at 10 years of age. Environ Health Perspect 122:521–528

Kolpin DW, Furlong ET, Meyer MT et al (2002) Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999–2000: a national reconnaissance. Environ Sci Technol 36:1202–1211

Kolšek K, Gobec M, Mlinarič Raščan I et al (2015) Screening of bisphenol A, triclosan and paraben analogues as modulators of the glucocorticoid and androgen receptor activities. Toxicol in Vitro 29:8–15

Koniecki D, Wang R, Moody RP et al (2011) Phthalates in cosmetic and personal care products: Concentrations and possible dermal exposure. Environ Res 111:329–336
Kougias DG, Sellinger EP, Willing J et al (2018) Perinatal exposure to an environmentally relevant mixture of phthalates results in a lower number of neurons and synapses in the medial prefrontal cortex and decreased cognitive flexibility in adult male and female rats. J Neurosci 38:6864–6872
Krishnan AV, Stathis P, Permuth SF et al (1993) Bisphenol A: an estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology 132:2279–2286
Ku HY, Su PH, Wen HJ et al (2015) Prenatal and postnatal exposure to phthalate esters and asthma: a 9-year follow-up study of a Taiwanese birth cohort. PLoS ONE 10:e0123309–e0123309
Kumar V, Chakraborty A, Kural MR et al (2009) Alteration of testicular steroidogenesis and histopathology of reproductive system in male rats treated with triclosan. Reprod Toxicol 27:177–185
Kurosawa T, Hiroi H, Tsutsumi O et al (2002) The activity of bisphenol A depends on both the estrogen receptor subtype and the cell type. Endocr J 49:465–471
Kwak ES, Just A, Whyatt R et al (2009) Phthalates, pesticides, and bisphenol-A exposure and the development of nonoccupational asthma and allergies: how valid are the links? Open Allergy J 2:45–50
Lange A, Sebire M, Rostkowski P et al (2015) Environmental chemicals active as human antiandrogens do not activate a stickleback androgen receptor but enhance a feminising effect of oestrogen in roach. Aquat Toxicol 168:48–59
Leung YK, Govindarajah V, Cheong A et al (2017) Gestational high-fat diet and bisphenol A exposure heightens mammary cancer risk. Endocr Relat Cancer 24:365–378
Lien YJ, Ku HY, Su PH et al (2015) Prenatal exposure to phthalate esters and behavioral syndromes in children at 8 years of age: Taiwan maternal and infant cohort study. Environ Health Perspect 123:95–100
Ma Y, Liu H, Wu J et al (2019) The adverse health effects of bisphenol A and related toxicity mechanisms. Environ Res 176:108575
Maserejian NN, Trachtenberg FL, Hauser R et al (2012a) Dental composite restorations and neuropsychological development in children: Treatment level analysis from a randomized clinical trial. Neurotoxicology 33:1291–1297
Maserejian NN, Trachtenberg FL, Hauser R et al (2012b) Dental composite restorations and psychosocial function in children. Pediatrics 130:328–338
Matsushima A, Kakuta Y, Teramoto T et al (2007) Structural evidence for endocrine disruptor bisphenol A binding to human nuclear receptor ERRγ. J Biochem 142:517–524
Miodovnik A, Engel SM, Zhu C et al (2011) Endocrine disruptors and childhood social impairment. Neurotoxicology 32:261–267
Miodovnik A, Edwards A, Bellinger DC et al (2014) Developmental neurotoxicity of ortho-phthalate diesters: review of human and experimental evidence. Neurotoxicology 41:112–122
Molina-Molina JM, Amaya E, Grimaldi M et al (2013) In vitro study on the agonistic and antagonistic activities of bisphenol-S and other bisphenol-A congeners and derivatives via nuclear receptors. Toxicol Appl Pharmacol 272:127–136
Monteiro SC, Boxall A (2009) Factors affecting the degradation of pharmaceuticals in agricultural soils. Environ Toxicol Chem 28:2546–2554
Moore RW, Rudy TA, Lin TM et al (2001) Abnormalities of sexual development in male rats with in utero and lactational exposure to the antiandrogenic plasticizer Di(2-ethylhexyl) phthalate. Environ Health Perspect 109:229–237
Moriyama K, Tagami T, Akamizu T et al (2002) Thyroid hormone action is disrupted by bisphenol A as an antagonist. J Clin Endocrinol Metab 87:5185–5190
Nakajima Y, Goldblum RM, Midoro-Horiuti T (2012) Fetal exposure to bisphenol A as a risk factor for the development of childhood asthma: an animal model study. Environ Health 11:8
Nieminen P, Lindström-Seppä P, Juntunen M et al (2002) In vivo effects of bisphenol A on the polecat (mustela putorius). J Toxicol Environ Health A 65:933–945

Nishizawa H, Imanishi S, Manabe N (2005) Effects of exposure in utero to bisphenol A on the expression of aryl hydrocarbon receptor, related factors, and xenobiotic metabolizing enzymes in murine embryos. J Reprod Dev 51:593–605

Panagiotidou E, Zerva S, Mitsiou DJ et al (2014) Perinatal exposure to low-dose bisphenol A affects the neuroendocrine stress response in rats. J Endocrinol 220:207–218

Paul KB, Hedge JM, Devito MJ et al (2010) Short-term exposure to triclosan decreases thyroxine in vivo via upregulation of hepatic catabolism in young long-evans rats. Toxicol Sci 113:367–379

Paul KB, Hedge JM, Bansal R et al (2012) Developmental triclosan exposure decreases maternal, fetal, and early neonatal thyroxine: A dynamic and kinetic evaluation of a putative mode-of-action. Toxicology 300:31–45

Perera F, Vishnevetsky J, Herbstman JB et al (2012) Prenatal bisphenol A exposure and child behavior in an inner-city cohort. Environ Health Perspect 120:1190–1194

Petchiappan A, Chatterji D (2017) Antibiotic resistance: current perspectives. ACS. Omega 2:7400–7409

Philippat C, Wolff MS, Calafat AM et al (2013) Prenatal exposure to environmental phenols: concentrations in amniotic fluid and variability in urinary concentrations during pregnancy. Environ Health Perspect 121:1225–1231

Philippat C, Nakiwala D, Calafat AM et al (2017) Prenatal exposure to nonpersistent endocrine disruptors and behavior in boys at 3 and 5 years. Environ Health Perspect 125:097014

Poimenova A, Markaki E, Rahiotis C et al (2010) Corticosterone-regulated actions in the rat brain are affected by perinatal exposure to low dose of bisphenol A. Neuroscience 167:741–749

Pope L, Boxall A, Corsing C et al (2009) Exposure assessment of veterinary medicines in terrestrial systems. In: Crane M, Boxall ABA, Barrett K (eds) Veterinary Medicines in the Environment. CRC Press, Boca Raton, p 224

Prasanth GK, Divya LM, Sadasivan C (2010) Bisphenol-A can bind to human glucocorticoid receptor as an agonist: an in silico study. J Appl Toxicol 30:769–774

Prins GS, Patisaul HB, Belcher SM et al (2019) CLARITY-BPA academic laboratory studies identify consistent low-dose Bisphenol A effects on multiple organ systems. Basic Clin Pharmacol Toxicol 25:14–31

Provencher G, Bérubé R, Dumas P et al (2014) Determination of bisphenol A, triclosan and their metabolites in human urine using isotope-dilution liquid chromatography–tandem mass spectrometry. J Chromatogr A 1348:97–104

Rochester JR (2013) Bisphenol A and human health: a review of the literature. Reprod Toxicol 42:132–155

Roen EL, Wang Y, Calafat AM et al (2015) Bisphenol A exposure and behavioral problems among inner city children at 7–9 years of age. Environ Res 142:739–745

Roy P, Salminen H, Koskimies P et al (2004) Screening of some anti-androgenic endocrine disruptors using a recombinant cell-based in vitro bioassay. J Steroid Biochem Mol Biol 88:157–166

Rubin BS (2011) Bisphenol A: an endocrine disruptor with widespread exposure and multiple effects. J Steroid Biochem Mol Biol 127:27–34

Serra H, Brion F, Porcher JM et al (2018) Triclosan lacks (anti-)estrogenic effects in zebrafish cells but modulates estrogen response in zebrafish embryos. Int J Mol Sci 19:1175

Sheng ZG, Tang Y, Liu YX et al (2012) Low concentrations of bisphenol a suppress thyroid hormone receptor transcription through a nongenomic mechanism. Toxicol Appl Pharmacol 259:133–142

Shigeyuki K, Tomoharu S, Seigo S et al (2005) Comparative study of the endocrine-disrupting activity of bisphenol A and 19 related compounds. Toxicol Sci 84:249–259

Shin HM, Schmidt RJ, Tancredi D et al (2018) Prenatal exposure to phthalates and autism spectrum disorder in the MARBLES study. Environ Health 17:85

Smit LAM, Lenters V, Høyer BB et al (2015) Prenatal exposure to environmental chemical contaminants and asthma and eczema in school-age children. Allergy 70:653–660

Spanier AJ, Kahn RS, Kunselman AR et al (2012) Prenatal exposure to bisphenol A and child wheeze from birth to 3 years of age. Environ Health Perspect 120:916–920

Spanier AJ, Kahn RS, Kunselman AR et al (2014) Bisphenol A exposure and the development of wheeze and lung function in children through age 5 years. JAMA Pediatr 168:1131–1137
Sussman RG, Schatz AR, Kimmel TA et al (2016) Identifying and assessing highly hazardous drugs within quality risk management programs. Regul Toxicol Pharmacol 79:S11–S18
Takayanagi S, Tokunaga T, Liu X et al (2006) Endocrine disruptor bisphenol A strongly binds to human estrogen-related receptor γ (ERRγ) with high constitutive activity. Toxicol Lett 167:95–105
Takeda Y, Liu X, Sumiyoshi M et al (2009) Placenta expressing the greatest quantity of bisphenol A receptor ERRγ among the human reproductive tissues: predominant expression of type-1 ERRγ isoform. J Biochem 146:113–122
Takeuchi T, Tsutsumi O (2002) Serum bisphenol A concentrations showed gender differences, possibly linked to androgen levels. Biochem Biophys Res Commun 291:76–78
Tan BL, Kassim NM, Mohd MA (2003) Assessment of pubertal development in juvenile male rats after sub-acute exposure to bisphenol A and nonylphenol. Toxicol Lett 143:261–270
Tarnow P, Tralau T, Hunecke D et al (2013) Effects of triclocarban on the transcription of estrogen, androgen and aryl hydrocarbon receptor responsive genes in human breast cancer cells. Toxicol in Vitro 27:1467–1475
Teng C, Goodwin B, Shockley K et al (2013) Bisphenol A affects androgen receptor function via multiple mechanisms. Chem Biol Interact 203:556–564
Thomas P, Dong J (2006) Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: A potential novel mechanism of endocrine disruption. J Steroid Biochem Mol Biol 102:175–179
Tohmé M, Prud'homme SM, Boulahtouf A et al (2014) Estrogen-related receptor γ is an in vivo receptor of bisphenol A. FASEB J 28:3124–3133
Toms LML, Allmyr M, Mueller JF et al (2011) Triclosan in individual human milk samples from Australia. Chemosphere 85:1682–1686
Vandenberg LN (2014) Non-monotonic dose responses in studies of endocrine disrupting chemicals: bisphenol a as a case study. Dose Response 12:259–276
Vernet C, Pin I, Giorgis-Allemand L et al (2017) In utero exposure to select phenols and phthalates and respiratory health in five-year-old boys: a prospective study. Environ Health Perspect 125:097006
Villar-Pazos S, Martinez-Pinna J, Castellano-Muñoz M et al (2017) Molecular mechanisms involved in the non-monotonic effect of bisphenol-a on Ca2+ entry in mouse pancreatic β-cells. Sci Rep 7:11770
Watson CS, Bulayeva NN, Wozniak AL et al (2007) Xenoestrogens are potent activators of nongenomic estrogenic responses. Steroids 72:124–134
Wazir U, Mokbel K (2019) Bisphenol A: a concise review of literature and a discussion of health and regulatory implications. In Vivo 33:1421–1423
Weatherly LM, Gosse JA (2017) Triclosan exposure, transformation, and human health effects. J Toxicol Environ Health B Crit Rev 20:447–469
Welshons WV, Nagel SC, Saal FSV (2006) Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure. Endocrinology 147:S56-69
Wetherill YB, Alejandro BT, Kanno J et al (2007) In vitro molecular mechanisms of bisphenol A action. Reprod Toxicol 24:178–198
World Health Organization (2002) International programme on chemical safety. Assessment of the state-of-the-science of endocrine disruptors, Geneva. Available: http://www.who.int/ipcs/publications/new_issues/endocrine_disruptors/en/
Whyatt RM, Perzanowski MS, Just AC et al (2014) Asthma in inner-city children at 5 years of age and prenatal exposure to phthalates: the Columbia center for children's environmental health cohort. Environ Health Perspect 122:1141–1146
Williams RT (2005) Human health pharmaceuticals in the environment-an introduction

Witorsch RJ, Thomas JA (2010) Personal care products and endocrine disruption: a critical review of the literature. Crit Rev Toxicol 40:1–30

Wu Y, Beland FA, Fang JL (2016) Effect of triclosan, triclocarban, 2,2',4',-tetrabromodiphenyl ether, and bisphenol A on the iodide uptake, thyroid peroxidase activity, and expression of genes involved in thyroid hormone synthesis. Toxicol in Vitro 32:310–319

Yin J, Wei L, Shi Y et al (2016) Chinese population exposure to triclosan and triclocarban as measured via human urine and nails. Environ Geochem Health 38:1125–1135

Yueh MF, Li T, Evans RM et al (2012) Triclocarban mediates induction of xenobiotic metabolism through activation of the constitutive androstane receptor and the estrogen receptor alpha. PLoS One 7:e37705

Zenobio JE, Sanchez BC, Archuleta LC et al (2014) Effects of triclocarban, N, N-diethyl-meta-toluamide, and a mixture of pharmaceuticals and personal care products on fathead minnows (Pimephales promelas). Environ Toxicol Chem 33:910–919

Zhang P, Yang M, Zeng L et al (2018) P38/TRHr-dependent regulation of TPO in thyroid cells contributes to the hypothyroidism of triclosan-treated rats. Cell Physiol Biochem 45:1303–1315

Zhang Q, Chen XZ, Huang X et al (2019) The association between prenatal exposure to phthalates and cognition and neurobehavior of children-evidence from birth cohorts. Neurotoxicology 73:199–212

Zhou Z, Yang J, Chan KM (2017) Toxic effects of triclosan on a zebrafish (Danio rerio) liver cell line, ZFL. Aquat Toxicol 191:175–188

Zoeller RT, Bansal R, Parris C (2005) Bisphenol-A, an environmental contaminant that acts as a thyroid hormone receptor antagonist in vitro, increases serum thyroxine, and alters RC3/neurogranin expression in the developing rat brain. Endocrinology 146:607–612

Zsarnovszky A, Le HH, Wang HS et al (2005) Ontogeny of rapid estrogen-mediated extracellular signal-regulated kinase signaling in the rat cerebellar cortex: potent nongenomic agonist and endocrine disrupting activity of the xenoestrogen bisphenol A. Endocrinology 146:5388–5396

Chapter 12
Mechanisms of Action of Emerging Contaminants: Disinfection Byproducts

Ting Xu and Daqiang Yin

Abstract Although disinfection byproducts (DBPs) can be detected in surface water, most of these emerging contaminants exist in the drinking water system, which means their long-term risks to human health are much greater than to wildlife. About 700 types of DBPs have gradually been identified and confirmed in drinking water or reclaimed water, however, this is believed to be just the tip of the iceberg for numerous unknown members of the family. Multiple experimental models including cell lines, rodents, and zebrafish are employed in toxicological studies to uncover the adverse effects induced by DBPs, but only a fraction of DBPs have been regulated by the standards of public health authorities. Furthermore, current data indicates that some unregulated DBPs which are not regulated or even discovered may pose more severe threats than regulated ones. In general, studies concerning the toxicity and health risks of DBPs need to provide more data for the assessment and regulation, and more efforts are also required for understanding their underlying mechanisms.

Introduction

A number of disinfectants, such as chlorine, chloramine, chlorine dioxide, ozone and ultraviolet radiation are applied to water supply systems. Chlorine is the most widely-applied disinfectant at drinking water treatment plants (DWTPs) due to its cost-effectiveness. Chlorination disinfection is a reliable and efficient way to remove pathological bacteria and viruses in drinking water, but it produces multiple carbonaceous disinfection byproducts (C-DBPs) primarily including trihalomethanes (THMs) and haloacetic acids (HAAs). After THMs and HAAs along with bromate and chlorite were identified as DBPs and reported in the drinking water system, the regulation and control of the generation of DBPs have been urgent topics in drinking water safety (Xie 2004). THMs and HAAs account for

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nearly 25% of all halogenated DBPs of which chloroform, bromodichloromethane, dichloroacetic acid, and trichloroacetic acid have been classified as contaminants possibly carcinogenic to humans (Group 2B) by the International Agency for Research on Cancer (IARC 1999, 2004, 2014). To reduce the yields of C-DBPs, chloramination disinfection was implemented. At the same time, because of the introduction of excess nitrogen, various nitrogenous functional groups including amino, cyano, and nitro groups are formed during the process and the amounts of nitrogenous DBPs (N-DBPs) significantly increased. Ironically, some emerging DBPs, though not yet regulated, compared with the dominant chlorinated C-DBPs, N-DBPs are now generally considered to possess higher risks to human health (Muellner et al. 2007), for example, N-nitrosodimethylamine (NDMA) and halonitromethanes (HNMs). The legacy and emerging DBPs are associated with diseases such as bladder and colon cancer, asthma, irritation of the eyes and mucous membrane, and reproductive function. Thus, the health effects of DBPs cannot be ignored. This chapter mainly introduces the environmental toxicology research and potential mechanism of key DBPs, to provide a theoretical basis for the assessment and supervision of DBPs toxicity and health risks.

Haloacetamides

One major group of compounds produced during the water disinfection process are haloacetamides (HAcAms). Studies have shown that regardless of the disinfection type (chlorination or chloramination), the order of production of HAcAms with different halogenation degrees by quantity was di-HAcAms» tri-HAcAms > mono-HAcAms and the order of production of HAcAms with different types of halogenation was Cl-HAcAms» Cl-Br-HAcAms > Br-HAcAms (Yang et al. 2014). Overall, the concentration of HAcAms produced by chloramination was higher than that produced by chlorination, even when the production of THMs was lower in the case of chloramination (Chu et al. 2013). The different DBPs could potentially induce different levels of toxicity and health outcomes, which require appropriate toxicological methodologies for characterization.

Influenced by the pioneering works of Dr. Plewa's team, the toxicological studies of DBPs have adapted *in vitro* cellular tests (Kargalioglu et al. 2002; Plewa et al. 2002, 2004, 2008), which, compared with conventional animal experiments, have the advantages of being high-throughput, having a relatively short test period and no conflicts in animal ethics. The establishment of a mature system for cell lines saves much laborious work in primary cells, which is beneficial to the further popularization of the tests. The range of different cellular models employed in DBP toxicity testing is broad, including, for example, Chinese hamster ovary (CHO) cells and human embryonic kidney (HEK293) cells. The toxicological tests so far have mainly focused on cytotoxicity and genotoxicity. Only a few *in vivo* studies have been conducted to assess the developmental toxicity of HAcAms (Ding et al. 2020), but these are not described in detail in this chapter.

Cytotoxicity

Cytotoxicity tests can indicate the impacts of DBPs on cellular physiology including cell proliferation, cell cycle, and cell death. The commonly used tests for cell proliferation include 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay), 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT assay), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS assay) and 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8 assay). The latter (WST-8) assay is gaining popularity due to its higher sensitivity compared to other tetrazolium salts such as MTT, XTT or MTS and its solubility in the culture medium. However, researchers should exercise caution when comparing the results of the WST-8 assay to the results obtained with other tetrazolium salts as there may be differences due to the varying levels of sensitivities of the dies.

Thirteen different HAcAms, which included chlorinated (Cl-), brominated (Br-), and iodinated (I-) HAcAms, were tested for their effects on cell proliferation using the CHO cell line AS52 (Plewa et al. 2008). The results showed that the general order of cytotoxicity was I-HAcAms > Br-HAcAms > Cl-HAcAms, and multihalogenated HAcAms had generally stronger toxic effects than monohalogenated HAcAms. The MTT assay conducted with normal rat kidney (NRK) 52^{E} cells demonstrated higher cytotoxicity of dichloroacetamide (DCAcAm) compared with dichloromethane (DCM) (Yang et al. 2014). However, the LC_{50} values derived using NRK cells (6390 mg/L for DCAcAm) were higher than obtained with CHO cells (250 mg/L for DCAcAm) (Plewa et al. 2008), which highlights the issue that attention should be paid to the influences caused by the choice of cell models. Other studies using HepG-2 cells (a human liver cancer cell line) and CCD 841 CoN cells (immortalized normal human colon epithelial cells) reported similar toxicity order for HAcAms, though the LC_{50} values were distinctly higher than in the study using CHO cells (Hong et al. 2018; Sayess et al. 2017).

Except for the proliferation, most cytological indicators now are commonly detected using flow cytometry (FCM). With easily usable commercial kits, FCM provides more rapid and sensitive detection than classical toxicological methods. It also provides superior quantitative data, which increases the statistical quality of the results. Apoptosis assay and cell cycle assay are two frequently applied tests. Apoptosis as programmed cell death can be distinguished from necrosis by Annexin V-PI double staining. This approach was used to determine that the exposure to DCAcAm significantly induced the apoptosis of NRK cells in a dose-dependent way (Yang et al. 2014).

The cell cycle consists of the following phases: G1 (Gap 1), S (synthesis), and G2 (Gap 2), and M (mitosis). During the process, the DNA content in cells periodically oscillates, which can be used for cell cycle measurement. Cell cycle analysis which is widely applied in diagnosing tumors and assessing cellular damage has also been used to assess the effects of DCAcAm and DCM in NRK cells (Yang et al. 2014).

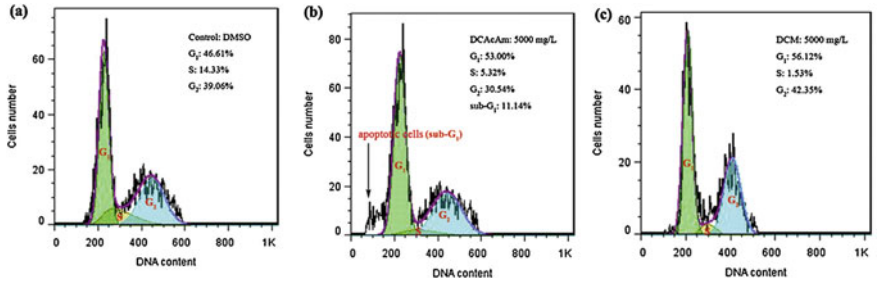

Fig. 12.1 Cell cycle analysis of NRK cells exposed to DCAcAm and DCM for 4 h (at the highest DBP concentration of 5000 mg/L). G1, S and G2 indicate cell cycle phases. Sub-G1 peak expresses apoptotic cells (reprinted from Yang et al. 2014, copyright © 2014 Elsevier B.V.)

When comparing the effects of the two DBPs, DCAcAm and DCM, G1 phase population was increased by DCAcAm after 4-h exposure, accompanied by a concomitant decrease of cell numbers in S phase and G2 phase (Fig. 12.1). However, DCM caused no significant arrest in G1 phase ($p \geq 0.05$). Therefore, DCAcAm was more potent than DCM in inducing the cell cycle arrest in the G1 phase. Furthermore, it was also found that sub-G1 phase arrest could be induced by DCAcAm in the highest concentration. This was indicated by the increased number of apoptotic cells (Fig. 12.1b). The results, thus, suggested that a high concentration (5000 mg/L) of DCAcAm, but not DCM, induced apoptosis in NRK cells.

Genotoxicity

The results of the classical test methods of genotoxicity such as sister chromatid exchange (SCE) assay and micronucleus assay have been correlated to cytotoxicity, thus can provide also information about general cell health. In addition, single-cell gel electrophoresis (SCGE), i.e., comet assay, is widely used to assess the damage of genomic DNA (Rundell et al. 2003). The advantages of SCGE assay include speed and small sample amounts, which suit well for high-throughput screening. The indicator of the tail moment (tail length × DNA density) makes the method better quantitative than other alternatives. Therefore, SCGE assay has become the preferred option for the genotoxicity of DBPs.

Based on the SCGE assay and significance analysis of differences among tail moments, the rank order of genotoxic potency for common HAcAms was tribromoacetamide (TBAcAm) > diiodoacetamide (DIAcAm) ≈iodoacetamide (IAcAm) > bromoacetamide (BAcAm) > dibromochloroacetamide (DBCAcAm) > bromoiodoacetamide (BIAcAm) > bromodichloroacetamide (BDCAcAm) > chloroiodoacetamide (CIAcAm) > bromochloroacetamide (BCAcAm) > dibromoacetamide (DBAcAm) > chloroacetamide (CAcAm) > trichloroacetamide (TCAcAm) > > dichloroacetamide (DCAcAm) using CHO cell model (Plewa et al. 2008).

According to this ranking there appears to be no clear pattern in the genotoxicity order of HAcAms. In addition, DCAcAm did not exhibit acute genotoxicity in the tested concentration range of 1.28 to 1280 mg/L. However, in another comparison between DCAcAm and DCM using NRK cell model, the lowest genotoxic concentration of DCAcAm for the induction of acute genomic DNA damage was 100 mg/L, and DCAcAm was distinctly more genotoxic than DCM. The results were another indication that the choice of cell models would possibly have a significant impact on the test outcome.

Underlying Mechanisms

Mitochondria are the primary suppliers of energy (i.e., ATP) and reactive oxygen species (ROS) in all eukaryotic cells, and thus their impairment may influence various cellular events involving energy conversion, calcium homeostasis, and regulation of cellular proliferation and apoptosis (Chen et al. 2019; Voet et al. 2006). The principles of most cytotoxicity tests rely on the function or physiological condition of mitochondria. For instance, the common tests of cellular proliferation are based on the formation of a formazan dye by the dehydrogenases in mitochondria. Thus, the DBPs which show significant cytotoxicity in the formazan-based assays have the potential to disturb the mitochondria.

Oxidative stress is a universal toxicity mechanism for most pollutants and ROS play an important role in this process. In a screening study of 50 DBPs, 98% of DBPs activated the Nrf2-ARE mediated oxidative stress response. Nrf2 is a transcription factor that is a major transactivator of cytoprotective genes in response to oxidative stress and xenobiotic electrophiles. Nrf2 binds to ARE, an antioxidant responsive element, to activate the protective pathway. Among the tested DBPs, HAcAms were among the most potent chemicals, in particular di-HAcAms (Stalter et al. 2016). In a study about the toxicity mechanisms of four I-HAcAms, conducted with HepG2 cells, different mechanisms for the cytotoxicity of I-HAcAms species (BIAcAm, CIAcAm, DIAcAm and IAcAm) were identified (Hong et al. 2018). BIAcAm and CIAcAm induced apoptosis through a ROS-independent pathway, while the combination of ROS and the mitochondrial pathway was attributed to the apoptosis induced by DIAcAm and IAcAm, which was considered as the reason for their higher potency compared to BIAcAm and CIAcAm. Similarly, DCAcAm caused a continuous increase in ROS levels in HEK293 cells, along with the upregulated expressions of three caspase genes (caspase-3, -7, and -8) and the decrease in the transcript of the Bcl-2 gene, which demonstrated the mitochondria-related mechanism of apoptosis induced by the exposure (Chen et al. 2019). The exposure of male mice to mono-HAcAms decreased the activities of antioxidant enzymes including catalase, superoxide dismutase, and glutathione peroxidase, but increased the level of 8-hydroxy-2-deoxyguanosine, a marker for DNA adducts, in mice liver (Deng et al. 2014). The study confirmed that the rank order of oxidative stress induction by monoHAcAms (IAcAm > BAcAm > CAcAm, determined by the leaving tendency

of the halogens) was consistent with the results from *in vitro* studies. Furthermore, the metabolomic analysis showed that all mono-HAcAms influenced amino acid metabolism, energy metabolism and lipid metabolism. IAcAm exposure has also been shown to lead to thiol depletion and lipid peroxidation in porcine kidney epithelial cell line LLC-PK1, while the mitigation of the effects by antioxidants indicated the ROS-mediated action of IAcAm (Chen and Stevens 1991).

Energy metabolism is the foundation of all life activities, and its disturbance would have profound effects, including the development of metabolic disorders and cancer. Since ATP is the basic unit of energy storage and transfer, its levels are commonly used to characterize the status of energy metabolism. For example, 24-h exposure to DCAcAm was shown to cause a significant dose-dependent decrease in the ATP content on HEK293 cells. This decrease was correlated with the down-regulated expression of the ADP/ATP carrier SLC25A6 gene (Chen et al. 2019). The combined transcriptomic and metabonomic analysis also found that TCAcAm influenced the expression of genes encoding NADH dehydrogenase, cytochrome c oxidase, and ATP synthase, which are all members of oxidative phosphorylation and mitochondrial respiratory chain, the primary source of ATP (Zhang et al. 2013). Though the authors did not highlight the glycolysis pathway, the increased levels of pyruvate and lactate, two essential end products of glycolysis (Rogatzki et al. 2015), implied that glycolysis was activated by the exposure. Glycolysis is an important route of energy production, secondary to oxidative phosphorylation; hence the activation of glycolysis can compensate for disrupted oxidative phosphorylation and consequent ATP deficiency. However, iodinated HAcAms acted as inhibitors of glycolysis because they inactivated the key enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by alkylating the cysteine residue in GAPDH (Schmidt and Dringen 2009). Therefore, the influence of HAcAms on cellular energy metabolism remains controversial.

The endoplasmic reticulum (ER) is another essential organelle that acts as the transportation system of the eukaryotic cell and executes many important cellular functions. For example, ER-mediated apoptosis is an additional apoptotic pathway to mitochondria- and death receptor-dependent apoptotic pathways. ER signaling, through ER stress proteins such as glucose-regulated proteins, was shown to be involved in the apoptosis caused by IAcAm exposure in pig kidney epithelial cells (LLC-PK1) (van de Water et al. 1999). IAcAm acted through ER-signaling because of its alkylating properties. It is unclear if other HAcAms have a similar effect on ER, thus this toxicity pathway remains to be studied with other DBPs.

N-nitrosodimethylamines

Chloramination can also promote the generation of N-nitrosamines (NAs), an emerging class of non-halogenated N-DBPs. Among them, N-nitrosodimethylamine (NDMA), a typical degradation product of NAs, has attracted increasing attention due to its frequent occurrence and high toxicity (Krasner et al. 2013). There is a

considerable amount of toxicity data available for NDMA compared to the other non-halogenated N-DBPs, hence NDMA will be the focus of this section.

According to available literature, NDMA exists in various environmental media and can be involved in multiple exposure pathways, which would potentially increase human health and ecological risks. NDMA has been detected in drinking water in many countries, with most data available, for example, from Canada, the United States, and China (Mitch et al. 2003; Zhao et al. 2008; Ma et al. 2012). In addition, it has been reported that NDMA was more abundant than other NAs in the atmosphere (Farren et al. 2015) and swimming pools (Walse and Mitch 2008). NDMA can enter the human body via direct intake of drinking water or via food and medicine where it is formed unintentionally. Therefore, the body burdens of NDMA have been concerning for a long time. In a recent study, NDMA was detected in the blood of Russian children at levels ranging from 100 to 900 ng/L (Zaitseva et al. 2018). NDMA was also detected at the highest frequency among nine NAs in the urine of the residents of two Chinese cities, and its average concentration was the second-highest after N-nitrosodiethylamine (NDEA) (Zhao et al. 2019). NDMA is the most concerning DBP in the family of NAs. Due to its established occurrence in the public water system, NDMA was placed in the drinking water contaminant candidate list (CCL 4) by the US EPA. The World Health Organization (WHO) set a guideline value of 100 ng/L for NDMA in drinking water, while the California Department of Public Health has lowered the allowed NDMA level to 10 ng/L.

Hepatotoxicity and Carcinogenicity

NDMA-associated health concerns have been known for longer than for most other DBPs. Because of its unintentional production in the cured meat, fish, vegetables, and during smoking (Herrmann et al. 2015; Tricker and Preussmann 1991), NDMA was known for its hepatotoxicity and potential carcinogenicity before NAs were regarded as DBPs. Hepatic necrosis from exposure to a high dose of NDMA can cause lethality, while there is no specific agent for the intoxication of NDMA. Intraperitoneal injection of NDMA caused cirrhosis-like symptoms in male Wistar rats, including collagen fiber deposition, severe centrilobular necrosis, focal fatty changes, bile duct proliferation, bridging necrosis and fibrosis surrounding the central veins (George et al. 2001). The cumulative exposure to NDMA through diet is suspected to be a cause of the high incidence of liver cancer in certain regions.

NDMA was labeled as Group 2A “Probably carcinogenic to humans (limited evidence of carcinogenicity in humans)” by the International Agency for Research on Cancer, and Group B2 “Probably carcinogenic to humans with little or no human data” by the US EPA. Both classifications have taken into consideration the data indicating NDMA carcinogenicity in lab animals. In addition, there exists some human data that indicate the connection between NDMA exposure and cancer risk. As mentioned above, the tumors induced by NDMA often occur in the liver, but also the whole digestive and excretory system including the esophagus, stomach,

kidney, and gut are the targets (White 2020). The epidemiological investigations have revealed that exposure to NDMA in food is linked with a higher risk of colorectal cancer, exclusively rectal carcinoma (Zhu et al. 2014). Also, the high incidence of esophageal cancer in some regions of China was confirmed to correlate with long-term dietary nitrosamines including NDMA (Tricker and Preussmann 1991). Besides, the carcinogenicity of NDMA has been demonstrated in many *in vivo* toxicological studies. For example, rats exposed to NDMA via inhalation developed nasal, hepatic, pulmonary and renal tumors (Klein et al. 1991; Pottegard et al. 2018) and mice exposed via drinking water developed hepatic and renal tumors (Clapp and Toya 1970). The administration of NDMA to pregnant mice increased the frequency of hepatic and pulmonary tumors in the offspring (Anderson et al. 1989). US EPA has assigned an estimated specified cancer risk level (10^{-6}) for NDMA in drinking water at 0.7 ng/L, and WHO suggests that 0.1 μg/L of NDMA in drinking water would be associated with 10^{-5} cancer risk.

Genotoxicity

NDMA has been demonstrated to be genotoxic in many *in vivo* and *in vitro* studies. This is connected to the carcinogenic properties of NDMA in rodents and probably humans. A detailed summary concerning the genotoxicity of NDMA has been prepared by the Agency for Toxic Substances and Disease Registry (ATSDR 1989). In *in vitro* tests, NDMA causes the increased frequencies of gene mutations, DNA damage, and genetic material aberration in a wide variety of cell lines and mammalian primary cells, including without metabolic activation, suggesting the structural properties of NDMA cause mutagenicity. The genotoxicity for five nitrosamine DBPs, N-nitrosopiperidine (NPIP), N-nitrosopyrrolidine (NPYR), N-nitrosomorpholine (NMOR), N-nitrosodiphenylamine (NDPhA), and NDMA, in drinking water, were analyzed and compared using *Salmonella typhimurium* strain YG7108 and Chinese hamster ovary (CHO) cells (Fujita and Kamataki 2001; Wagner et al. 2012). The descending rank order of mutagenicity was NDMA > NPIP > NMOR > NDPhA > NPYR. Since NDPhA induced genotoxicity only at one concentration and NPYR was not genotoxic, it was suggested that the generation and occurrence of NDMA, NPIP and NMOR should be the focus of future studies. However, in another study with HepG2 cells, NDMA exhibited genotoxic activity only at very high concentrations as tested with micronucleus and comet assays (Valentin-Severin et al. 2003). Similar results were also obtained for NDMA and NDEA using the human lymphoblastoid TK6 cell line (Liviac et al. 2011).

Genotoxic effects of NDMA have also been established in *in vivo* studies early on. For instance, the analysis of SCE was used as a predictive assay for the carcinogenicity of NDMA and other four alkylating agents (Neft and Conner 1989). The study harvested multiple murine tissues including bone marrow, alveolar macrophages, regenerating and intact liver, and kidney. NDMA (0.03–0.27 mmol/kg) exposures significantly increased SCE in all tissues. Classic clastogenic

effects including SCE, micronuclei, and chromosome aberrations were also found in hepatocytes of F334 rats after *in vivo* exposure to hepatocarcinogens, whereas the genotoxicity induced by NDMA was overall higher than in the case of known mutagens carbon tetrachloride (CCl_4) and 2-acetylaminofluorene (2-AAF) (Sawada et al. 1991). However, in other studies, NDMA was regarded as a weak inducer of micronuclei in many tissues, including bone marrow and peripheral blood (Sato et al. 1992), but its effect was more significant in mouse testicular cells (Cliet et al. 1993). In a recent study, 5 mg/kg NDMA treatment significantly increased the frequency of micronuclei in bone marrow cells, and elevated DNA fragments in the liver and kidney of rats (Adeleke and Adaramoye 2016). The contradictory results may be caused, in part, by the methodological challenges involved in the application of genotoxicity assays. For example, in marine mussel *Mytilus edulis* L., the DNA damage responses to NDMA were much weaker than to H_2O_2, indicating that the comet assay was possibly insensitive to NDMA (Wilson et al. 1998).

The epigenetic effects of NDMA have been studied for the past four decades, mostly focusing on the measurements of abnormal methylation of bases in the genome (Liteplo and Meek 2001). A rodent study showed that N^7-methylguanine (N7-meG) and O^6-methylguanine (O^6-meG) were the main DNA adducts formed following the exposure to NDMA (Diaz Gomez et al. 1986), and a primate study also highlighted the formation of O^6-meG (Chhabra et al. 1995). Small amounts of N^3-methyladenine and O^4-methylthymine were concurrently induced in animals during the exposure to NDMA.

Reproductive and Developmental Toxicity

Reproductive and developmental toxicity are essential criteria in chemical risk assessment. The reproductive and developmental toxicity of NDMA was established by the studies conducted around the 1980s. For example, NDMA was found to induce reproductive and developmental toxicity in Holtzman rats, and the toxic effects of NDMA were greater in pregnant than nonpregnant rats (Nishie 1983). When the drinking water containing 0.1 mg NDMA/L was administrated to female, Swiss CD-1 mice before and during pregnancy, the perinatal death of their offspring in treated mice was significantly higher than in the controls (Anderson et al. 1978). In another study, conducted with male rats, a single intraperitoneal injection of 30 or 60 mg NDMA/kg body weight induced testicular damage (necrosis or degeneration of the seminiferous epithelium) (Hard and Butler 1970). The fact that tests of DNA fragmentation for NDMA were more sensitive in germ cells than bone marrow cells could also reflect its reproductive toxicity (Cliet et al. 1993).

In a comparative, transplacental study with mice exposed to two kinds of nitrosamines (NDMA and NDEA), a single intraperitoneal injection of 37 mg NDMA/kg body weight on day 16 or 19 of gestation resulted in the deaths of the fetuses in all exposed dams; lethality was not significantly observed following the administration of 7.4 mg NDMA/kg body weight (Anderson et al. 1989). NDEA

had no effect when given on day 16 of gestation. In the previous study performed by the authors, groups of 20 female mice were provided with drinking water containing 0 or 0.1 mg NDMA/L for 75 days before mating and throughout pregnancy and lactation. The proportion of deaths was increased twofold ($p < 0.05$) in the NDMA-exposed animals compared with controls (i.e., 20% and 9.9%, respectively) (Anderson et al. 1978). The intraperitoneal injection of NDMA to pregnant mice significantly increased the frequency of hepatic tumors in the (especially female) offspring (Anderson et al. 1989). The fetal body weight was significantly ($p < 0.05$) reduced after a single oral dose of 20 mg NDMA/kg body weight was administered to pregnant rats on day 15 or 20 of gestation (Nishie 1983). In an aquatic model zebrafish, NDMA exhibited the highest mortality rate (40% at 0.9 mg/L) in embryos among several unregulated DBPs (in Portugal), and the morphological abnormalities (max. 38% at 0.4 mg/L) caused by NDMA were prominent (Chaves et al. 2020).

Neurobehavioral Effects

The neural and behavioral effects of NDMA have been explored in a limited number of studies using conventional rodent models, although the neurotoxicity of NAs has been demonstrated (Konstantinou et al. 2018). Since neurodevelopment and behavioral phenotypes modulated by neurodevelopment are highly susceptible to external stimuli during animal early life stages (e.g., zebrafish larvae), this is a sensitive endpoint for NDMA-induced toxicity which can provide valuable information in addition to traditional developmental effects (e.g., teratogenesis and developmental retardation) (Kienle et al. 2009; Powers et al. 2010). Therefore, studying the potential effects of NDMA on the behavior of larvae of aquatic organisms is expected to be beneficial for the risk assessment of NDMA.

Locomotion is the basic element for animal behavior which can provide information about biochemical and physiological responses of animals to environmental contaminants (Kane et al. 2004). Compared with certain other behavioral endpoints, the locomotion can be quantified, thus has the advantage over those indicators which can only provide qualitative or "yes/no" information. Additionally, the characteristics of ontogenic origin, synchronous development, and optical transparency make zebrafish larvae an excellent model for high-throughput locomotion tests. With the recent rapid development of video-based movement tracking systems and matched protocols, locomotion test based on zebrafish larvae has rapidly developed and gained great success especially in environmental toxicology (Chen et al. 2011; Zhao et al. 2014). The protocol has also been adjusted for detecting earthworm locomotion (Xu et al. 2020). To further improve the assessment of neurobehavioural effects of chemicals, a detection system consisting of multiple behavioral tests in zebrafish larvae has been proposed, which includes three kinds of endpoints, fundamental locomotion, path angle, and two-fish interaction (Zhang et al. 2017). In addition to locomotion assessment, the test of path angle indicates zebrafish responses to sensory stimuli and the test of two-fish interaction (social activity) provides an aspect of cognition.

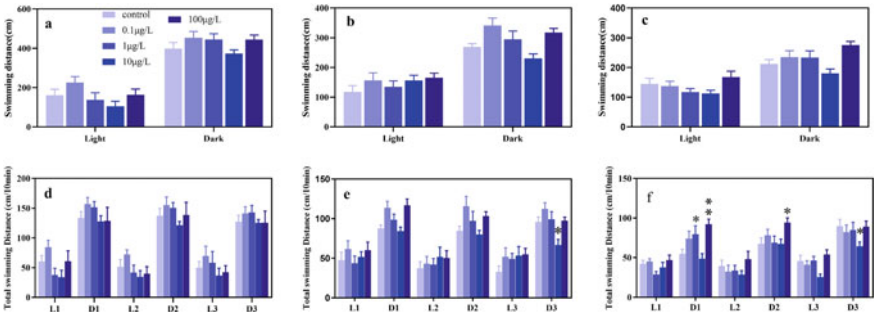

Fig. 12.2 Locomotion alternation of zebrafish larvae under the light–dark stimulus upon low-dose NDMA exposures. a, d: 5 dpf; b, e: 6 dpf; c, f: 7 dpf. Data are expressed as mean ± SEM (n = 8). The significant difference between the control group and the exposure groups is indicated by the asterisks (*$p < 0.05$, **$p < 0.01$) (reprinted from Zhang et al. 2021, copyright © 2021 Elsevier B.V.)

Following this idea, the behavioral effects of low-dose NDMA exposure on zebrafish larvae have been studied (Zhang et al. 2021). The indicators including locomotion (swim distance), path angel, and social activity were used and compared to investigate the safety of low-dose (0, 0.1, 1, 10, 100 μg/L) NDMA exposure to the embryo-larval stage of zebrafish. The effects of NDMA were tested on neurobehavioral changes in zebrafish larvae 5, 6, and 7 days post fertilization (dpf). Exposure to different concentrations of NDMA caused hypoactivity and hyperactivity in locomotion during dark periods (Fig. 12.2). The swimming distance in four NDMA exposure groups at 5 dpf had no significant differences compared to the control group. In contrast, the swimming distance of 7 dpf larvae exposed to 1 μg/L NDMA was longer than in the control group ($p < 0.05$), especially in the first dark periods. 100 μg/L NDMA exposure also caused locomotor hyperactivity compared with the control group in the first two dark periods at 7 dpf. Interestingly, 10 μg/L NDMA exposure caused locomotor hypoactivity in the last dark period both at 6 dpf and 7 dpf. In addition, the swimming activity was not considerably changed in any of the NDMA exposure groups relative to the control group during the light periods. As the system detected locomotion and path angle synchronously, the data of locomotion was interpreted in light of the path angle. In the straight motion, larvae displayed a distinct preference to turn right (+) rather than left (−) in both relative and absolute path angle values. However, during the average and responsive turns, larvae did not show a distinct preference in orientation. Nevertheless, the absolute value of average turns of larvae in the 1 μg/L exposure group was significantly higher than in the control group in the first dark period at 5 dpf and 6 dpf, but there was no significant difference in the relative value. Moreover, the absolute value of average turns of larvae in the 100 μg/L exposure group was significantly higher than in the control group across all dark periods at 5 dpf, while being significantly different only in the first dark period at 6 dpf and 7 dpf. However, the relative value showed significance in the straight motion at 5 dpf and average turns at 5 dpf and 6 dpf during the first

dark period. On the contrary, the absolute value of the average turning in the 10 μg/L group was significantly lower than in the control group in the last dark period at 6 dpf, and a similar phenomenon also appeared in the relative value in the last two dark periods at 6 dpf. The numbers of responsive turns had no significant differences in any of the exposure groups. In the social activity test, 1 μg/L NDMA exposure adversely impacted the interaction of the larvae by shortening the duration of contact at 7 dpf, which was an opposite effect in comparison with the results of locomotion and path angle tests. Meanwhile, the social activity of larvae exposed to 10 μg/L of NDMA was moderately inhibited during the whole test. On the contrary, a slight increase in the duration of contact between two fish was observed at 7 dpf in the 100 μg/L group, although it was not significantly different from the control. In general, exposure to NDMA did not cause an obvious tendency for reduced social activity with increased exposure concentrations.

By contrast, the tests for developmental toxicity, which are commonly utilized for toxicity and risk assessment, have not shown a similar level of sensitivity as the behavioral tests. The two high-dose groups (10 μg/L and 100 μg/L) of NDMA exposure slightly inhibited the hatching process of zebrafish embryos at 72 hpf. Furthermore, there were no significant differences in the survival rates in any of the exposure groups compared with the control group regardless of the exposure time. The survival rate was higher than 90% even in the group exposed to NDMA at the maximum concentration (0.1 mg/L), which was higher than the 86% survival rate at 0.08 mg/L NDMA reported previously (Chaves et al. 2020). The comparison demonstrated that the behavioral test is expected to be a convenient method for screening and assessing the toxicity of trace levels of DBPs in drinking water. Besides the sensitivity of behavioral responses, the precise measurement of larval locomotion is the reason for the superior performance of behavioral tests compared to the developmental toxicity tests which use poorly quantifiable morphological phenotype as an endpoint.

Underlying Mechanisms

The hepatotoxicity and carcinogenicity of NDMA are closely connected with its liver metabolism. Because NDMA intake is often through the diet and metabolic activation of NDMA occurs in the liver, hepatic damage is the most concerning effect of NDMA, which is different from other types of DBPs. Via the cytochrome P450 (CYP2E1)-dependent Phase I (oxidation) metabolism and a series of subsequent chemical transformations NDMA transforms to methyl diazonium, an alkylating agent. According to the theory of chemical carcinogenesis, alkylating agents contribute to the induction of tumors. NDMA is the intermediate product in the metabolism of methylamines, and this is the primary reason why methylamines are carcinogenic (Smith et al. 1994).

Many toxicological outcomes, especially carcinogenicity of NAs, including NDMA, can be attributed to the compounds' alkylating properties (Tricker and

Preussmann 1991). As mentioned before, regarding DNA alkylation by NDMA, the proportion of the produced O^6-meG was the second highest after N7-meG in alkylated bases (Diaz Gomez et al. 1986), and the methyl group in O^6-meG could efficiently inactivate the alkyltransferase which is in charge of removing the adducts from DNA (Pegg et al. 1995). The attack at the O6 position of guanine is regarded as the primary mechanism for a wide range of biological effects including cell death, mutation, and cancer (Margison et al. 2002). In rat liver cells, only the alkylating agents which give rise to substantial DNA O-alkylation induced persistent genotoxicity (Tates et al. 1986). The accumulation of N7-meG, as well as O^6-meG, were associated with the hepatocarcinogenesis of NDMA (Souliotis et al. 2002). Besides, the simple and symmetrical structure of NDMA and NDEA favor maintaining a common, high metabolic activity among various animal tissues (Tricker and Preussmann 1991). The above reasons possibly make NDMA more potent in metabolic activation than most of its derivatives. The primary mechanism for the carcinogenicity of NDMA has been proposed to be the transition of GC-AT as a result of O^6-meG in the codon 12 of K-*ras* protooncogene (Belinsky et al. 1989). This mechanism has also been demonstrated in nonmammalian vertebrates. Exposure of Japanese medaka (*Oryzias latipes*) to NDMA induced hepatic tumors, while a point mutation at codon 16 of *ras* was reported in addition to the mutation in the previously reported codon 12 (Liu et al. 2003).

Oxidative stress as a universal toxicity mechanism is also involved in the adverse effects of NAs, which mainly manifests as the loss of mitochondrial membrane potential, lipid peroxidation, depletion in antioxidants, and ROS generation (Oliveira et al. 2013). For the case of NDMA, upregulated expression of Bcl-2 and nuclear p53 in NDMA-exposed male Wistar rats indicated the adaptive mechanism for the ROS-related apoptotic activity of NDMA (Adeleke and Adaramoye 2016). NDMA was shown to induce a high ratio of apoptotic cells in polymorphonuclear neutrophils (PMNs) via both death-receptor-mediated and mitochondrial apoptotic pathways (Iwaniuk et al. 2019). Since PMNs naturally generate ROS and reactive nitrogen species (RNS) including nitric oxide through enhancing the expression of inducible nitric oxide synthase (iNOS), which possibly, in turn, promotes *in vivo* synthesis of NDMA (Jabłoński et al. 2006; Dubey et al. 2016). Besides, oxidative stress has been identified as a contributing factor to the carcinogenicity of NDMA. NDMA exposure induced histopathological symptoms of developing hepatocarcinoma, along with impacting the levels of mitochondrial ROS, lipid peroxidation, cytochrome C, and iNOS expression (Ghosh et al. 2012). The toxicological effects of NDMA associated with the induction of oxidative stress are believed to be related to the alkylating properties of the compound (Ahotupa et al. 1987).

Other Typical DBPs

The definition of DBPs, in theory, includes any chemical species which exists in the drinking water matrix and is influenced by different disinfectants. Thus, it can

be estimated that DBPs include a large number of various compounds. However, only a small fraction of DBPs have been identified and are regulated. Most of them are halogenated methanes (primarily chlorinated and brominated THMs), and the rests are chlorinated HAAs, NDMA, trichloroacetaldehyde, dichloro- and dibromoacetonitriles, and some disinfection-related inorganic salts.

Trihalomethanes

THMs (except iodinated THMs) are not emerging DBPs; they were the first DBPs to be identified in disinfected water (Landi et al. 2003). For example, trichloromethane or chloroform ($CHCl_3$) which has been regarded as a toxicant and carcinogen for a long time, is the most prevalent compound among DBPs and can contribute up to 85% to total THMs (Monarca et al. 2004; Lodhi et al. 2017). In the guidelines of WHO and China, the limits for four kinds of chlorinated and brominated THMs have been set, but for European Union, the US, and Canada, the total level of all THMs is used as an overall indicator for potentially harmful compounds in the disinfected water. Therefore, THMs are so far the DBPs that attract the most concerns in regulation and scientific research, although their adverse health effects have not still been fully elucidated.

Studies have shown that exposure to THMs may lead to colon, bladder, and rectal cancers, reproductive disorders, birth defects, and miscarriage (Hildesheim et al. 1998; Villanueva et al. 2004; Wright et al. 2004). For example, one of the major THMs, bromodichloromethane (BDCM) is found almost exclusively in the disinfected drinking water, while only less than 1% of BDCM is naturally formed by algae in the ocean. BDCM is believed to be a more potent acute toxicant than $CHCl_3$ based on the study where BDCM and $CHCl_3$ were administered by gavage to male Fischer 344 rats (Keegan et al. 1998). After studying the hepatic toxicity and renal toxic effects in the rats, it was found that kidneys were more sensitive target organs to BDCM than liver, which was consistent with previous epidemiological studies regarding the relationship between the THMs in drinking water and urinary tract cancer (Cantor et al. 1978). Both BDCM and $CHCl_3$ are metabolized in the organism into similar transformation products, mainly free radicals of phosgene and dichloromethyl. BDCM has been shown to have a greater potential for forming free radicals and binding to macromolecules than $CHCl_3$ (Waller and McKinney 1993). In addition, the difference in their toxicity is suggested to be driven by the different formation rates of free radicals. Consumption of drinking water containing BDCM possibly caused mortality and some clinical signs in CRL SD rats, while the no-observed-adverse-effect level (NOAEL) value of BDCM was at 50 mg/L which was 5125–15,750 times of the human adult exposure level. This result suggested that BDCM possesses a low risk to human reproduction and development (Christian et al. 2002). Furthermore, in another study of joint toxicity of regulated THMs (including DCM) and HAAs, the mixture did not induce significant toxicity in SD rats (Narotsky et al. 2015). However, among 300 chemicals identified in

drinking water in the US, BDCM, along with other brominated methanes, including bromoform, bromomethane, dibromomethane, bromochloromethane, and chlorodibromomethane, showed dose-dependent mutagenic responses in *S. typhimurium* TA100 assay (Simmon et al. 1978).

The existence of iodinated THMs mainly depends on the iodine substances in the source water, and in most cases, they account for only a small proportion in total THM residues (Cancho et al. 2000). During the chlorination, naturally occurring iodine in water can be quickly oxidized to iodide (in the form of highly reactive hypoiodous acid or HOI), which can be further oxidized to iodate (IO_3^-) (Bichsel and von Gunten 1999). However, in chloramination, HOI is not transformed to less toxic IO_3^-. This is the reason why chloramination disinfection leads to the accumulation of I-DBPs (Krasner et al. 2006; Liu et al. 2019). As a class of emerging DBPs, the toxicological studies concerning I-THMs are relatively few, but they are widely believed to possess higher cytotoxic and genotoxic potentials than the chlorinated and brominated analogs (Wagner and Plewa 2017).

Haloacetic Acids

HAAs are formed in drinking water during various disinfection processes, including chlorination, chloramination, and ozonation, while slightly acidic source water and high organic matter content contribute to the elevated amounts of HAAs. HAAs, along with THMs, are used as indicators for harmful levels of DBPs in drinking water, whereas chlorinated HAAs have been the focus of the health effect studies.

Two kinds of chlorinated HAAs, trichloroacetic acid (TCA) and dichloroacetic acid (DCA), are ubiquitously found in disinfected drinking water up to 87.5–170 μg/L (Parvez et al. 2011). DCA or TCA in drinking water at a concentration of 5 g/L induced hepatocellular carcinomas in 81 and 32% of tested male B6C3Fl mice, respectively (Herren-Freund et al. 1987). After a 90-day subchronic exposure, both DCA and TCA produced substantial systemic organ toxicity to the liver and kidney. Nevertheless, the doses used in these studies were much greater than those expected to occur in the environment. Also, high doses of DCA and TCA have been associated with teratogenicity and adverse effects in pregnant rats. For example, exposure to DCA during the pregnancy resulted in teratogenic effects, particularly fetal heart malformations, in Long-Evans rats (Epstein et al. 1992). Fetal death, weight loss and deformities (cardiovascular and orbital deformities) were observed in the pregnant Long-Evans rats exposed to TCA in the drinking water (Smith et al. 1989). In addition, erythrocyte damage and intravascular hemolysis, spleen and kidney weight increased in the pregnant rats. The studies, although conducted with relatively high doses of TCA, suggested that in case TCA is transported to and retained long-term in the fetoplacental unit, and swallowed or skin-absorbed by the fetus, the exposure can potentially lead to adverse effects to the fetus.

Brominated HAAs are also prevalent DBPs in drinking water usually accompanied with chlorinated HAAs. A study with CD-1 mice indicated that the embryonic

toxicity of dibromoacetic acid (DBA) and bromochloroacetic acid (BCA) may be mediated in part by protein kinase C inhibition and apoptosis modulation (Ward et al. 2000). Epidemiology studies are now underway to relate haloacid exposure with compromised semen quality in men. Dibromoacetic acid (DBA), dichloroacetic acid (DCA), and BCA have all been shown to delay spermiation in the testis. As demonstrated in multiple rodent experiments, DBA can affect the male reproductive system through reduced sperm quality. For example, this effect was found after administrating DBA at a dose of 2 mg/kg by gavage to rats (Kaydos et al. 2004; Klinefelter et al. 2004) and a dose of 1 mg/kg by drinking water to rabbits (Veeramachaneni et al. 2007). In vitro experiments with newly matured ovarian follicles of Sprague–Dawley rats, exposed to DBA, showed decreased progesterone secretion (Goldman and Murr 2002). The toxicological effects were caused by the disturbance of mitochondrial cholesterol transport by steroidogenic acute regulatory protein, along with a possible impact triggered by human chorionic gonadotropin added to the rat preovulatory follicles to assess progesterone secretion. DBA exposure in the drinking water (20–161 mg/kg/day) to adolescent male and female Fischer 344 rats for 6 months produced neuromuscular toxicity including concentration-dependent effects (limb weakness, mild gait abnormalities, hypotonia, sensorimotor depression and decreased responses to a tail-pinch and click) and effects only at the highest concentration of 161 mg/kg/day (decreased activity and chest clasping) (Moser et al. 2004). In neuropathological evaluation, the major symptom was the degeneration of spinal cord nerve fibers and cellular vacuolization in spinal cord gray matter and white matter tracts.

The concentrations of iodinated HAAs in drinking water are commonly lower than other kinds of HAAs; for example, iodoacetic acid (IAA) has a maximum value of 2.18 μg/L in Shanghai, China (Wei et al. 2013). Studies have shown that, at these concentrations, IAA can induce significant toxicological consequences and potentially pose risks to human health over lifetime exposure. IAA and iodoform (IF), the tri-iodinated THM, exhibited cytotoxicity and genotoxicity to the primary mouse embryonic fibroblast cells (NIH3T3 cell line) with $LC_{50} = 2.77$ μM and 83.37 μM, respectively (Wei et al. 2013). Both compounds induced significantly increased DNA damage as quantified using SCGE and γ-H2AX phosphorylation assay, while IAA also promoted *in vitro* malignant transformations in NIH3T3 cells. After transplantation into Balb/c nude mice, these IAA-transformed cells produced aggressive fibrosarcomas. Toxicogenomic studies using nontransformed human small intestinal epithelial cells (line FHs 74 Int) demonstrated that a set of mono-HAAs, including IAA, modulated the expression of genes involved in DNA repair and cell cycle progression even within 30 min exposure (Attene-Ramos et al. 2010). Plewa et al. demonstrated that iodoacetic acid (IAA) was more mutagenic, genotoxic, and cytotoxic in *S. typhimurium* and mammalian cells than bromoacetic acid or chloroacetic acid (Plewa et al. 2004). Compared with chlorinated and brominated HAAs, IAA exerted stronger impacts on cell cycle regulation and apoptosis control.

Aromatic DBPs

Almost 700 types of DBPs have gradually been identified and confirmed in drinking water or reclaimed water by now, most of which are aliphatic DBPs (Yang and Zhang 2016). Nevertheless, it is estimated that there are still numerous unknown DBPs remaining to be identified, and among them, there is possibly a large number of aromatic DBPs. The presence of benzene rings confers unique physical and chemical characteristics on aromatic DBPs, which can cause different toxicological effects compared to aliphatic DBPs (Liu and Zhang 2014). For example, the newly identified halophenolic DBPs were significantly more toxic than classical THMs and HAAs which are regulated by the US EPA.

Halogenated phenols and benzoquinones are earlier discovered aromatic DBPs, with more potent toxicity than halohydroxybenzaldehydes and halohydroxybenzoic acids (Zhang et al. 2020). Using T24 bladder cancer cells, four kinds of halobenzoquinones were tested and the results showed that halobenzoquinones triggered the generation of intracellular ROS, genomic DNA damage, and protein carbonylation (Du et al. 2013). High-throughput microarray analysis revealed that 4-h dibromobenzoquinone exposure (4.6×10^{-5} mol/L, 10% maximal inhibitory concentration) to nontransformed human cells significantly impacted the inflammatory response pathways, possibly through ROS production (Prochazka et al. 2019). Before being identified as a new class of DBPs, halogenated phenols as legacy pollutants have been a topic of toxicological studies for decades because of their occurrence in industrial, agricultural, and medical wastewater. This new wave of research has focused on using *in vitro* toxicological tests for elucidating the mechanisms of action of halogenated phenols. For example, experiments with human extended pluripotent stem (EPS) cells indicated that the phenolic halogenated DBPs possibly have specific effects on human embryo development in the early stage of pregnancy (Liu et al. 2021).

Similar to aliphatic DBPs, the addition of nitrogenous groups or bromine/iodine atoms will enhance the toxicity of aromatic DBPs (Liu et al. 2020). The functional groups of phenyl DBPs determine their site of binding in antioxidant enzymes (e.g., catalase) and modulate the binding energy, which influences their toxic potency (Zhang et al. 2020). Based on the results of *in vitro* and *in vivo* toxicity studies, it can be concluded that nitrogenous phenyl DBPs are generally more toxic than non-nitrogenous phenyl DBPs and the toxicity follows the order of halonitrophenols > halophenylacetonitriles > halophenols > halohydroxybenzaldehydes > halohydroxybenzoic acids (Zhang et al. 2018, 2020). In the comparison between iodinated phenyl and iodinated aliphatic DBPs using HepG2 cells, overall, iodinated phenyl DBPs exerted higher cytotoxicity with the exception that IAA was the most cytotoxic in this study (Hu et al. 2018b). 2,4,6-triiodophenol and 2,6-diiodo-4-nitrophenol also exhibited relatively severe developmental toxicity on marine polychaete *Platynereis dumerilii* (Yang and Zhang 2013).

Risk Assessment of DBPs

The health risks of environmental pollutants, including DBPs, encompass two factors: hazards (i.e., the toxicity of the pollutants) and exposure (i.e., the concentration of pollutants in the human body or environmental matrix). Compared with the concentration of pollutants which can be precisely determined by various physical, chemical, and biochemical means, much more uncertainty and variability are associated with the toxicity testing, especially establishing dose–response (or effect) relationships of pollutants. Some of the issues associated with the assessment of the potential risks of DBPs include:

1. Since DBPs often do not exert acute effects on cell viability, which is the most common endpoint in *in vitro* studies, it is challenging to establish the standard measures of toxicity, such as EC_{50} or IC_{50}. The difficulties in establishing the full dose–response are caused by the need to use very high concentrations of DBPs where their water-solubility is limited. To overcome this experimental obstacle, EC_{50} or IC_{50} values are sometimes obtained by extrapolation which is not a good practice in toxicology. Clearly, more sensitive toxicity endpoints for the assessment of DBP toxicity are needed to enable the hazard evaluation and categorization of the compounds.
2. Although for regulatory purposes experiments in rodent models are required, *in vitro* cell models are useful for preliminary and rapid toxicity evaluation. However, the test results from different cell models often vary significantly, especially for a large and diverse group of chemicals such as DBPs. Thus, to enable universal comparison and maximum benefit of the obtained toxicity data, decisions about suggested and most appropriate *in vitro* test systems for DBP hazard assessment are necessary.

The most commonly used *in vitro* test system for cytotoxicity and genotoxicity assessment of DBPs are the rodent-derived CHO cells (Plewa et al. 2002; Richardson et al. 2007; Wagner and Plewa 2017). To date, almost all known DBPs have been tested in this cell model. An indicator named cytotoxicity index (CTI), for the calibration and comparison of the toxicity potentials of various DBPs has been established (Wagner and Plewa 2017). For a certain DBP, the CTI mathematically equals the reciprocal of %C1/2, which was adopted in the original article of Plewa et al. (2002) and close to the meaning of the median lethal concentration (LC_{50}) or the half-maximal inhibitory concentration (IC_{50}). Here we used a more common concept LC_{50} in the formula.

$$\mathrm{CTI} = \frac{1}{LC_{50}}$$

The advantage of the indicator is that it allows the comparison of the cytotoxicities of different DBPs. The larger CTI indicates the potential to induce more significant cytotoxicity. Similarly, the genotoxicity of DBPs has been evaluated by calculating the genotoxicity index (GTI), which equals the reciprocal of 50% DNA tail moment.

However, both CTI and GTI are the functions of a single factor of toxicity. Since the increasing demand for drinking water safety assessment requires the evaluation of actual health risks of DBPs, which take into account the environmental concentrations of DBPs, an approach has been applied where the CTI or GTI values for individual DBPs in the drinking water of a specific location are summarized:

$$CTI = \sum \frac{DBP\ concentration\ (M)}{LC_{50}}$$

Such calculation has been applied in the studies regarding the evaluation of drinking water safety, especially in the Yangtze River Delta of China (Hu et al. 2018a; Wu et al. 2020).

In some studies, the sum of CTI and GTI was adopted to reflect the total potential influences of DBPs on human health. This can be regarded as an effort expected to improve the accuracy of risk evaluation by integrating the risks from cytotoxicity and genotoxicity of DBPs, but the defects in the idea need to be indicated. First, the basic form of the above formulas of CTI calculation is acceptable. It is similar to the risk quotient (exposure/toxicity) used by the US EPA to assess the ecological risk of pesticides (Backhaus and Faust 2012). Second, the strategy of simple summation ("CTI + GTI") is problematic because the connotations of the two indices have some overlaps. The mechanisms of genotoxicity tests (e.g., DNA damage) will usually lead to cell death, the primary reason for inducing the cytotoxicity. The summing is not only unnecessary but can introduce new errors into the calculation. In the ecological risk assessment based on in vivo invertebrate data, toxicity metrics for risk calculation only includes lethality and does not integrate it with developmental or reproductive toxicity. Therefore, cytotoxicity (LC_x/EC_x of cell proliferation) should suffice for characterizing the risks. Third, LC_{50} (or %C1/2) has been applied in many cases when calculating CTI values, however, it may not the most suitable even when no extrapolation has been used in establishing the LC_{50}. LC_{50}/EC_{50} lies in the middle section of the dose–response curve and does not reflect the low-level toxicity (the beginning of the curve). Traditionally LC_{50}/EC_{50} is not a recommended threshold for chronic toxicity; the thresholds including EC_{10}, EC_{20}, or NOEC (no-observable-effect concentration) are optimal. Considering that drinking water intake constitutes lifetime exposure to the micropollutants in water, lower-level toxicity thresholds (instead of LC_{50}/EC_{50}) should be applied both in chronic or acute toxicity assessments.

Regarding the choice of suitable *in vitro* cell models for DBP testing, despite the popularity of CHO cells so far, models which more closely represent the *in vivo* targets of DBPs such as kidney and bladder would be more suitable. The kidney is a primary organ for removing waste from the blood and excreting the waste in the form of urine. It is also essential for the detoxification and the regulation of water and salt homeostasis, which establishes the plausible logical relationship between kidney damage and the intake of DBPs in drinking water. Human embryonic kidney HEK293 cells are well known for their application as vectors in transfection and have also been used in functional, biochemical, and toxicological studies (Stepanenko and Dmitrenko

2015). In addition, they are human-derived. Therefore, HEK293 cells are possibly a good model for studying the adverse effects of DBPs on human health. For example, in a comparative cytotoxicity study with HEK293 cells, 10 DBPs were included, five iodo-THMs containing chlorodiiodomethane (CDIM), dichloroiodomethane (DCIM), bromochloroiodomethane (BCIM), dibromoiodomethane (DBIM), and bromodiiodomethane (BDIM), three HAcAms containing CAcAms, DCAcAms, and TCAcAms, and two haloacetaldehydes containing tribromoacetaldehyde (TBAL) and trichloroacetaldehyde (TCAL) (Chen et al. 2019). The toxicity order according to the EC_{10} values was TBAL > CDIM > CAM > DBIM ≈ DCIM > BCIM > DCAM ≈ TCAM > TCAL > BDIM. The results were different from these obtained with the CHO cells (line AS52) in another study, which reported the order: BDIM > DBIM > BCIM ≈ CDIM > DCIM (Richardson et al. 2008). However, although CHO cell is a successful cell model in genetics, it is controversial to apply CHO models to identify the human health risks of DBPs because they have basic metabolic processes that differ from those of the cell lines from a human source, which can potentially affect the results of toxicologic experiments using CHO cell. Therefore, in the study of the relationship between DBP intake in drinking water and human health, CHO may be gradually replaced by the cell model derived from human cells.

The risk assessment of DBPs can be presented in the form of an environmental potential risk quotient (PRQ):

$$\mathrm{PRQ} = \frac{PEC}{EC_{10}}$$

where the PRQ is calculated as the predicted environmental concentration (PEC) of each DBP divided by its corresponding EC_{10} value in the cell proliferation test.

According to the study concerning the concentrations of DBPs in drinking water systems of the Yangtze River Delta, most of them ranged from the level of ng/L to μg/L (Wang et al. 2015). After being divided by EC_{10} values obtained based on the cell viability tests, the calculated PRQ of most DBPs ranged from 10^{-2} to 10^{-4}, except for that of TBAL due to the lack of monitoring data (Table 12.1). Obviously, the choice of toxicity indicator will influence the range of PRQ. NOEC is theoretically the best parameter to use in PRQ calculation, however, there are challenges primarily from the difficulties in acquiring the accurate NOEC values. In the case of using EC_x value in PRQ equation, it has been suggested that EC_{10} values have certain advantages over other EC_x values (Beasley et al. 2015). Considering that, through drinking water, humans are exposed to low doses of DBP mixtures over a long time, the EC_{10} is more suitable than an acute toxicity threshold like EC_{50}. The total risk of DBPs was further estimated using the sum of the PRQs (ΣPRQs). The accuracy of ΣPRQs remains controversial because the modes of joint exposure are too complex. In addition, summarizing individual PRQs does not necessarily reflect accurately the effects of DBPs with different toxic mechanisms. However, when mechanisms of DBP mixtures are unknown or unfit for standard models, the addition is unavoidable. It can be expected that the large number of DBPs in the mixture will possibly reduce the error derived from the addition.

Table 12.1 The calculated predictive risk quotients (PRQs) of DBPs in drinking water systems, reprinted from Chen et al. (2019), copyright © 2019 Elsevier B.V.

DBPs	PRQ	EC_{10} (μg/L)	PEC (μg/L)	Water Source	Reference
CAM	8.16E−04	79.64	0.065	Taihu Lake (Chlorination), China	Chu et al., 2013
	2.67E−03		0.213	Taihu Lake (Chloramination), China	
	8.66E−04		0.069	Huangpu River (Chlorination), China	
	1.80E−03		0.143	Huangpu River (Chloramination), China	
DCAM	5.00E−02	142.68	7.13	Taihu Lake (Chlorination), China	
	6.68E−02		9.535	Taihu Lake (Chloramination), China	
	1.03E−02		1.472	Huangpu River (Chlorination), China	
	8.38E−03		1.196	Huangpu River (Chloramination), China	
TCAM	1.05E−02	149.04	1.571	Taihu Lake (Chlorination), China	
	6.24E−03		0.93	Taihu Lake (Chloramination), China	
	4.78E−03		0.712	Huangpu River (Chlorination), China	
	1.01E−03		0.15	Huangpu River (Chloramination), China	
TCAL	1.03E−02	456.14	0.016–0.062	Southern Jiangsu, China	Wu et al., 2013
	1.10E−03–4.60E−03		0.5–2.1	Surface water from Bogdanka, Warta and Cybina River, Poland	Dabrowska and Nawrocki, 2009
	3.73E−0.3–6.58E−03		1.7–3.0	United States	Krasner et al., 1989
	1.32E−03–2.30E−02		0.6–10.5	Canada	Lebel et al., 1997
TBAL	N/A	0.43	N/A		
CDIM	1.23E−02	8.16	0.1	Barcelona water treatment plant, Spain	Cancho et al., 2000
BDIM	N/A	591.7	N.D.	Barcelona water treatment plant, Spain	Cancho et al., 2000
DBIM	2.31E−03	86.42	0.2	Barcelona water treatment plant, Spain	Cancho et al., 2000
DCIM	1.73E−03–6.57E−02	86.82	0.15–5.7	Water treatment plants, United States	Richardson et al., 2008
	2.30E−03		0.2	Barcelona water treatment plant, Spain	Cancho et al., 2000
BCIM	4.30E−04–3.74E−02	144.23	0.062–5.4	Water treatment plants, United States	Richardson et al., 2008
	4.16E−03		0.6	Barcelona water treatment plant, Spain	Cancho et al., 2000

Conclusions

The toxicological studies regarding the chemical compounds now considered as DBPs have historically proceeded in two phases. At the early stage, some compounds, included currently among DBPs, were of concern primarily because they were present in food and medicines, or posed a threat in occupational settings. For toxicity assessment, animal experiments were common at this stage and the focus was on the mechanisms of single DBPs. Recently, the legacy pollutants have been identified also as DBPs in drinking water and their toxicity has been assessed along with the toxicity of emerging DBPs in many comparative toxicity studies. Recent studies are focused on rapid and high-throughput cell-based analysis, generating large amounts of dose–response data based on established toxicity endpoints, while large knowledge gaps still remain regarding the mechanisms of action of emerging DBPs. There is also an urgent need for assessment of the potential risks of co-exposures to multiple DBPs in drinking water and for the development of suitable methods for this analysis. From the perspective of risk assessment, future work is expected to focus on developing and universal application of suitable *in vitro* tests for assessment of the adverse effects of each group of DBPs considering their specific mechanism of action and the biological target.

References

Adeleke GE, Adaramoye OA (2016) Modulatory role of betulinic acid in N-nitrosodimethylamine-induced hepatorenal toxicity in male Wistar rats. Hum Exp Toxicol 36:734–743

Ahotupa M, Bereziat JC, Bussacchini-Griot V et al (1987) Lipid peroxidation induced by N-nitrosodimethylamine (NDMA) in rats in vivo and in isolated hepatocytes. Free Radical Res Comm 3:285–291

Anderson LM, Giner-Sorolla A, Ebeling D (1978) Effects of imipramine, nitrite, and dimethylnitrosamine on reproduction in mice. Res Commun Chem Pathol Pharmacol 19:311–327
Anderson LM, Hagiwara A, Kovatch RM (1989) Transplacental initiation of liver, lung, neurogenic, and connective tissue tumors by N-nitroso compounds in mice. Fundam Appl Toxicol 12:604–620
Attene-Ramos MS, Wagner ED, Plewa MJ (2010) Comparative human cell toxicogenomic analysis of monohaloacetic acid drinking water disinfection byproducts. Environ Sci Technol 44:7206–7212
Backhaus T, Faust M (2012) Predictive environmental risk assessment of chemical mixtures: A conceptual framework. Environ Sci Technol 46:2564–2573
Beasley A, Belanger SE, Brill JL et al (2015) Evaluation and comparison of the relationship between NOEC and EC10 or EC20 values in chronic Daphnia toxicity testing. Environ Toxicol Chem 34:2378–2384
Belinsky SA, Devereux TR, Maronpot RR et al (1989) Relationship between the formation of promutagenic adducts and the activation of the K-ras protooncogene in lung tumors from A/J mice treated with nitrosamines. Cancer Res 49:5305–5311
Bichsel Y, von Gunten U (1999) Oxidation of iodide and hypoiodous acid in the disinfection of natural waters. Environ Sci Technol 33:4040–4045
Cancho B, Ventura P, Galceran M (2000) Determination, synthesis and survey of iodinated trihalomethanes in water treatment processes. Water Res 34:3380–3390
Cantor KP, Hoover R, Mason TJ et al (1978) Associations of cancer mortality with halomethanes in drinking water. J Natl Cancer Inst 61:979–985
Chaves RS, Guerreiro CS, Cardoso VV et al (2020) Toxicological assessment of seven unregulated drinking water Disinfection By-products (DBPs) using the zebrafish embryo bioassay. Sci Total Environ 742:140522
Chen Q, Stevens JL (1991) Inhibition of iodoacetamide and t-butylhydroperoxide toxicity in LLC-PK1 cells by antioxidants: a role for lipid peroxidation in alkylation induced cytotoxicity. Arch Biochem Biophys 284:422–430
Chen TH, Wang YH, Wu YH (2011) Developmental exposures to ethanol or dimethylsulfoxide at low concentrations alter locomotor activity in larval zebrafish: implications for behavioral toxicity bioassays. Aquat Toxicol 102:162–166
Chen YW, Xu T, Yang XY et al (2019) The toxic potentials and focus of disinfection byproducts based on the human embryonic kidney (HEK293) cell model. Sci Total Environ 664:948–957
Chhabra SK, Souliotis VL, Harbaugh JW et al (1995) O6-methylguanine DNA adduct formation and modulation by ethanol in placenta and fetal tissues after exposure of pregnant patas monkeys to N-nitrosodimethylamine. Cancer Res 55:6017–6020
Christian MS, York RG, Hoberman AM et al (2002) Oral (drinking water) two-generation reproductive toxicity study of bromodichloromethane (BDCM) in rats. Int J Toxicol 21:115–146
Chu WH, Gao NY, Yin DQ et al (2013) Formation and speciation of nine haloacetamides, an emerging class of nitrogenous DBPs, during chlorination or chloramination. J Hazard Mater 260:806–812
Clapp NK, Toya RE (1970) Effect of cumulative dose and dose rate on dimethylnitrosamine oncogenesis in RF mice. J Natl Cancer Inst 45:495–498
Cliet I, Melcion C, Cordier A (1993) Lack of predictivity of bone marrow micronucleus test versus testis micronucleus test: comparison with four carcinogens. Mutat Res 292:105–111
Dabrowska A, Nawrocki J (2009) Controversies about the occurrence of chloral hydrate in drinking water. Water Res 43:2201–2208
Deng YF, Zhang Y, Zhang R et al (2014) Mice in vivo toxicity studies for monohaloacetamides emerging disinfection byproducts based on metabolomic methods. Environ Sci Technol 48:8212–8218
Diaz Gomez MI, Tamayo D, Castro JA (1986) Administration of N-nitrosodimethylamine, N-nitrosopyrrolidine, or N'-nitrosonornicotine to nursing rats: their interactions with liver and kidney nucleic acids from sucklings. J Natl Cancer Inst 76:1133–1136

Ding XL, Zhu JY, Zhang J et al (2020) Developmental toxicity of disinfection by-product monohaloacetamides in embryo-larval stage of zebrafish. Ecotoxicol Environ Safety 189:110037
Du HY, Li JH, Moe B et al (2013) Cytotoxicity and oxidative damage induced by halobenzoquinones to T24 bladder cancer cells. Environ Sci Technol 47:2823–2830
Dubey M, Nagarkoti S, Awasthi D et al (2016) Nitric oxide-mediated apoptosis of neutrophils through caspase-8 and caspase-3-dependent mechanism. Cell Death Dis 7:e2348
Epstein D, Nolen G, Randall J et al (1992) Cardiopathic effects of dichloroacetate in the fetal Long-Evans rat. Teratology 46:225–235
Farren NJ, Ramirez N, Lee JD (2015) Estimated exposure risks from carcinogenic nitrosamines in urban airborne particulate matter. Environ Sci Technol 49:9648–9656
Fujita K, Kamataki (2001) Role of human cytochrome P450 (CYP) in the metabolic activation of N-alkylnitrosamines: application of genetically engineered Salmonella typhimurium YG7108 expressing each form of CYP together with human NADPH-cytochrome P450 reductase. Mutat Res/Fund Mol M 483:35–41
George J, Rao KR, Stern R et al (2001) Dimethylnitrosamine-induced liver injury in rats: the early deposition of collagen. Toxicology 156:129–138
Ghosh D, Choudhury ST, Das N (2012) Nanocapsulated curcumin: oral chemopreventive formulation against DEN-induced hepatocellular carcinoma in rats. Chem-Biol Interact 195:206–214
Goldman JM, Murr AS (2002) Alterations in ovarian follicular progesterone secretion by elevated exposures to the drinking water disinfection by-product dibromoacetic acid: examination of the potential site(s) of impact along the steroidogenic pathway. Toxicology 171:83–93
Hard GC, Butler WH (1970) Toxicity of dimethylnitrosamine for the rat testis. J Pathol 102:201–207
Herren-Freund SL, Pereira MA, Khoury MD et al (1987) The carcinogenicity of trichloroethylene and its metabolites, trichloroacetic acid and dichloroacetic acid, in mouse liver. Toxicol Appl Pharmacol 90:183–189
Herrmann SS, Duedahl-Olesen L, Christensen T (2015) Dietary exposure to volatile and non-volatile N-nitrosamines from processed meat products in Denmark. Food Chem Toxicol 80:137–143
Hildesheim ME, Cantor KP, Lynch CF et al (1998) Drinking water source and chlorination byproducts II. Risk of colon and rectal cancers. Epidemiology 9:29–35
Hong HC, Wu H, Chen J et al (2018) Cytotoxicity induced by iodinated haloacetamides via ROS accumulation and apoptosis in HepG-2 cells. Environ Pollut 242:191–197
Hu JL, Chu WH, Sui MH et al (2018a) Comparison of drinking water treatment processes combinations for the minimization of subsequent disinfection by-products formation during chlorination and chloramination. Chem Eng J 335:352–361
Hu SY, Gong TT, Xian QM et al (2018b) Formation of iodinated trihalomethanes and haloacetic acids from aromatic iodinated disinfection byproducts during chloramination. Water Res 147:254–263
IARC, 1999. IARC monographs on the evaluation of carcinogenic risks to humans. Some chemicals that cause tumours of the kidney or urinary bladder in rodents and some other substances, vol. 73. World Health Organization. International Agency for Research on Cancer
IARC, 2004. IARC monographs on the evaluation of carcinogenic risks to humans. In: Some drinking-water disinfectants and contaminants, including arsenic, vol. 84 World Health Organization. International Agency for Research on Cancer
IARC, 2014. IARC monographs on the evaluation of carcinogenic risks to humans. trichloroethylene, tetrachloroethylene, and some other chlorinated agents, vol. 106. World Health Organization. International Agency for Research on Cancer
Iwaniuk A, Grubczak K, Ratajczak-Wrona W (2019) N-nitrosodimethylamine (NDMA) induced apoptosis dependent on Fas/FasL complex in human leukocytes. Hum Exp Toxicol 38:578–587
Jabłoński J, Jabłońska E, Iwanowska J et al (2006) The influence of human neutrophils on N-nitrosodimethylamine (NDMA) synthesis. Immunopharmacol Immunotoxicol 28:93–102
Kane AS, Salierno JD, Gipson GT et al (2004) A video-based movement analysis system to quantify behavioral stress responses of fish. Water Res 38:3993–4001

Kargalioglu Y, McMillan BJ, Minea RA et al (2002) Analysis of the cytotoxicity and mutagenicity of drinking water disinfection by-products in Salmonella typhimurium. Teratog Carcinog Mutagen 22:113–128

Kaydos EH, Suarez JD, Roberts NL et al (2004) Haloacid induced alterations in fertility and the sperm biomarker SP22 in the rat are additive: validation of an ELISA. Toxicol Sci 81:430–442

Keegan TE, Simmons JE, Pegram RA (1998) NOAEL and LOAEL determinations of acute hepatotoxicity for chloroform and bromodichloromethane delivered in an aqueous vehicle to F344 rats. J Toxicol Environ Health Part A 55(1):65–75

Kienle C, Kohler HR, Gerhardt A (2009) Behavioural and developmental toxicity of chlorpyrifos and nickel chloride to zebrafish (Danio rerio) embryos and larvae. Ecotoxicol Environ Safety 72:1740–1747

Klein RG, Janowsky I, Pool-Zobel BL (1991) Effects of long-term inhalation of N-nitrosodimethylamine in rats. IARC Sci Publ 105:322–328

Klinefelter GR, Strader LF, Suarez JD et al (2004) Continuous exposure to dibromoacetic acid delays pubertal development and compromises sperm quality in the rat. Toxicol Sci 81:419–429

Konstantinou E, Fotopoulou F, Drosos A et al (2018) Tobacco-specific nitrosamines: a literature review. Food Chem Toxicol 118:198–203

Krasner SW, Mcguire MJ, Jacangelo JG (1989) The occurrence of disinfection by-products in US drinking water. J AWWA 81:41–53

Krasner SW, Mitch WA, McCurry DL, Hanigan D, Westerhoff, P (2013) Formation, precursors, control, and occurrence of nitrosamines in drinking water: A review. Water Res 47:4433–4450

Krasner SW, Weinberg HS, Richardson SD et al (2006) Occurrence of a new generation of disinfection byproducts. Environ Sci Technol 40:7175–7185

Landi S, Naccarati A, Ross MK et al (2003) Induction of DNA strand breaks by trihalomethanes in primary human lung epithelial cells. Mutat Res/gen Tox En 538:41–50

Liteplo RG, Meek ME (2001) N-nitrosodimethylamine: hazard characterization and exposure-response analysis. J Environ Sci Heal C 19:281–304

Liu C, Ersan MS, Plewa MJ et al (2019) Formation of iodinated trihalomethanes and noniodinated disinfection byproducts during chloramination of algal organic matter extracted from Microcystis aeruginosa. Water Res 162:115–126

Liu JQ, Zhang XR (2014) Comparative toxicity of new halophenolic DBPs in chlorinated saline wastewater effluents against a marine alga: halophenolic DBPs are generally more toxic than haloaliphatic ones. Water Res 65:64–72

Liu XY, Chen L, Yang MT et al (2020) The occurrence, characteristics, transformation and control of aromatic disinfection by-products: a review. Water Res 184:116076

Liu YJ, Zhu DC, Zhao ZH et al (2021) Comparative cytotoxicity studies of halophenolic disinfection byproducts using human extended pluripotent stem cells. Chemosphere 263:127899

Liu Z, Kullman SW, Bencic DC et al (2003) Ras oncogene mutations in diethylnitrosamine-induced hepatic tumors in medaka (Oryzias latipes), a teleost fish. Mutat Res/gen Tox En 539:43–53

Liviac D, Creus A, Marcos R (2011) Genotoxic evaluation of the non-halogenated disinfection by-products nitrosodimethylamine and nitrosodiethylamine. J Hazard Mater 185:613–618

Lodhi A, Hashmi I, Nasir H et al (2017) Effect of trihalomethanes (chloroform and bromoform) on human haematological count. J Water Health 15:367–373

Ma FJ, Wan Y, Yuan GX et al (2012) Occurrence and source of nitrosamines and secondary amines in groundwater and its adjacent jialu river basin, China. Environ Sci Technol 46:3236–3243

Margison GP, Koref MFS, Povey AC (2002) Mechanisms of carcinogenicity/chemotherapy by O6-methylguanine. Mutagenesis 17:483–487

Mitch WA, Sharp JO, Trussell R et al (2003) N-nitrosodimethylamine (NDMA) as a drinking water contaminant: a review. Environ Eng Sci 20:389–404

Monarca S, Zani C, Richardson SD et al (2004) A new approach to evaluating the toxicity and genotoxicity of disinfected drinking water. Water Res 38:3809–3819

Moser VC, Phillips PM, Levine AB et al (2004) Neurotoxicity produced by dibromoacetic acid in drinking water of rats. Toxicol Sci 79:112–122

Muellner MG, Wagner ED, McCalla K et al (2007) Haloacetonitriles vs. regulated haloacetic acids: are nitrogen-containing DBPs more toxic? Environ Sci Technol 41:645–651
Narotsky MG, Klinefelter GR, Goldman JM (2015) Reproductive toxicity of a mixture of regulated drinking-water disinfection by-products in a multigenerational rat bioassay. Environ Health Persp 123:564–570
Neft RE, Conner MK (1989) Induction of sister chromatid exchange in multiple murine tissues in vivo by various methylating agents. Teratogen Carcinogen Mutagen 9:219–237
Nishie K (1983) Comparison of the effects of N-nitrosodimethylamine on pregnant and nonpregnant Holtzman rats. Food Chem Toxicol 21:453–462
Oliveira MM, Teixeira JC, Vasconcelos-Nobrega C et al (2013) Mitochondrial and liver oxidative stress alterations induced by N-butyl-N-(4-hydroxybutyl)nitrosamine: relevance for hepatotoxicity. J Appl Toxicol 33:434–443
Parvez S, Rivera-Núñez Z, Meyer A et al (2011) Temporal variability in trihalomethane and haloacetic acid concentrations in Massachusetts public drinking water systems. Environ Res 111:499–509
Pegg AE, Dolan ME, Moschel RC (1995) Structure, function, and inhibition of O6-alkylguanine-DNA alkyltransferase. In: Cohn WE, Moldave K (eds) Progress in nucleic acid research and molecular biology, vol 51. Elsevier, Amsterdam, pp 167–223
Plewa MJ, Kargalioglu Y, Vankerk D et al (2002) Mammalian cell cytotoxicity and genotoxicity analysis of drinking water disinfection by-products. Environ Mol Mutagen 40:134–142
Plewa MJ, Muellner MG, Richardson SD (2008) Occurrence, synthesis, and mammalian cell cytotoxicity and genotoxicity of haloacetamides: an emerging class of nitrogenous drinking water disinfection byproducts. Environ Sci Technol 42:955–961
Plewa MJ; Wagner ED; Richardson SD et al (2004) Chemical and biological characterization of newly discovered iodoacid drinking water disinfection byproducts. Environ Sci Technol 38:4713–4722
Pottegard A, Kristensen KB, Ernst MT et al (2018) Use of N-nitrosodimethylamine (NDMA) contaminated valsartan products and risk of cancer: Danish nationwide cohort study. BMJ-Brit Med J 362:k3851
Powers CM, Yen J, Linney EA et al (2010) Silver exposure in developing zebrafish (Danio rerio): persistent effects on larval behavior and survival. Neurotoxicol Teratol 32:391–397
Prochazka E, Melvin SD, Escher BI (2019) Global transcriptional analysis of nontransformed human intestinal epithelial cells (FHs 74 Int) after exposure to selected drinking water disinfection by-products. Environ Health Persp 127:117006
Richardson SD, Fasano F, Ellington JJ et al (2008) Occurrence and mammalian cell toxicity of iodinated disinfection byproducts in drinking water. Environ Sci Technol 42:8330–8338
Richardson SD, Plewa MJ, Wagner ED et al (2007) Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water: a review and roadmap for research. Mutat Res/rev Mutat 636:178–242
Rogatzki MJ, Ferguson BS, Goodwin ML (2015) Lactate is always the end product of glycolysis. Front Neurosci 9:22
Rundell MS, Wagner ED, Plewa MJ (2003) The comet assay: Genotoxic damage or nuclear fragmentation? Environ Mol Mutagen 42:61–67
Sato S, Taketomi M, Morita T (1992) Simplified mouse peripheral reticulocyte micronucleus test with dimethylnitrosamine. Mutat Res/gen Tox En 278:103–107
Sawada S, Yamanaka T, Yamatsu K et al (1991) Chromosome aberrations, micronuclei and sister-chromatid exchanges (SCEs) in rat liver induced in vivo by hepatocarcinogens including heterocyclic amines. Mutat Res/fund Mol M 251:59–69
Sayess R, Khalil A, Shah M et al (2017) Comparative cytotoxicity of six iodinated disinfection byproducts on nontransformed epithelial human colon cells. Environ Sci Technol Lett 4:143–148
Schmidt MM, Dringen R (2009) Differential effects of iodoacetamide and iodoacetate on glycolysis and glutathione metabolism of cultured astrocytes. Front Neuroenerget 1:1

Simmon VF, Kauhanen K, Mortelmans K et al (1978) Mutagenic activity of chemicals identified in drinking water. Mutat Res 53:262–262
Smith JL, Wishnok JS, Deen WM (1994) Metabolism and excretion of methylamines in rats. Toxicol Appl Pharmacol 125:296–308
Smith MK, Randall JL, Read EJ et al (1989) Teratogenic activity of trichloroacetic acid in the rat. Teratology 40:445–451
Souliotis VL, Hennemanc JR, Reed CD et al (2002) DNA adducts and liver DNA replication in rats during chronic exposure to N-nitrosodimethylamine (NDMA) and their relationships to the dose-dependence of NDMA hepatocarcinogenesis. Mutat Res/fund Mol M 500:75–87
Stalter D, O'Malley E, von Gunten U et al (2016) Fingerprinting the reactive toxicity pathways of 50 drinking water disinfection by-products. Water Res 91:19–30
Stepanenko AA, Dmitrenko VV (2015) HEK293 in cell biology and cancer research: phenotype, karyotype, tumorigenicity, and stress-induced genome-phenotype evolution. Gene 569:182–190
Tates AD, Neuteboom I, Rotteveel AHM et al (1986) Persistence of preclastogenic damage in hepatocytes of rats exposed to ethylnitrosourea, diethylnitrosamine, dimethylnitrosamine and methyl methanesulphonate. Correlation with DNA O-Alkylation. Carcinogenesis 7:1053–1058
Tricker AR, Preussmann R (1991) Carcinogenic N-nitrosamines in the diet: occurrence, formation, mechanisms and carcinogenic potential. Mutat Res/gen Tox En 259:277–289
Valentin-Severin I, Le Hegarat L, Lhuguenot JC et al (2003) Use of HepG2 cell line for direct or indirect mutagens screening: comparative investigation between comet and micronucleus assays. Mutat Res/gen Tox En 36:79–90
van de Water B, Wang YP, Asmellash S et al (1999) Distinct endoplasmic reticulum signaling pathways regulate apoptotic and necrotic cell death following iodoacetamide treatment. Chem Res Toxicol 12:943–951
Veeramachaneni D, Palmer J, Klinefelter G (2007) Chronic exposure to low levels of dibromoacetic acid, a water disinfection by-product, adversely affects reproductive function in male rabbits. J Androl 28:565–577
Villanueva CM, Cantor KP, Cordier S et al (2004) Disinfection byproducts and bladder cancer: a pooled analysis. Epidemiology 15:357–367
Voet D, Voet JG, Pratt CW (2006) Fundamentals of biochemistry, 2nd edn. John Wiley & Sons, New Jersey
Wagner ED, Hsu KM, Lagunas A et al (2012) Comparative genotoxicity of nitrosamine drinking water disinfection byproducts in Salmonella and mammalian cells. Mutat Res/gen Tox En 741:109–115
Wagner ED, Plewa MJ (2017) CHO cell cytotoxicity and genotoxicity analyses of disinfection by-products: an updated review. J Environ Sci 58:64–76
Waller CL, McKinney JD (1993) Theoretical investigation into the potential of halogenated methanes to undergo reductive metabolism. J Comput Chem 14:1575–1579
Walse SS, Mitch WA (2008) Nitrosamine carcinogens also swim in chlorinated pools. Environ Sci Technol 42:1032–1037
Wang XM, Mao YQ, Tang S et al (2015) Disinfection byproducts in drinking water and regulatory compliance: a critical review. Front Environ Sci Eng 9:3–15
Ward KW, Rogers EH, Hunter ES III (2000) Comparative pathogenesis of haloacetic acid and protein kinase inhibitor embryotoxicity in mouse whole embryo culture. Toxicol Sci 53:118–121
Wei X, Wang S, Zheng W et al (2013) Drinking water disinfection byproduct iodoacetic acid induces tumorigenic transformation of NIH3T3 cells. Environ Sci Technol 47:5913–5920
White CM (2020) Understanding and preventing (N-nitrosodimethylamine) NDMA contamination of medications. Ann Pharmacother 54:611–614
Wilson JT, Pascoe PL, Parry JM et al (1998) Evaluation of the comet assay as a method for the detection of DNA damage in the cells of a marine invertebrate, Mytilus edulis L. (Mollusca: Pelecypoda). Mutat Res/fund Mol M 399:87–95
Wright JM, Schwartz J, Dockery DW (2004) The effect of disinfection by-products and mutagenic activity on birth weight and gestational duration. Environ Health Persp 112:920–925

Wu QY, Yan YJ, Lu Y et al (2020) Identification of important precursors and theoretical toxicity evaluation of byproducts driving cytotoxicity and genotoxicity in chlorination. Front Environ Sci Eng 14:25

Xie YF (2004) Disinfection byproducts in drinking water formation, analysis and control, 1st edn. Lewis Publishers, Boca Raton

Xu T, Zhao WT, Miao JJ et al (2020) A sensitive optical-based test method for the locomotor activity of earthworms. Sci Total Environ 715:136966

Yang F, Zhang J, Chu WH et al (2014) Haloactamides versus halomethanes formation and toxicity in chloraminated drinking water. J Hazard Mater 274:156–163

Yang MT, Zhang XR (2013) Comparative developmental toxicity of new aromatic halogenated DBPs in a chlorinated saline sewage effluent to the Marine polychaete platynereis dumerilii. Environ Sci Technol 47:10868–10876

Yang MT, Zhang XR (2016) Current trends in the analysis and identification of emerging disinfection byproducts. Trends Environ Anal Chem 10:24–34

Zaitseva NV, Ulanova TS, Dolgikh OV (2018) Diagnostics of early changes in the immune system due to low concentration of N-nitrosamines in the blood. B Exp Biol Med 164:334–338

Zhang B, Chen XL, Pan RJ et al (2017) Effects of three different embryonic exposure modes of 2,2',4,4'-tetrabromodiphenyl ether on the path angle and social activity of zebrafish larvae. Chemosphere 169:542–549

Zhang D, Chu WH, Yu Y et al (2018) Occurrence and stability of chlorophenylacetonitriles: A new class of nitrogenous aromatic DBPs in chlorinated and chloraminated drinking waters. Environ Sci Technol Lett 5:394–399

Zhang Y, Zhang ZY, Zhao YP et al (2013) Identifying health effects of exposure to trichloroacetamide using transcriptomics and metabonomics in mice (Mus musculus). Environ Sci Technol 47:2918–2924

Zhang YJ, Zhang B, Xu T et al (2021) Developmental and neurobehavioral assessment of low-dose N-nitrosodimethylamine (NDMA) using zebrafish embryo bioassay. Sci Total Environ 770C:144748

Zhang ZX, Zhu QY, Huang C et al (2020) Comparative cytotoxicity of halogenated aromatic DBPs and implications of the corresponding developed QSAR model to toxicity mechanisms of those DBPs: Binding interactions between aromatic DBPs and catalase play an important role. Water Res 170:115283

Zhao C, Lu Q, Gu Y et al (2019) Distribution of N-nitrosamines in drinking water and human urinary excretions in high incidence area of esophageal cancer in Huai'an, China. Chemosphere 235:288–296

Zhao J, Xu T, Yin DQ (2014) Locomotor activity changes on zebrafish larvae with different 2,2',4,4'-tetrabromodiphenyl ether (PBDE-47) embryonic exposure modes. Chemosphere 94:53–61

Zhao Y, Boyd JM, Woodbeck M, Andrews RC, Qin F, Hrudey SE, Li X (2008) Formation of N-nitrosamines from eleven disinfection treatments of seven different surface waters. Environ Sci Technol 42:4857– 4862

Zhu Y, Wang PP, Zhao J et al (2014) Dietary N-nitroso compounds and risk of colorectal cancer: a case-control study in Newfoundland and Labrador and Ontario, Canada. Brit J Nutr 111:1109–1117

Part III
Modeling and Risk Assessment

Chapter 13
Adverse Outcome Pathway Network-Based Chemical Risk Assessment Using High-Throughput Transcriptomics

Pu Xia, Pingping Wang, Wendi Fang, and Xiaowei Zhang

Abstract The lack of adequate toxicity data for the vast majority of chemicals in the environment has spurred the development of high-throughput transcriptomics (HTT) to support pathway-based screening of chemicals. The main challenge is how to decipher molecular response into adverse effects from omics data. This chapter describes an adverse outcome pathway (AOP) network-based approach for chemical screening using HTT in a compendium of human cells. First, the methodology for conducting HTT, concentration-dependent modeling analysis and AOP network analysis is introduced. Two case studies are presented: (1) cross-species comparison of transcriptomic dose–response of short-chain chlorinated paraffins and (2) high-throughput transcriptomics screening of chemicals with various known modes of action using human cells, which demonstrate the ability of HTT for chemical screening, classification and tiered chemical risk assessment by HTT-based AOP network profiles. In summary, the AOP network-based chemical screening provides a rapid and efficient omics-based approach for ranking, clustering and assessment of chemical hazards.

Introduction

A major challenge for chemical risk assessment is the lack of sufficient toxicological information for thousands of chemicals. The numbers of registered chemicals are approximately 140,000, 85,000 and 45,600 in Europe (ECHA 2016), USA (EPA 2016) and China (MEE 2013), respectively, while the majority of chemicals have inadequate toxicity data. Toxicology in the twenty-first century has envisioned a shift from traditional animal-based experiments to the mechanistic understanding of biological pathways via high-throughput screening (HTS) (Collins et al. 2008; Dix et al. 2007). In the last decade, in vitro bioassays (e.g., US EPA ToxCast and Tox21

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programs), were extensively applied to characterize the concentration responses of >10,000 chemicals on hundreds of molecular targets (Richard et al. 2016). However, in vitro bioassays are limited to the targeted biological endpoints within known toxicological pathways, which cannot capture the molecular signals over comprehensive biological space (Gaytán and Vulpe 2014; Huang et al. 2018; North and Vulpe 2010).

High-throughput transcriptomics (HTT) that can measure global gene expressions in cellular systems is a transformative phase of HTS to allow large-scale screening of chemicals (Dai 2018; Harrill et al. 2019; Mav et al. 2018; Zhang et al. 2018). Concentration-dependent HTT is a powerful approach to characterize chemical concentration-dependent responses of comprehensive biological pathways, which can be used to derive transcriptional point-of-departure (POD_T) as potency thresholds and estimate putative molecular mechanisms (Farmahin et al. 2017; Thomas et al. 2007, 2013). Our previous works have developed and applied HTT platforms in both human cells and zebrafish embryos for the screening of environmental chemicals (Fang et al. 2020; Wang et al. 2018, 2020a, b; Xia et al. 2017, 2020b; Zhang et al. 2018, 2020). US EPA ToxCast Phase III recently demonstrated the ability of HTT to yield POD_T aligned with previous ToxCast high-throughput in vitro screening assays (Harrill et al. 2021; Ramaiahgari et al. 2019). However, omics has a longstanding limitation in translating molecular perturbations into apical toxicity, which relies heavily on expert-based interpretation (Herwig et al. 2016).

The adverse outcome pathway (AOP) framework uses a modular structure to organize existing knowledge concerning the linkage between a molecular-level perturbation of a biological system and the adverse outcome(s) that the perturbation by chemicals may cause (Doering et al. 2018). An AOP describes a consecutive chain of key events (KEs) that link a molecular initiating event (MIE) to an adverse outcome (AO) across different levels of biological organization (Ankley et al. 2010). Efforts have been made to incorporate omics data into the description of KEs (Labib et al. 2015; Martens et al. 2018; Nymark et al. 2018). Genome annotations (e.g., Gene Ontology or GO terms) can be manually curated and assigned to each KE in the AOPs, and these gene-KE assignments can be used to link AOPs with omics data. Current applications of using omics data to decipher AOP events have been limited to only a few specific AOPs (Nymark et al. 2018). However, the application of deciphering omics data in the context of the entire AOP knowledge base is scarce.

Assemblages of AOPs that share one or more KEs can be interconnected to generate an AOP network (Knapen et al. 2018). AOP network can capture and extend the diversity of biological perturbations that may occur in different species and target organs (Villeneuve et al. 2018). For instance, multiple MIEs may contribute to the same AO within an AOP network. Importantly, incorporation of concentration or time-response data into the AOP network can help define the potency values of KEs that can be ranked to identify KEs sequentially affected across dose and time, which can quantitatively inform the most plausibly impacted pathways by particular chemicals (Pollesch et al. 2019; Song et al. 2020). To date, the use of the AOP network for chemical screening is still in its infancy.

Here, an AOP network-based approach for chemical risk assessment using HTT is proposed (Fig. 13.1). Briefly, concentration-dependent HTT was conducted to

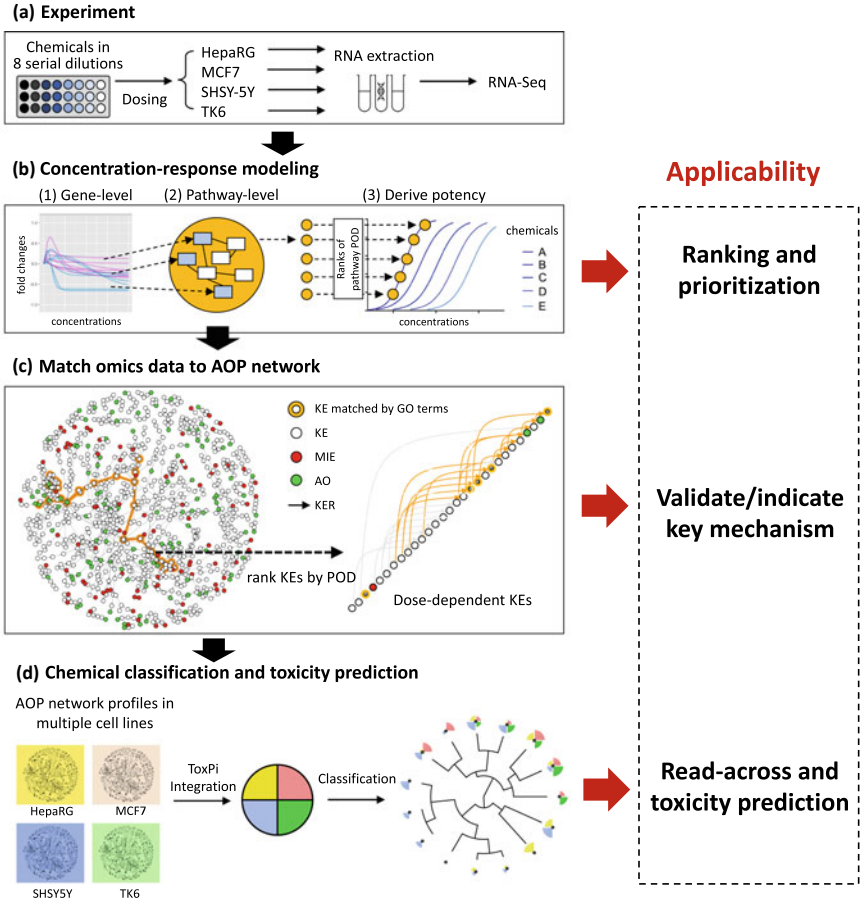

Fig. 13.1 Workflow for AOP network-based analysis of high-throughput transcriptomics (HTT) data (*Note* AOP, adverse outcome pathway; POD, point of departure; KE, key event; MIE, molecular initiating event; AO, adverse outcome; KER, key event relationship)

profile the concentration–response of thousands of genes and pathways, followed by deriving POD_T values to estimate the transcriptional potency of chemicals. The perturbed GO terms identified by HTT were matched to AOP KEs to visualize the specific patterns of perturbed AOP network by chemicals, which can be used to examine the KEs that were perturbed in a concentration-dependent manner as potential key molecular mechanisms. Lastly, the AOP network profiles of chemicals can be used for chemical classification/read-across. In the following part of this chapter, we elaborate on the details of conducting, analyzing and applying AOP network-based chemical risk assessment using high-throughput omics data.

Pipeline for AOP-Network Chemical Risk Assessment by HTT

HTT technologies can be categorized into three types by the breadth of genes measured, including quantitative reverse transcription-polymerase chain reaction (qRT-PCR) arrays for dozens of genes, e.g., ToxChip array (Crump et al. 2016; Xia et al. 2020a; Zahaby et al. 2021), reduced transcriptomics using targeted RNA-Seq on customized panels of hundreds or thousands of genes, e.g., L1000 (Subramanian et al. 2017), S1500+ (Mav et al. 2018) and whole transcriptome analysis using microarrays or RNA-Seq (Yeakley et al. 2017). The reduced transcriptomics approach has been proposed as a cost-effective proxy to whole transcriptome analysis, and it is based on the principle that a small set of key genes can represent the expression of whole gene networks (Bild et al. 2006; Dai 2018). Currently, reduced transcriptomics have been primarily developed for testing chemicals on human cells (Xia et al. 2017) and zebrafish embryos (Wang et al. 2018). The following sections describe the design, experimental setup and analysis of reduced transcriptomics for chemical screening.

Design of Reduced Gene Panels

The reduced gene panel should include maximal coverage of biological pathways and toxicologically relevant genes (Mav et al. 2018; Zhang et al. 2018). To cover comprehensive biological pathways, a data-driven approach is employed by selecting genes from biological pathway databases (e.g., GO and KEGG), followed by bioinformatic network analysis to extract key genes that play central roles (e.g., biologically-connected to a majority of genes in a pathway). To select toxicologically relevant genes, a toxicological-driven approach is used by retrieving genes from existing toxicology testing databases, such as the gene-based endpoints tested in ToxCast and the genes associated with KEs in AOP Wiki. All the genes collected from data-driven and toxicological-driven approaches are merged as a reduced gene panel, followed by validation of the coverage of biological pathways (e.g., whether >95% pathways were covered by at least three genes of the reduced gene panel). Furthermore, the ability of the reduced gene panel to represent the whole transcriptomics profiles should be evaluated by using exiting whole transcriptomics data (e.g., comparing the performance for clustering different samples by using gene expressions of reduced genes or whole genome genes). Finally, the panel of reduced genes is submitted to the targeted RNA-Seq platform to design primers available for next-generation sequencing. For instance, in the case of amplicon sequencing technology, thousands of primers for the reduced genes can be synthesized, followed by mixing in one tube. The mixed primers are optimized for multiplex PCR amplification and the following transcriptomics sequencing (Li et al. 2015).

Chemicals and Biologicals

Stock solutions of chemicals are prepared in vehicles (e.g. dimethyl sulfoxide (DMSO), methanol or water) and stored at −80 °C until used. First, the cytotoxic concentrations of chemicals need to be determined to ensure that the highest concentrations used for HTT testing do not induce secondary effects in cellular systems (e.g., apoptosis and cytotoxicity). For human cells, cell viability assays are commonly conducted on cells exposed to chemicals for 24 h. For zebrafish, embryonic toxicity assays are conducted on zebrafish embryos exposed to chemicals for 120 h.

Concentration-Dependent HTT Experiment

HTT conducted in a broad concentration-dependent manner is necessary to characterize comprehensive concentration–response of genes and pathways for quantitative estimation of the potency values of chemicals (Fig. 13.1a) (Farmahin et al. 2017; Thomas et al. 2007, 2013). Serial dilutions (e.g., 5x and 10x) of chemicals in six to ten concentrations were used to expose cells/embryos for a short time (e.g., 6 h, 12 h or 24 h). After exposure, cells were collected for transcriptomics analysis using RNA-Seq technology. Currently, a targeted RNA-Seq platform (e.g., amplicon-seq technology) (Xia et al. 2017) is the primary approach for the HTT experiment. US EPA has been applying Tempo-Seq technology that can directly measure gene expression using cell lysis without RNA extraction (Bushel et al. 2018, 2020). Targeted RNA-Seq has advantages in measuring mRNA expression with extremely low input RNA, in pg or ng level, by hybridization and sequencing with highly specific detector oligos. After sequencing, the read counts of each gene can be automatically generated during genome annotation and quality control (removing low-quality/expressed genes). A list of expressed genes in a matrix format (each column represents a sample and each row represents a gene) is used in further analysis.

Concentration–Response Modeling Analysis

Concentration–response modeling analysis is used to characterize the concentration–response curves of genes to derive gene-level POD, followed by deriving pathway-level POD and transcriptional potency of chemicals (Fig. 13.1b). To avoid confusion with the AOP pathway, in this section, the pathways are referred to as molecular pathways in the pathway databases (e.g., GO and KEGG). First, gene-level concentration–response modeling is conducted to identify the trends of genes perturbed at different toxicant concentrations. The concentration–response models of genes are fitted into two types, including monotonic and non-monotonic models (Smetanová et al. 2015). For the monotonic model, linear and non-linear curves are the two

major graphs that represent the concentration–response relationships of genes. For the non-monotonic model, gaussian and log-transformed curves are commonly used for concentration–response modeling. Multiple tools can be used for concentration–response modeling, including R language-based packages, e.g., drc (Ritz et al. 2015) and DRomics (Larras et al. 2018) and benchmark dose software developed by US EPA (Yang et al. 2007). The best-fitted concentration–response model is assigned to each gene according to the pre-set criteria (e.g. the model that has the lowest Akaike's Information Criterion (AIC) value). Then the gene-level POD values are derived from the best fitted concentration–response model by plotting toxicant concentration against the benchmark response (e.g., the mean value + 3 times the standard derivation of vehicle controls of that gene; 1.5-fold changes).

Pathway-level POD values can be calculated by matching genes to pathways (GO terms or KEGG pathways), which is important to translate gene-level changes to higher biological level information of perturbed pathways. The criterium to define a pathway as a potentially perturbed pathway is that the number of genes matched to that pathway is at least three. The three-gene cut-off has been widely used because three is the minimum number required to define the mean value and SD for a pathway (Thomas et al. 2007). The matched pathways can be ranked by pathway-level POD values to identify potentially sensitive pathways perturbed by chemical exposure. Moreover, the biological potency of chemicals can be estimated from the pathway-based profiles, such as the concentrations against the top 20% perturbed pathways, and the concentrations against the top number of perturbed pathways (Farmahin et al. 2017). The transcriptional potency of chemicals can relatively well distinguish their low and high potency. However, the accuracy of transcriptional potency needs further validation, for example, by using an in vivo experiment. In addition, the ability of transcriptional potency to estimate the potency of apical endpoints is unclear. Some studies have demonstrated the consistency between transcriptional potency and apical potency by in vivo testing (e.g., liver transcriptomics vs liver histopathology in rats) (Thomas et al. 2012). However, a limited number of studies have evaluated the ability of in vitro omics-derived potency to predict in vivo-based potency. The in vitro omics-derived potency value is usually lower than in vivo-based potency because the molecular-level responses happen earlier and at lower concentrations than apical effects. Whether there exists an uncertainty factor between in vitro omics-derived potency and in vivo-based potency remains to be established.

AOP Network Analysis

AOP network analysis can integrate the above pathway-level information into a systematic and topological framework. Briefly, the identified pathways (e.g., GO terms) can be matched to KEs via GO-KE annotation database in AOP Wiki using the R package AOP (Burgoon 2015). KEs that meet the concentration-dependent ranks in a connected path in the AOP network indicate a potential key molecular mechanism of that chemical (Fig. 13.1c). The concentration-dependent KEs may

be present in an existing AOP, and may also indicate a putative AOP that was not previously curated in AOP Wiki database. The identified putative AOP may support the investigation of new potential AOPs, which need further evaluation and validation such as using in vitro or in vivo bioassays. Moreover, rich network-based information can be extracted, such as the central KEs that are connected to the largest number of matched KEs, and the longest path in AOP network that has the largest number of matched KEs. For instance, the longest path between an MIE and AO may suggest the most detailed mechanistic description (Pollesch et al. 2019). Lastly, the weight of edges and nodes in the AOP network can be assigned according to AOP Wiki database. This is due to the fact that, during the development of AOPs, the KEs or key event relationships (KERs) are based on sources with different weight-of-evidence (e.g., in vivo or in vitro studies; validated or not). By assigning weight values, the identified critical paths in AOP network can be grouped into different levels of confidence.

The AOP network profiles of chemicals can be integrated into ToxPi for chemical classification. ToxPi is an interactive graphical user interface developed by the US EPA, which is a powerful tool for visual interpretation and transparent weight-of-evidence analysis (Reif et al. 2013). The AOP network profiles can be deconstructed into a matrix of all possible linear paths and each path can be scored by the mean values of POD of matched KEs (if there are no matched KEs, the score of that path is set as 'NA', i.e., 'not available'). The scored AOP network profiles can be submitted to ToxPi to generate a pie plot. If multiple cell lines are used for HTT testing, the AOP network profiles from multiple cell lines can be used to generate an integrated plot (Fig. 13.1d) (Grimm et al. 2016). The AOP network-based ToxPi profiles can be used for clustering analysis. Chemicals clustered into the same group are assumed to present similar molecular modes of action, which can be used to guide future evaluations of the toxicity of these chemicals.

Examples of AOP Network-Based Chemical Screening

Cross-Species Comparison of Transcriptomic Dose–Response of Short-Chain Chlorinated Paraffins

Short-chain chlorinated paraffins (SCCPs) have attracted ever-increasing attention because of their toxicological potential in humans and wildlife at environmentally relevant doses. However, limited information is available regarding mechanistic differences across species in terms of the biological pathways that are impacted by SCCP exposure. Here, a concentration-dependent reduced human transcriptome (RHT) analysis approach was used to evaluate fifteen SCCPs in HepG2 cells and compared with our previous results using a reduced zebrafish transcriptome (RZT) analysis approach in zebrafish embryos (ZFE) (Xia et al. 2021). Generally, SCCPs induced a broader suite of biological pathways in ZFE than HepG2, while all fifteen

SCCPs were more potent in HepG2 compared to ZFE. Despite these general differences, the transcriptional potency of SCCPs in both model systems showed a significant linear relationship ($p = 0.0017$, $r^2 = 0.57$). $C_{10}H_{14}Cl_8$ was the most potent SCCP, while $C_{10}H_{17}Cl_5$ was the least potent in both ZFE and HepG2. An AOP network-based analysis demonstrated model-specific responses, such as xenobiotic metabolism that may be mediated by different nuclear receptor-mediated pathways between HepG2 (e.g., activation of the constitutive androstane receptor or CAR and the aryl hydrocarbon receptor or AhR) and ZFE (e.g., activation of the pregnane X receptor or PXR) (Fig. 13.2a). Moreover, induced transcriptional changes in ZFE

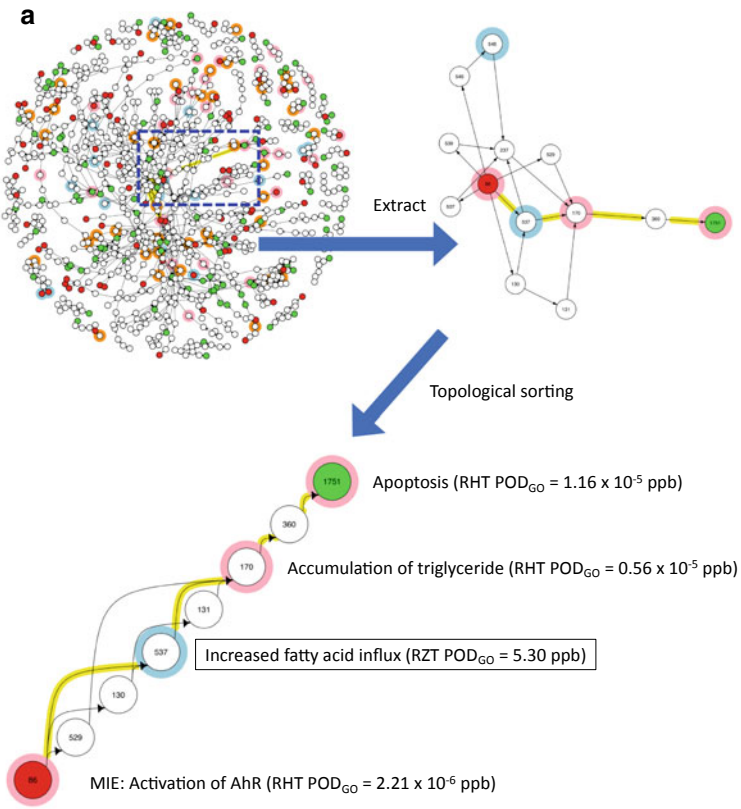

Fig. 13.2 A demonstration of AOP networks of SCCPs covered by both reduced human transcriptome (RHT) and reduced zebrafish transcriptome (RZT) analysis (Xia et al. 2021). **a** 2,3,4,5,6,7,8,9-Octachlorodecane ($C_{10}H_{14}Cl_8$) **b** 1,2,5,6,9,10-Hexachlorodecane ($C_{10}H_{16}Cl_6$). Red, green and white dots represent molecular initiating events (MIE), adverse outcomes (AO) and key events (KE), respectively. Dots encircled with pink, light blue and orange represent AOP-associated events matched by only RHT, only RZT, and both RHT and RZT, respectively. Edges in yellow represent an extracted path starting from an MIE to an AO. AhR, aryl hydrocarbon receptor. nAChR, nicotinic acetylcholine receptor

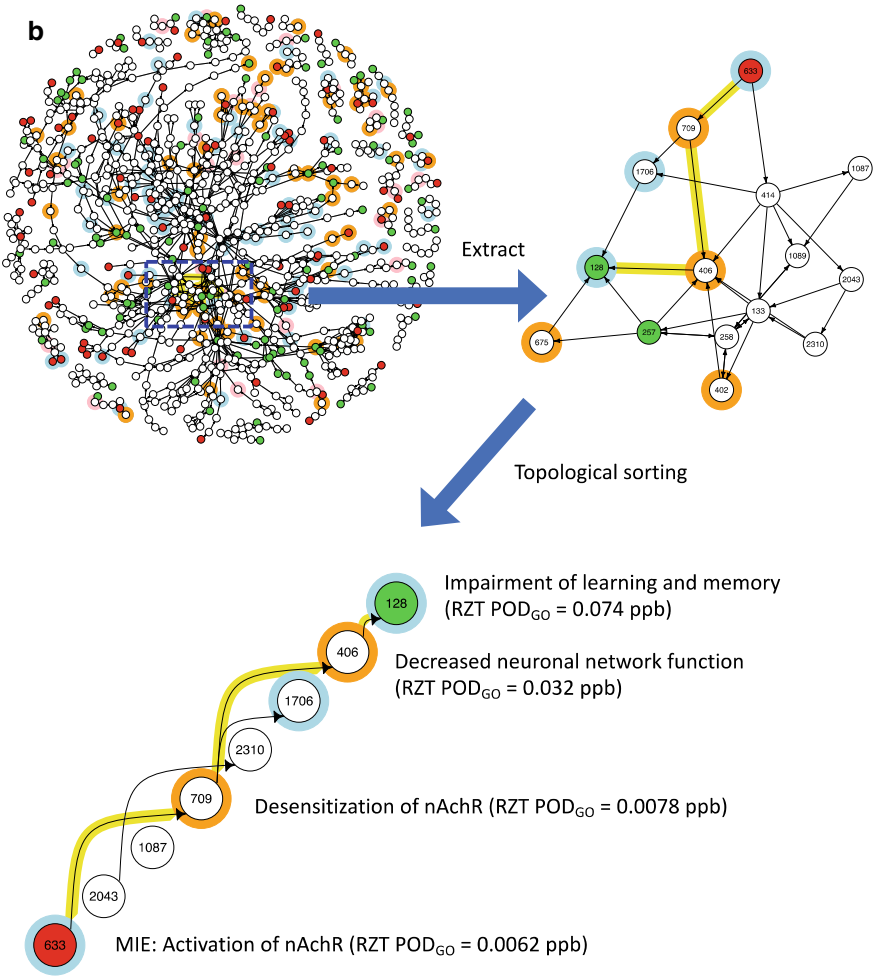

Fig. 13.2 (continued)

associated with pathways and molecular initiating events (e.g., activation of nicotinic acetylcholine receptor or nAChR) suggest that SCCPs may disrupt neural development processes (Fig. 13.2b). This study demonstrated that the cross-model comparison of concentration-dependent transcriptomics represents a promising approach to assess and prioritize SCCPs.

High-Throughput Transcriptomics Screening of Chemicals with Various Known Modes of Action Using Human Cells

The current application of HTT is limited due to the lack of systematic evaluation of its performance for chemical screening. Concentration-dependent transcriptomics of 32 chemicals with different modes of action (i.e. genotoxicity, endocrine disruption and metabolic activity) (Table 13.1) were conducted on HepG2 and MCF7 cells using RHT approach. The pathway-based profiles identified by RHT were used to group chemicals generally consistent with their known modes of action. Comparison of the RHT and ToxCast in vitro bioassay profiles demonstrated that POD values of the pathways associated with DNA repair (i.e., GO:0000729 and GO:0006287) had a significant linear correlation (p-value < 0.05). Furthermore, the identified pathways were matched to KEs in an AOP network that arranged biological pathways into topological structures, which showed that RHT and ToxCast indicated different potentially perturbed KEs (DNA damage-associated events for RHT; hormone disruption-associated events for ToxCast). For concentration-dependently perturbed KEs, RHT and ToxCast both identified paths starting from MIE of AhR for most chemicals (Fig. 13.3), while RHT specifically identified paths involved in cellular stress processes, including suppression of constitutive androstane receptor, activation of phosphatidylinositol-3 kinase (PI3K), an increase in insulin and activation of transcription factor NRF2 (nuclear factor erythroid-2-related factor 2). The ToxPi clustering of AOP network-based profiles for chemicals tested in both HepG2 and MCF7 cells showed distinct groups of chemicals with different known modes of action (genotoxic, endocrine disrupting and metabolic activity) (Fig. 13.4). This study demonstrated that RHT can provide a novel approach for chemical screening and classification, which can be complementary to in vitro bioassays.

Challenges and Perspectives

The emergent need for new approach methodologies (NAMs) has been proposed to accelerate the pace of chemical risk assessment (Harrill et al. 2019; Kavlock et al. 2018). NAMs aim to provide efficient large-scale information on chemical hazards by HTS alternatives to animal testing approaches, including a battery of high-throughput in vitro bioassays and computational models for prioritization and screening of chemicals (EPA 2018). HTT performed in in vitro test systems is considered a novel type of NAM, but its application is still in its infancy. The validity of omics-identified KEs to explain potential apical effects needs to be evaluated by comparing them to traditional in vitro bioassays or in vivo assays. Besides, multiple omics approaches (e.g., proteomics and metabolomics) are encouraged to investigate the AOP network profiles of chemicals across broad biological levels, which also requires the development of a system for biological analysis pipeline to interpret the multiple-omics data. Moreover, omics analysis at different time points is

Table 13.1 Thirty-two chemicals used for concentration-dependent transcriptomics analysis in human cells

Chemicals	Full names	CAS	Maximal dose (μM)	Minimal dose (μM)	Known MOA[a]	Tested cells[b]
2,4-D	2,4-dichlorophenoxyacetic acid	94-75-7	8.79E+01	8.79E-06	Metabolic	HepG2
4NQO	4-nitroquinoline 1-oxide	56-57-5	1.41E-02	1.41E-09	Genotoxic	HepG2, MCF7
ATZ	Atrazine	1912-24-9	3.88E+01	3.88E-06	Metabolic	HepG2, MCF7
BaP	Benzo(a)pyrene	50-32-8	3.96E-01	3.96E-08	Genotoxic	HepG2, MCF7
BbF	Benzo[B]Fluoranthene	205-99-2	9.91E+00	9.91E-07	Genotoxic	HepG2, MCF7
BPA	Bisphenol A	80-05-7	1.00E+01	1.00E-06	EDC	HepG2, MCF7
BPB	Bisphenol B	77-40-7	2.00E+01	2.00E-06	EDC	HepG2
BPF	Bisphenol E	77-40-7	8.00E+01	8.00E-06	EDC	HepG2
BPS	Bisphenol S	80-09-1	8.00E+01	8.00E-06	EDC	HepG2
CDZ	Carbendazim	10605-21-7	2.42E+00	2.42E-07	Metabolic	HepG2
CIS	Cisplatin	15663-27-1	3.93E-01	3.93E-08	Genotoxic	HepG2, MCF7
CLP	Chlorophene	120-32-1	1.54E+01	1.54E-06	Unspecific	MCF7
CPD	Cyprodinil	121552-61-2	2.21E+00	2.21E-07	Metabolic	MCF7
DCA	Dichloroacetic acid	79-43-6	5.00E+01	5.00E-06	Metabolic	HepG2
DCF	Diclofenac	15307-86-5	4.85E+01	4.85E-06	Metabolic	MCF7
DIR	Diuron	330-54-1	6.90E+01	6.90E-06	Metabolic	MCF7

(continued)

Table 13.1 (continued)

Chemicals	Full names	CAS	Maximal dose (μM)	Minimal dose (μM)	Known MOA[a]	Tested cells[b]
DIZ	Diazinon	333-41-5	6.31E+01	6.31E-06	Neuroactive	MCF7
E1	Estrone	53-16-7	1.33E+02	1.33E-05	EDC	HepG2
FIP	Fipronil	120068-37-3	3.04E+01	3.04E-06	EDC	MCF7
FSK	Forskolin	66575-29-9	4.00E+01	4.00E-06	EDC	HepG2, MCF7
GES	Genistein	446-72-0	3.70E+00	3.70E-07	EDC	HepG2, MCF7
H2O2	Hydrogen peroxide	7722-84-1	3.00E+01	0.000003	Genotoxic	HepG2, MCF7
ISZ	Isoniazid	54-85-3	1030	0.000103	Metabolic	MCF7
MMC	Mitomycin C	50-07-7	9.87E-02	9.87E-09	Genotoxic	HepG2
MTC	Metolachlor	51218-45-2	4.36E+01	0.00000436	Metabolic	HepG2
5-Cl-6-HO-BDE-47	5-Chloro-6-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether	NA	1.50E+00	0.00000015	Metabolic	HepG2
PCZ	Propiconazole	60207-90-1	5.42E+01	0.000005422	EDC	MCF7
PFOA	Perfluorooctanoic acid	335-67-1	9.39E+01	0.00000939	Metabolic	HepG2, MCF7
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin	1746-01-6	6.00E-03	6.00E-10	AHR	HepG2
TCS	Triclosan	3380-34-5	1.00E+01	0.000001	Metabolic	HepG2
TEB	Tebuconazole	107534-96-3	7.15E+01	0.000007154	Metabolic	HepG2
TPP	Triphenylphosphate	115-86-6	4.98E+01	0.000004978	Neuroactive	MCF7

[a]MOA, mode of action; EDC, endocrine-disrupting chemicals; AHR, agonist of aryl hydrocarbon receptor. [b]HepG2, human liver cancer cell lines; MCF7, human breast cancer cell lines

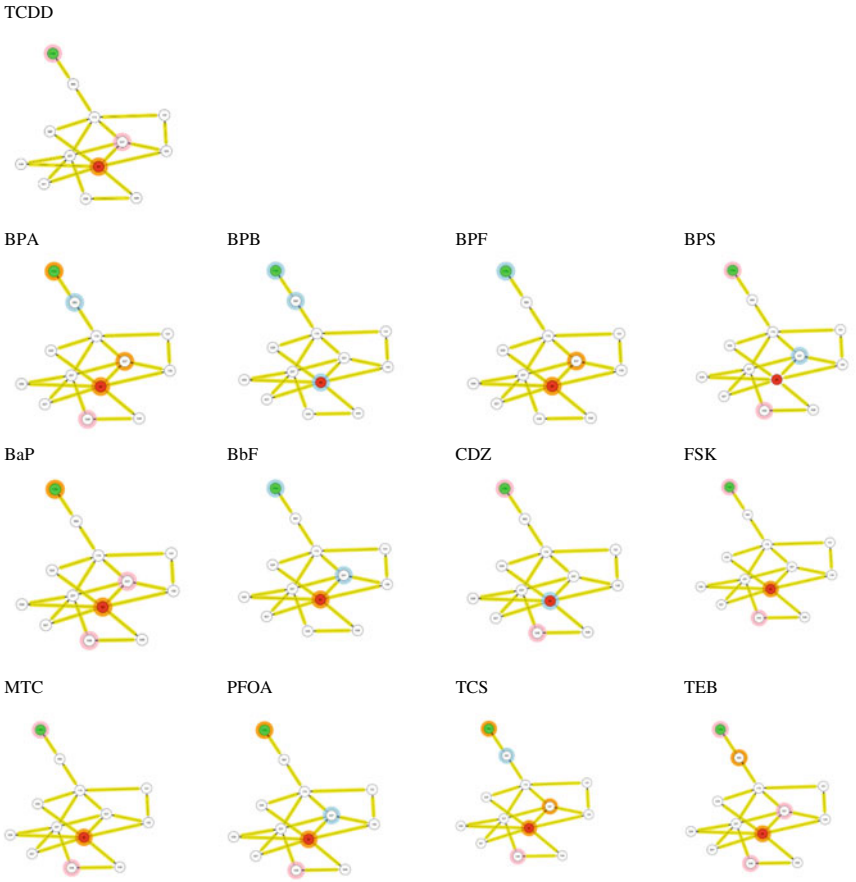

Fig. 13.3 Plotting of an AOP network-extracted pathway starting from MIE (AhR activation-associated KE) to AO (apoptosis-associated KE) that was matched by both RHT and ToxCast for 13 chemicals. Dots encircled with pink, light blue and orange represent AOP-associated events matched by only RHT, only ToxCast and both by RHT and ToxCast, respectively. Red, green and white dots represent molecular initiating events (MIE), adverse outcomes (AO) and key events (KE), respectively. For an explanation of abbreviations see Table 13.1

needed to identify KEs that become perturbed early. The time-dependent results can be combined with concentration-dependent omics data to obtain three-dimension patterns of AOP network profiles. The AOP Wiki database is still being updated and newly developed AOPs are being added, which will improve the quality of the currently available AOP network.

Lastly, HTT is proposed to be incorporated into the tiered testing of chemicals (Fig. 13.5). First, concentration-dependent HTT is used to profile the disrupted biological pathways and transcriptional potency of a group of reference chemicals with well-known toxicity information, which helps to establish a reference database.

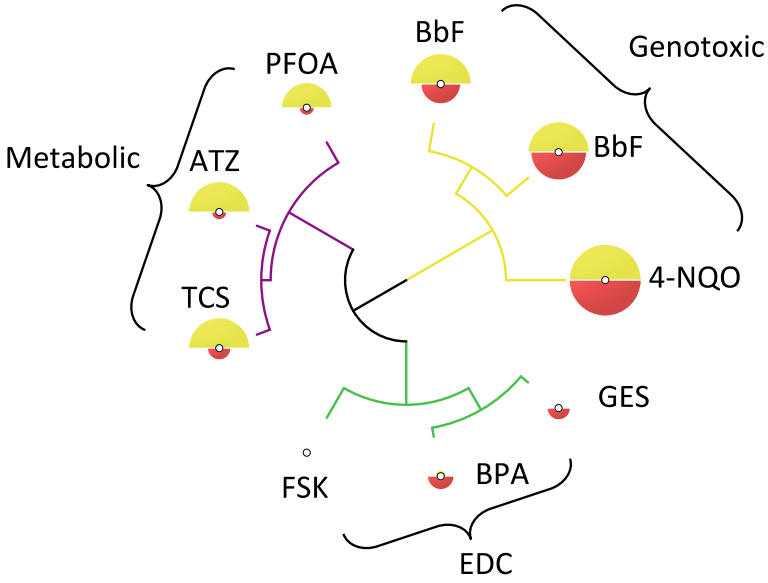

Fig. 13.4 ToxPi clustering plot of scores for AOP network-based profiles of chemicals tested by high-throughput transcriptomics (HTT) in HepG2 and MCF7 cells. The sectors in yellow and red colors represent the ToxPi scores of HTT pathway profiles in HepG2 and MCF7, respectively. For an explanation of abbreviations of chemicals see Table 13.1

Then, the effects of emerging chemicals for which there is no toxicological data are analyzed using HTT, and the results are compared to the HTT-based reference database to prioritize chemicals with similar profiles to reference chemicals. The prioritized chemicals are further evaluated by a set of in vitro bioassays that cover multiple endpoints, including hepatotoxicity, immunotoxicity, developmental toxicity, mitochondrial toxicity, and developmental neurotoxicity as proposed by the US EPA (Patlewicz et al. 2019). The chemicals that are validated to be able to induce in vitro toxicity are submitted to risk assessment by evaluating the margin of exposure (Buesen et al. 2017), which is finally applied to chemical management. In conclusion, HTT can provide a novel approach for NAM-based chemical risk assessment.

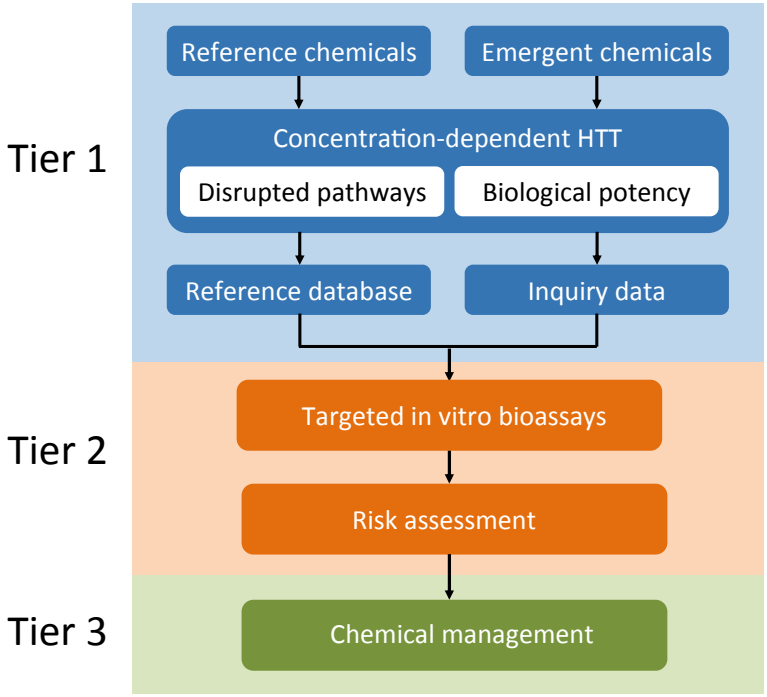

Fig. 13.5 Tiered approach for HTT-based chemical risk assessment

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References

Ankley GT, Bennett RS, Erickson RJ et al (2010) Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. Environ Toxicol Chem 29(3):730–741

Bild AH, Yao G, Chang JT et al (2006) Oncogenic pathway signatures in human cancers as a guide to targeted therapies. Nature 439(7074):353–357

Buesen R, Chorley BN, da Silva LB et al (2017) Applying 'omics technologies in chemicals risk assessment: report of an ECETOC workshop. Regul Toxicol Pharmacol 91:S3–S13

Burgoon LD (2015) AOP: a biocondcutor package for sufficient causal analysis in pathway-based screening of drugs and chemicals for adversity. BioRxiv 029694

Bushel PR, Ferguson SS, Ramaiahgari SC et al (2020) Comparison of normalization methods for analysis of TempO-Seq targeted RNA sequencing data. Front Genet 11:594

Bushel PR, Paules RS, Auerbach SS (2018) A comparison of the TempO-Seq S1500+ platform to RNA-Seq and microarray using rat liver mode of action samples. Front Genet 9:485

Collins FS, Gray GM, Bucher JR (2008) Transforming environmental health protection. Sci 319(5865):906
Crump D, Farhat A, Chiu S et al (2016) Use of a novel double-crested cormorant ToxChip PCR array and the EROD assay to determine effects of environmental contaminants in primary hepatocytes. Environ Sci Technol 50(6):3265–3274
Dai J (2018) Reduced transcriptomic approach for screening and prediction of chemical toxicity. Chem Res Toxicol 31(7):532–533
Dix DJ, Houck KA, Martin MT et al (2007) The ToxCast program for prioritizing toxicity testing of environmental chemicals. Toxicol Sci 95(1):5–12
Doering JA, Wiseman S, Giesy JP et al (2018) A cross-species quantitative adverse outcome pathway for activation of the aryl hydrocarbon receptor leading to early life stage mortality in birds and fishes. Environ Sci Technol 52(13):7524–7533
ECHA (2016) Guidance on information requirements and chemical safety assessment Chapter R.7b: endpoint specific guidance. Available at: https://echa.europa.eu/guidance-documents/guidance-on-information-requirements-and-chemical-safety-assessment
EPA (2016) About the TSCA chemical substance inventory. Available at: https://www.epa.gov/tsca-inventory/about-tsca-chemical-substance-inventory
EPA (2018) Strategic plan to promote the development and implementation of alternative test methods within the TSCA program. USEPA, Washington, DC
Fang W, Peng Y, Yan L et al (2020) A tiered approach for screening and assessment of environmental mixtures by omics and in vitro assays. Environ Sci Technol 54(12):7430–7439
Farmahin R, Williams A, Kuo B et al (2017) Recommended approaches in the application of toxicogenomics to derive points of departure for chemical risk assessment. Arch Toxicol 91(5):2045–2065
Gaytán BD, Vulpe CD (2014) Functional toxicology: tools to advance the future of toxicity testing. Front Genet 5:110
Grimm FA, Iwata Y, Sirenko O et al (2016) A chemical–biological similarity-based grouping of complex substances as a prototype approach for evaluating chemical alternatives. Green Chem 18(16):4407–4419
Harrill JA, Everett LJ, Haggard DE et al (2021) High-throughput transcriptomics platform for screening environmental chemicals. Toxicol Sci 181(1):68–89
Harrill JA, Shah I, Setzer RW et al (2019) Considerations for strategic use of high-throughput transcriptomics chemical screening data in regulatory decisions. Curr Opin Toxicol 15:64–75
Herwig R, Hardt C, Lienhard M et al (2016) Analyzing and interpreting genome data at the network level with ConsensusPathDB. Nat Protoc 11(10):1889
Huang R, Xia M, Sakamuru S et al (2018) Expanding biological space coverage enhances the prediction of drug adverse effects in human using in vitro activity profiles. Sci Rep 8(1):1–12
Kavlock RJ, Bahadori T, Barton-Maclaren TS et al (2018) Accelerating the pace of chemical risk assessment. Chem Res Toxicol 31(5):287–290
Knapen D, Angrish MM, Fortin MC et al (2018) Adverse outcome pathway networks I: development and applications. Environ Toxicol Chem 37(6):1723–1733
Labib S, Williams A, Yauk CL et al (2015) Nano-risk science: application of toxicogenomics in an adverse outcome pathway framework for risk assessment of multi-walled carbon nanotubes. Part Fibre Toxicol 13(1):15
Larras F, Billoir E, Baillard V et al (2018) DRomics: a turnkey tool to support the use of the dose–response framework for Omics data in ecological risk assessment. Environ Sci Technol 52(24):14461–14468
Li W, Turner A, Aggarwal P et al (2015) Comprehensive evaluation of AmpliSeq transcriptome, a novel targeted whole transcriptome RNA sequencing methodology for global gene expression analysis. BMC Genom 16(1):1–13
Martens M, Verbruggen T, Nymark P et al (2018) Introducing WikiPathways as a data-source to support adverse outcome pathways for regulatory risk assessment of chemicals and nanomaterials. Front Genet 9:661

Mav D, Shah RR, Howard BE et al (2018) A hybrid gene selection approach to create the S1500+ targeted gene sets for use in high-throughput transcriptomics. PLoS ONE 13(2):e0191105
MEE (2013) List of existing chemical substances in China. Available at: https://www.mee.gov.cn/ywgz/gtfwyhxpgl/hxphjgl/wzml/
North M, Vulpe CD (2010) Functional toxicogenomics: mechanism-centered toxicology. Int J Mol Sci 11(12):4796–4813
Nymark P, Rieswijk L, Ehrhart F et al (2018) A data fusion pipeline for generating and enriching adverse outcome pathway descriptions. Toxicol Sci 162(1):264–275
Patlewicz G, Richard AM, Williams AJ et al (2019) A chemical category-based prioritization approach for selecting 75 per-and polyfluoroalkyl substances (PFAS) for tiered toxicity and toxicokinetic testing. Environ Health Perspect 127(01):014501
Pollesch NL, Villeneuve DL, O'Brien JM (2019) Extracting and benchmarking emerging adverse outcome pathway knowledge. Toxicol Sci 168(2):349–364
Ramaiahgari SC, Auerbach SS, Saddler TO et al (2019) The power of resolution: contextualized understanding of biological responses to liver injury chemicals using high-throughput transcriptomics and benchmark concentration modeling. Toxicol Sci 169(2):553–566
Reif DM, Sypa M, Lock EF et al (2013) ToxPi GUI: an interactive visualization tool for transparent integration of data from diverse sources of evidence. Bioinformatics 29(3):402–403
Richard AM, Judson RS, Houck KA et al (2016) ToxCast chemical landscape: paving the road to 21st century toxicology. Chem Res Toxicol 29(8):1225–1251
Ritz C, Baty F, Streibig JC et al (2015) Dose-response analysis using R. PLoS ONE 10(12):e0146021
Smetanová S, Riedl J, Zitzkat D et al (2015) High-throughput concentration–response analysis for omics datasets. Environ Toxicol Chem 34(9):2167–2180
Song Y, Xie L, Lee Y et al (2020) De novo development of a quantitative adverse outcome pathway (qAOP) network for ultraviolet B (UVB) radiation using targeted laboratory tests and automated data mining. Environ Sci Technol 54(20):13147–13156
Subramanian A, Narayan R, Corsello SM et al (2017) A next generation connectivity map: L1000 platform and the first 1,000,000 profiles. Cell 171(6):1437–1452
Thomas RS, Allen BC, Nong A et al (2007) A method to integrate benchmark dose estimates with genomic data to assess the functional effects of chemical exposure. Toxicol Sci 98(1):240–248
Thomas RS, Clewell HJ III, Allen BC et al (2012) Integrating pathway-based transcriptomic data into quantitative chemical risk assessment: a five chemical case study. Mutat Res 746(2):135–143
Thomas RS, Wesselkamper SC, Wang NCY et al (2013) Temporal concordance between apical and transcriptional points of departure for chemical risk assessment. Toxicol Sci 134(1):180–194
Villeneuve DL, Angrish MM, Fortin MC et al (2018) Adverse outcome pathway networks II: network analytics. Environ Toxicol Chem 37(6):1734–1748
Wang P, Wang Z, Xia P et al (2020a) Concentration-dependent transcriptome of zebrafish embryo for environmental chemical assessment. Chemosphere 245:125632
Wang P, Xia P, Wang Z et al (2020b) Evidence-based assessment on environmental mixture using a concentration-dependent transcriptomics approach. Environ Pollut 265:114839
Wang P, Xia P, Yang J et al (2018) A reduced transcriptome approach to assess environmental toxicants using zebrafish embryo test. Environ Sci Technol 52(2):821–830
Xia P, Crump D, Chiu S et al (2020a) Toxicogenomic assessment of complex chemical signatures in double-crested cormorant embryos from variably contaminated great lakes sites. Environ Sci Technol 54(12):7504–7512
Xia P, Peng Y, Fang W et al (2021) Cross-model comparison of transcriptomic dose-response of short-chain chlorinated paraffins. Environ Sci Technol 55(12):8149–8158
Xia P, Zhang H, Peng Y et al (2020b) Pathway-based assessment of single chemicals and mixtures by a high-throughput transcriptomics approach. Environ Int 136:105455
Xia P, Zhang X, Zhang H et al (2017) Benchmarking water quality from wastewater to drinking waters using reduced transcriptome of human cells. Environ Sci Technol 51(16):9318–9326
Yang L, Allen BC, Thomas RS (2007) BMDExpress: a software tool for the benchmark dose analyses of genomic data. BMC Genomics 8(1):1–8

Yeakley JM, Shepard PJ, Goyena DE et al (2017) A trichostatin A expression signature identified by TempO-Seq targeted whole transcriptome profiling. PLoS ONE 12(5):e0178302

Zahaby Y, Xia P, Crump D et al (2021) ToxChip PCR arrays for two arctic-breeding seabirds: applications for regional environmental assessments. Environ Sci Technol 55(11):7521–7530

Zhang X, Wang P, Xia P (2020) Dose-dependent transcriptomic approach for mechanistic screening in chemical risk assessment. In: Jiang G, Li X (eds) A new paradigm for environmental chemistry and toxicology. Springer, Singapore, pp 33–56

Zhang X, Xia P, Wang P et al (2018) Omics advances in ecotoxicology. Environ Sci Technol 52(7):3842–3851

Chapter 14
Advances in In Silico Toxicity Assessment of Nanomaterials and Emerging Contaminants

Xuehua Li, Yang Huang, and Jingwen Chen

Abstract Risk assessment of engineered nanomaterials (ENMs) and other emerging substances is essential to protect human health and the environment. Non-testing approaches in hazard assessment are necessary, considering cost and time efficiency to assess potential risks. This chapter reviews diverse in silico tools including machine learning models and molecular simulations for predicting toxicological properties or the adverse effects of nanomaterials and emerging contaminants during the last decade. In silico approaches used as alternatives to animal models are helping to address the ethical, economic, and time constraints of traditional toxicology while also advancing mechanistic understanding. In the future, comprehensive databases need to be built for substituting sparse literature data; co-exposure of nanomaterials and chemicals should be considered in risk assessment.

Machine Learning (ML) in Computational Toxicology: Unlocking and Empowering Toxicity Assessment

Artificial intelligence (AI) and machine learning (ML) have greatly contributed to the set of computational tools for enhancing and improving the simulation and modeling processes in toxicology. ML has already been successfully applied to simulate the biokinetics and interactions of nanomaterials and emerging contaminants in varying environments. The most studied approaches for the assessment of nanomaterial- and emerging contaminant-induced toxicity are structure-based mathematical models and such as quantitative structure–activity relationship (QSAR) models. ML techniques have been proven very helpful to gain an insight into features affecting toxicity, predicting potential adverse effects as part of proactive risk analysis, and informing safe design. ML models for predicting the toxicity of nanomaterials and emerging contaminants are discussed below.

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ML Models in Computational Nanotoxicology

The list of consumer goods developed in the nano-field is constantly increasing. A review of the 2021 version of the Nanotechnology Products Database (NPD) (https://product.statnano.com, accessed: May 2021) revealed more than 9000 commercially available nanomaterials distributed over 63 countries. A large number of nanoparticles and the variety of their characteristics including sizes and coatings suggest that the only rational approach that avoids testing of every single nanoparticle is to find relationships between nanoparticle properties (i.e., physicochemical characteristics) and their toxicity. Some computational tools such as QSAR are essential for increasing throughput, reducing the burden of animal testing, providing details of the toxicity mechanisms, and generating novel hypotheses for risk assessment.

While the potential health effects of ENMs in humans remain uncertain, evidence links the exposure to nanoscale particulates from ambient and occupational sources with increased susceptibility to lung infections, including pneumonia (Cho et al. 2010; JJ Li et al. 2010b). The main route of nanomaterial exposure is the inhalation of aerosols created during industrial processes (Christensen et al. 2010). Lung inflammation, the consequence of pulmonary deposition of pathogenic particles, can be seen as a rational priority toxicity endpoint since a spectrum of adverse pulmonary effects are to be anticipated (Cho et al. 2010; JJ Li et al. 2010b). In addition, inflammatory effects in the lungs generated by particle deposition could drive effects in secondary organs such as the blood vessel wall and the brain (JJ Li et al. 2010b; Mills et al. 2009). The increased risks of pneumonia and morbidity are also observed in welders exposed to fumes that are rich in metal oxide nanoparticles (MeONPs) (Andujar et al. 2014). Up to 80% of the airborne particulates from welding operations are submicron-sized particles (Dasch and D'Arcy 2008), and nano-scale particles (20–25 nm diameter) composed of iron, manganese and chromium oxides have been clearly identified within alveolar macrophages and in fibrous regions of the lung (Andujar et al. 2014). Some MeONPs were found to induce strong immune responses via inflammasome and Toll-like receptor activation in vitro (Li et al. 2014) and in vivo (Cho et al. 2010), evidenced by the substantial release of pro-inflammatory cytokine (IL-1β).

From the perspective of nanosafety assessment, ML models have been developed for predicting lung toxicity of nanomaterials and other nano-bio interactions (Table 14.1). Most of the published models were developed for a traditional toxicological endpoint: cell death. As the toxicities of nanoparticles beyond merely killing cells have been underlined recently, in silico models for predicting the interactions between ENMs and immune systems as well as reproductive systems have been developed.

Most models were developed for predicting cytotoxicity. For instance, Puzyn et al. (2011) developed a nano-QSAR model to describe the cytotoxicity of 16 different types of MeONPs in *Escherichia coli.* using two quantum chemical descriptors (ΔH: enthalpy of formation of a gaseous cation and LUMO: energy of the lowest unoccupied molecular orbital). Oh et al. (2016) established cellular toxicity models to predict the toxicity of 17 quantum dots using surface properties, diameters of the quantum dots, assay types and exposure times. Nano-QSARs were developed to predict the

Table 14.1 Machine learning (ML) models for predicting the toxicity of nanomaterials

Nanomaterials	Organism/cell	Key descriptors	Endpoint	Reference
16 MeONPs	*E. coli*	ΔH, LUMO	$\log(1/EC_{50})$	Puzyn et al. 2011
17 quantum dots	Epithelial cells Fibroblast cells	Shell, ligand, surface modifications, diameter, assay type and exposure time	LC_{50}	Oh et al. 2016
16 MeONPs	*E. coli*	ΔH, polarization force	EC_{50}	Mu et al. 2016
18 MeONPs	*E. coli*; HaCaT cells	ΔH Oxygen percent	EC_{50}	Basant and Gupta 2017
17 MeONPs	*E. coli*; HaCaT cells	SMILES	$\log(1/EC_{50})$	Toropova and Toropov 2017
21 MeONPs	A549 cells	Particle size and zeta potential	LC_{50}	Toropova and Toropov 2017
34 gold NPs	A549 and HEK293 cells	Hydrophobic potential	Cellular uptake	WY Wang et al. 2017a
20 MWCNTs	human lung cells (BEAS-2B, 16HBE14o-, WI-38, and HBE)	Diameter, length, surface area, and dose	CV	Trinh et al. 2018
30 MeONPs	THP-1 cells	Electronegativity, zeta potential, and cation charge	Inflammatory potential	Huang et al. 2020
250 data points for carbon nanotubes, graphene, Ag and TiO_2	Rats, mice	Surface ligand, size, NP type and exposure pathway	Reproductive toxicity	Ban et al. 2018

Note MWCNTs multiwalled carbon nanotubes; CV: cell viability %; EC_{50}: concentration for 50% of maximal effect; LC_{50}: median lethal concentration; MeONPs: metal oxide nanoparticles; LUMO: lowest unoccupied molecular orbital; ΔH: enthalpy of formation of a gaseous cation

cytotoxicity of 20 different types of multiwalled carbon nanotubes (MWCNTs) to human lung cells (Trinh et al. 2018). The model showed potential for use in the estimation of human lung cell viability after exposure to MWCNTs with the following properties: diameter, 12–4 nm; length, 0.19–20.25 μm; surface area, 11.3–380.0 m^2/g; and dose, 0–200 ppm.

Researches sought to develop intelligent strategies combining testing methods with non-testing predictive modeling to investigate the relationships between the structures and lung toxicity of nanomaterials (Cui et al. 2019; Gernand and Casman 2014). A study combining experimental and computational methods has been performed to estimate acute lung toxicity and develop predictive models for MeONPs (Cao et al. 2020). Nano-QSAR models were established for predicting the median

lethal concentration (LC_{50}) of MeONPs to human lung adenocarcinoma (A549) cells by considering the influence of particle size and zeta potential on the cytotoxicity. The models showed the toxic mechanism responsible for the cytotoxicity of MeONPs to A549 cells is related to reactive oxygen species release.

Recently, the interactions between ENMs and other systems (e.g., reproductive and immune systems) have been predicted in silico since nanoparticles have exhibited subtle effects at sub-lethal concentrations. To evaluate the reproductive toxicity of nanomaterials, the reported data from animal experiments were extracted for meta-analysis to identify the priority factors from highly heterogeneous data (Ban et al. 2018). Random forest (RF) classification models were built with an excellent classification accuracy based on hierarchical clustering with high vote rates (greater than 95%) and small error rates (less than 4%). Nano-QSAR models were also built for predicting the lung inflammatory potential of MeONPs (Huang et al. 2020). A comprehensive dataset of 30 MeONPs was built to screen a pro-inflammatory cytokine (IL-1β) release in a macrophage-like myeloid cell line THP-1 and validated in mouse lungs. Predictive models were developed for inflammatory potential with predictive accuracy exceeding 90%. Electronegativity, ζ-potential, and cation charge were identified as three key properties responsible for the inflammatory effects of MeONPs.

Although ML models can be used to predict nano-bio interactions, such a prediction is now hindered by the paucity of suitable nanodescriptors with applicable nanostructure annotation methods. Recently, inspired by face recognition technology, Yan et al. (2020) predicted nano-bio interactions through convolutional neural network analysis of nanostructure images, nanostructure features were directly learned from nanoparticle images without complicated nanodescriptor calculations. The constructed convolutional neural network models were successfully used to predict physicochemical properties, such as logarithms of the partition coefficient (logP) and zeta potential, and biological activities, such as cellular uptake and protein adsorption, of 147 unique nanoparticles, including 123 gold nanoparticles, 12 platinum nanoparticles and 12 palladium nanoparticles. This approach enables an efficient end-to-end deep learning modality suitable for the design of next-generation nanomaterials.

The predictive models for nanotoxicology can be used as alternative methods to animal models which helps to address the ethical, economic, and time constraints of traditional toxicology while also advancing mechanistic understanding. However, since the majority of the in silico studies are based on in vitro data. More efforts are needed for predicting in vivo toxicity. The development of nano-specific features is gaining momentum as classic QSARs have proven inadequate for predicting nanotoxicity. Comprehensive databases need to be built for substituting sparse, heterogeneous literature data.

ML Models for Predicting the Toxicity of Emerging Contaminants

In recent years, emerging contaminants (e.g., personal care and cleaning products, pesticides and their metabolites, pharmaceuticals, lifestyle products, food additives, industrial products and wastes) have become a problem to the environment. The cumulative use of a range of chemical substances in industrial activities, agriculture, and health care services has led to their recent appearance in soils, surface, and groundwater resources, with unpredictable consequences for these ecosystems (Gomes et al. 2017). There is limited data regarding the toxicity of emerging contaminants and their potential for bioaccumulation in biota. There are data available only for some representatives of the main groups of chemical substances, and for a limited number of species, making the risk assessment of emerging contaminants difficult. Therefore, it is necessary to develop computational models for screening the potential toxicity of emerging contaminants. Predictive models for screening potential endocrine-disrupting compounds (EDCs) and toxicity of drugs are discussed in this section.

EDCs, a major group of emerging contaminants, are compounds such as organochlorinated pesticides and industrial chemicals, plastics and plasticizers, fuels, and many other chemicals in the environment or are in widespread use that can interrupt the endocrine system (Diamanti-Kandarakis et al. 2009). Results from animal models, human clinical observations, and epidemiological studies indicated that EDCs are a significant concern to public health. There is growing interest in the possible health threat posed by EDCs (Diamanti-Kandarakis et al. 2009; Flint et al. 2012; Lyche et al. 2009). To alleviate this concern, computational models have been developed for screening potential EDCs (Table 14.2). QSAR models are widely used to predict the binding activity of chemicals to the estrogen, androgen, and thyroid receptors. Some most recent studies are discussed below.

Collaborative Estrogen Receptor Activity Prediction Project (CERAPP) combined multiple models developed in collaboration with 17 groups in the United States and Europe to predict estrogen receptor (ER) activity of a common set of 32,464 chemical structures (Mansouri et al. 2016). QSAR models and docking approaches were employed, mostly using a common training set of 1,677 chemical structures provided by the U.S. EPA, to build a total of 40 categorical and 8 continuous models for binding, agonist, and antagonist ER activity. All predictions were evaluated on a set of 7,522 chemicals curated from public databases including the US FDA's estrogenic activity database (EADB) (Shen et al. 2013) and the literature. To overcome the limitations of single models, consensus models were built by weighting individual models using the scores based on their evaluated accuracies. This project demonstrated the possibility to screen large libraries of chemicals using a consensus modeling of different in silico approaches.

In 2020, the Collaborative Modeling Project for Androgen Receptor Activity (CoMPARA), which follows the steps of the CERAPP was developed (Mansouri et al. 2020). TThe CoMPARA screened 55,450 chemicals. Computational toxicology

Table 14.2 Machine learning (ML) models for predicting the binding activity of EDCs

Endpoint	Method	Data points in modeling	Reference
Estrogen receptor	SVM, DT, NB, *k*NN	333	Chen et al. 2014
	Ensembled method	1677	Mansouri et al. 2016
	DNN, SVM	1677	Chierici et al. 2018
	SVM, LDA, CART	440	Zhang et al. 2017
Estrogen receptor α	RF	3308	Ng et al. 2015
	SVM	31	Hou et al. 2018
Estrogen receptor β	RF	2492	Sakkiah et al. 2017
Estrogen receptor α & β	NB, DT, RF, SVM, DNN	969 ~ 7351	Russo et al. 2018
Androgen receptor	kNN, RF, NB	1689	Grisoni et al. 2019
	ANN, SVM, DT	1689	Manganelli et al. 2019
	DNN, RF	7665	Idakwo et al. 2019
	MLR	14	Yang et al. 2016
Thyroid hormone receptor	MLR	18	F Li et al. 2010a
Thyroid hormone sulfotransferase	MLR	22	Jin et al. 2019
Thyroid hormone transporter	MLR	20	Yang et al. 2017
Androgen receptor	kNN, RF, ANN, SVM, etc	1662	Mansouri et al. 2020
Peroxisome proliferator-activated receptor γ (PPARγ)	Ridge regression, lasso regression, PLS regression, SVM, and RF	420	Wang et al. 2021

Note SVM: support vector machine, DT: decision tree, NB: Naive Bayes, KNN: k nearest neighbor, DNN: deep neural network, LDA: linear discriminant analysis, CART: classification and regression tree, RF: random forest, ANN: artificial neural network, MLR: multiple linear regression

scientists from 25 international groups contributed 91 predictive models for androgen receptor binding, agonist, and antagonist activity predictions. CoMPARA predictions were combined into consensus models that provided an averaged predictive accuracy of approximately 80% for the evaluation set. The developed models were evaluated using the data curated from different sources. After the CERAPP, which established the framework for such international collaborations, the CoMPARA was a more global collaboration with more participants and screened a larger set of chemicals. The success of these two projects and the eagerness of the participants for more collaborations have prompted the organization of new consortiums to model new endpoints with readily available high-quality data.

Recently, QSAR models were built to enable efficient screening of chemicals with peroxisome proliferator-activated receptor γ (PPARγ) binding activity, which is important for evaluating chemicals with potential endocrine-disrupting effects (Wang et al. 2021). Structure−activity landscapes of the training compounds were described by network-like similarity graphs (NSGs). Based on the NSGs, local discontinuity scores were calculated and found to be positively correlated with the cross-validation absolute prediction errors of the models using the different training sets, descriptors, and algorithms. The curated data sets and developed regression models could be useful for evaluating PPARγ-involved adverse effects of emerging contaminants.

Drugs are also a large group of emerging contaminants. Predicting drug-induced liver injury (DILI) is a challenge for drug developers and regulators. Despite many efforts to eliminate hepatotoxic drugs before they are tested in humans, hepatotoxic drugs often escape preclinical toxicity testing and are not identified as hepatotoxic until in a later stage of drug development and sometimes even after the approval. The US FDA's Liver Toxicity Knowledge Base (https://www.fda.gov/science-research/bioinformatics-tools/liver-toxicity-knowledge-base-ltkb) evaluated >1000 drugs for their likelihood of causing DILI in humans, of which >700 drugs were classified into three categories (most concern, less concern, and no concern). Based on this largest set of drugs, DILI prediction models were developed using a pattern recognition algorithm Decision Forest (Hong et al. 2017). In addition to 2-class prediction models, 3-class prediction models were also developed to further differentiate drugs with less concern from the ones with the most concern. The results suggested that the 3-class model presents a better option than the binary model (which most publications are focused on) for drug safety evaluation.

Peng et al. (2019) developed a computational systems toxicology approach to investigate the molecular mechanisms of DILI. In total 1478 DILI compounds were collected, together with 1067 known targets for 896 DILI compounds. Then, 173 new potential targets of these compounds were predicted by bSDTNBI (balanced substructure-drug-target network-based inference) method. After network analysis, 145 genes were found to be associated with hepatotoxicity and upregulated in the liver. DILI-Score was further proposed to assess the hepatotoxic severity of a given compound.

Because of the nature of the bioassays, the activity data are typically strongly imbalanced, with a small number of active compounds contrasting with a very large number of inactive compounds. Strategies need to be developed to efficiently build robust QSAR models from imbalanced data sets. Moreover, traditional QSAR models only predict a single endpoint at a time, namely, single-label models, which are insufficient for a comprehensive view of characteristics of various emerging contaminants. In silico models for the prediction of emerging contaminants with multiple targets need to be built in the future.

Quantum Chemical Calculation and Molecular Dynamics Simulation of Nano-Bio Interactions

The unprecedented advances in the synthesis and characterization of nanomaterials have opened a new era for nanoscience. Various novel nanomaterials with unique physicochemical properties are now being developed and extensively studied for potential applications in the fields of biomedicine (nanomedicine). Despite the advantages, the toxicity of nanomaterials (nanotoxicity) has also become the focus of the scientific community. The biological effects of nanomaterials are usually originated from the nano-bio interface interactions. Hence, an in-depth understanding of the nano-bio interface interactions is essential for revealing the molecular mechanisms underlying the biological effects of nanomaterials. The knowledge will be crucial for providing directions for the future functional design of nanomedicines/nanodevices. Benefitting from the rapid growth of molecular modeling theories and computer technologies, theoretical modeling has gradually become an economical and reliable method for elucidating the complex nano-bio interfaces (Ding and Ma 2015; Zhou 2014; Zhou et al. 2020). Theoretical studies can provide quantitative and comprehensive insight into the underlying mechanisms of the interactions at the nano-bio interface (Luan and Cheng 2020; Tsutsumi and Yoshioka 2011). Here we introduce the two most widely used theoretical approaches (quantum mechanical and force field methods) and review some of the recent advances in the simulations research on the nano-bio interface, with an emphasis on the molecular level understanding of the interface interactions between several types of typical nanomaterials and biological molecules blocks, including proteins/peptides, cell membranes, and nucleic acids.

Quantum Chemical Calculations and Molecular Dynamics Simulations

There are two major theoretical approaches involved in the molecular modeling of the nano-bio interface, i.e., quantum mechanical methods and force field methods. The basis of quantum mechanical approaches is to solve the Schrödinger equation which represents the quantum states of particles as wavefunctions. However, to date, it remains a challenge to solve the Schrödinger equation for atoms/molecules with more than one electron, due to many factors, including the extremely complicated "many-body problem" in mathematics and the description of electron spin (Leach and Leach 2001). A series of approximate methods have been thus developed for the solution of this equation, such as the Hartree–Fock (HF) based approach (Roothaan 1951) and density functional theory (DFT) (Koch and Holthausen 2001; Kohn 1999). HF equations treat the wavefunction of multiple electrons as a Slater determinant composed of a set of single-electron wavefunctions. The fundamental principle of DFT method is the relationship between electronic density and electronic energy. Therefore, this theory calculates the overall electronic density distribution to avoid

the complete solution for the many-body system. Since the ab initio HF method for large systems is quite expensive in terms of computing resources, there emerged semi-empirical methods, in which the solution of the HF equations is simplified and speeded up by approximating or omitting some of the integrals and/or certain terms in the Hamiltonian function (Leach and Leach 2001).

Although quantum mechanical methods provide a high modeling accuracy and allow the calculations of electronic properties, their applications are currently limited to relatively small systems even if the semi-empirical methods are adopted, mainly because numerous electrons must be taken into account (Leach and Leach 2001). Indeed, many of the issues at the nano-bio interface that need to be addressed by means of molecular modeling are too large/complicated to be tackled by quantum mechanical approaches. Force field methods, also known as molecular mechanics, ignore the electronic motions and lay their foundation on the Born–Oppenheimer approximation, which relies on the nuclear positions to describe the energy of systems (the so-called potential energy) (Leach and Leach 2001). A mathematical form of the potential function with empirically fitted parameters is called force field, and it usually includes contributions from representative processes, e.g., bond stretching, angle bending, single bond rotation and non-bonded interactions (Mayo et al. 1990; Wang et al. 2004). Moreover, a key feature of force fields is transferability, which enables a set of potential parameters to be transferable from relatively small molecules to other much larger systems (Leach and Leach 2001). Thereby, the force fields can be developed and validated by the conformational energies and other properties calculated by quantum mechanical methods on small molecules. For instance, many of the force fields for proteins and peptides are parameterized according to the potential energies and atomic charges that have been quantum mechanically obtained with small peptides. Compared with quantum mechanics, the molecular mechanical approach is less complicated and requires much lower computing resources. This method is thus able to deal with systems containing a large number of atoms. In particular, molecular mechanics are quite frequently employed to provide molecular insights into the bio-response of nanomaterials, including how the nanoparticles interact with biomolecules and the resulting effect on their biological functions. This knowledge is extremely important not only for assessing the toxicological risk but also for evaluating the potential treatment efficacy of trial nanomedicines (Selvaraj et al. 2018; Zhou 2014; Zhou et al. 2020).

Molecular dynamics (MD) simulation, among the most popular molecular mechanical approaches, is widely employed to investigate the properties/behaviors of nano-bio interfaces under dynamic conditions according to Newton's laws of motion (Gupta et al. 2020; Lei et al. 2020; Zhou 2014). The resulting motion of a system is called a trajectory, specifying the time evaluation of the positions and velocities of particles. The classical all-atom MD simulations entirely retained the atomic character compared with the methods using coarse-grained force fields. All-atom MD simulations up to hundreds of nanoseconds are becoming less and less expensive due to the development of high-performance computers (Perilla et al. 2015; Salomon-Ferrer et al. 2013). In many cases today, it is an ideal and reliable choice for theoretically investigating the nano-bio interactions. Extensive studies with such methods

have been performed and these have provided acceptable results for understanding the interactions between nanoparticles and biomolecules (proteins, membranes and nucleotides) at the atomic/molecular level (Liu et al. 2016; Nash et al. 2017; Yang et al. 2012). For instance, Yang et al. (2012) studied the binding of aromatic residue analogs to a single-walled carbon nanotube (CNT) using DFT calculations and MD methods based on different force fields. The binding energies and the distances between the aromatic residues and CNT calculated by the two theoretical methods were comparable. Liu et al. (2016) performed MD simulations to explore the interactions of twenty alpha-amino acids with a spherical TiO_2 nanoparticle. The results showed that, compared with non-charged amino acids, the charged residues more favorably adsorbed onto the TiO_2 surface, in good consistency with previous experimental findings. Additionally, Gu et al. (2015) used MD simulations to reveal the mechanism underlying the adsorption of blood proteins onto the graphene surface. They found that besides the aromatic rings, the basic residues also contributed to the formation of protein-graphene corona complexes.

Simulation of the Nano-Protein Interface Interactions

Proteins are important structural and functional units in cellular activities. The interface interactions between nanomaterials and proteins can trigger various biological outcomes, which can be either beneficial, such as therapeutic effects in nanomedicine, or harmful, causing nanotoxicity. For instance, carbon-based nanomaterials, e.g., graphene and graphene oxide (GO), have been studied for their potential use in treating various protein conformational diseases (PCDs). The aggregation and accumulation of intrinsically disordered proteins or peptides (IDPs) is a crucial pathological process of various PCDs. For example, β-amyloid (Aβ) and α-synuclein (α-Syn) peptides are the pathological hallmark of Alzheimer's disease (AD) and Parkinson's disease (PD), respectively. Therefore, inhibiting the aggregation of IDPs is a feasible prevention and treatment strategy for PCDs. Yang et al. (2015) showed, using MD simulations for the first time, that graphene can inhibit the aggregation of Aβ peptides, and can penetrate and extract peptides from the mature fibrils. These theoretical predictions were verified by using a series of experimental assays. The calculation results further showed that the inhibition of the peptide aggregation was caused by the exceptionally strong interactions between graphene and Aβ peptides in the dispersion. In addition, the strong π–π stacking interactions between the aromatic residues of Aβ peptides and the graphene surface further enhance the interface interactions. Going a step further, He et al. (2019) investigated the influence of surface inhomogeneity of GO (e.g., different degrees of oxidation) on the assembly of Aβ peptides using MD simulations. They found that the nonuniform GO sheet more strongly induced the dissociation of peptide assembly than its uniform counterpart. Kim et al. employed a combination of MD simulations and an experimental approach to investigate the role of graphene quantum dots (GQDs) in destructing the mature α-syn

fibrils (Mehra et al. 2019). Their simulation results demonstrated that the adsorption of GQDs to α-syn fibril was initiated by the electrostatic interactions, while the dissociation of mature fibril was caused by the hydrophobic interactions between GQDs and the hydrophobic residues in α-syn peptides.

Metallofullerenol $Gd@C_{82}(OH)_{22}$ is regarded as a potential anti-tumor nanodrug in treating pancreatic adenocarcinoma (Kang et al. 2012) and breast cancer (Meng et al. 2012). Zhao and coworkers showed that $Gd@C_{82}(OH)_{22}$ not only suppresses the expression of matrix metalloproteinases (MMPs, specifically, MMP-2 and MMP-9) but also reduces their activity. However, deciphering the inhibition specificity of $Gd@C_{82}(OH)_{22}$ to MMPs and the underlying mechanisms was a big challenge. Zhou and coworkers used large-scale MD simulations to provide mechanistic insight into the issue (Kang et al. 2012). They found that, driven by the nonspecific electrostatic, hydrophobic, and specific hydrogen-bonding interactions, $Gd@C_{82}(OH)_{22}$ is specifically bound to the ligand-specificity loop S1′ of MMP-9 rather than the well-known zinc catalytic site, thereby inhibiting its activity. Based on the reported surface recognition mechanisms, they moved one step further and theoretically designed a series of $Gd@C_{82}(OH)_{22}$ derivatives by substituting its surface hydroxyl groups with new groups (e.g., $–PO_4^{2-}$, $–CH_2CO_2^-$, $–CO_2^-$, $–NH_3^+$, or $–CONH_2$) aiming to strengthen the binding affinity for MMP-9 (Chen et al. 2018). They found that $Gd@C_{82}(OH)_{21}(PO_4)^{2-}$ bound more strongly to MMP-9 than $Gd@C_{82}(OH)_{22}$, due to the enhanced specific electrostatic interactions of $–PO_4^{2-}$ with basic residues at the binding sites. Besides, Ma et al. (2020) showed that negatively charged gold nanoclusters (AuNC) triggered protective effects in the cell model for Parkinson's disease by up-regulating the proteasome activity (20S proteasome which is a large protein complex with proteolytic activity). MD simulations revealed that negatively charged AuNC facilitated the opening of the central gate of proteasome for substrate access to the internal active site. These works shed light on the development of potential nanodrugs by tailoring the nano-protein interface interactions.

On the other hand, the protein conformational changes on the surface of nanomaterials have also been extensively simulated, because this may provide a theoretical prediction about the potential nanotoxicity of nanomaterials at the molecular level. In such cases, small model proteins (e.g., alpha-helical Villin HP35, β-sheet WW domain and SH3 domain) are usually adopted owing to their fast folding and unfolding dynamics. Multiple studies demonstrated that nanomaterials like graphene and its derivatives (Gu et al. 2019a, b; Li et al. 2019; Luan et al. 2015), MoS_2 (Gu et al. 2016), C_2N (Li et al. 2017), and certain platinum (Pt) nanocrystal surface (Liu et al. 2019) can destabilize the native structure of these proteins at different extent. It should be noted that the interface interactions between proteins and nanomaterials are very complicated and can be strongly influenced by multiple factors such as nanomaterial elemental composition, functional groups, defects, and interface water. For instance, Gu et al. (2019a, b) and Li et al. (2019) demonstrated that defects that appeared on the surface of graphene can reinforce its protein denaturation capacity. During the unfolding process, the protein can be tightly anchored to the defect edges by the strong electrostatic interactions between the basic residues of the protein and natively charged carboxyl groups on the defective sites of graphene. At the same

time, other parts of the protein attached to the graphene surface with aromatic and hydrophobic core residues of the protein via strong π–π stacking and hydrophobic interactions and accelerated the protein unfolding. Interestingly, Liu et al. (2019) found that the distinct water behavior on the surface of two Pt crystals (Pt(100) and Pt(111)) caused their different protein denaturation capabilities. Within the first solvation shell (FSS) of the Pt(100) crystal surface, water molecules formed a compact and stable monolayer via a highly uniform rhombic hydrogen-bond network. This water monolayer constituted a new interface in contact with protein which was favorable for the adsorption of acidic residues (Glu and Asp) and thus acted as a protective film that prevented the hydrophobic core residues from directly contacting with the metal surface. In comparison, the water hydrogen-bond network in the FSS of the Pt(111) crystal surface was sparse and distributed between many defects, making it easier to penetrate by various residues, particularly by those containing a planar side chain, e.g., Phe, Trp or Arg, due to their direct strong dispersion interactions, which eventually resulted in the subsequent protein unfolding.

Additionally, it has been shown that the competitive binding of nanomaterials to various proteins may disrupt the function of the target protein. For instance, Tian et al. (2017), using a combined computational and experimental approach, showed that GO nanosheets entered the gap of actin filaments and caused significant distortion of the actin cytoskeleton. Ma et al. (2018) showed that $Gd@C_{82}(OH)_{22}$ can competitively bind to the substrate recognition sites of the cytochrome P450 enzyme CYP2C8. Gu et al. (2018) theoretically predicted that a MoS_2 nanoflake can stably bind to ubiquitous potassium (K^+) channel, which may block the K^+ ion influx. The simulations were verified experimentally.

Simulation of the Nano-Membrane Interface Interactions

MD simulation studies on how nanoparticles interact with lipid membranes often offer a crucial basis for developing biomedical applications of nanomaterials (e.g., for antibacterial treatment, targeted drug delivery and bio-imaging), because the nano-membrane interactions are related to the cytotoxicity and cellular uptake of nanomaterials (Nel et al. 2009; Yang and Ma 2010). For example, Zhou and coworkers (Tu et al. 2013) studied the potential antibacterial activities of graphene via all-atom MD simulations and validated the results experimentally. They found that the nanosheets extracted a considerable number of phospholipid molecules from the membranes of *E. coli*, mainly as a result of the strong dispersion interactions of graphene and lipids. As a result, the bacterial viability was reduced. This extraction mechanism provided novel insights into the graphene's cytotoxicity and antibacterial activity, which can inspire strategies to manage the toxic effects of graphene-based nanomaterials (Duan et al. 2015; Zhou and Gao 2014). Specifically, subsequent investigations showed that both the adsorption of proteins (Chong et al. 2015; Duan et al. 2015) and polarized charge distribution (Tang et al. 2020) help to mitigate the cytotoxicity of graphene-based nanomaterials by reducing their ability to cause physical destruction to cell

membranes. In addition, based on the understanding that adverse nano-membrane interactions can lead to the formation of pores in cell membranes (Duan et al. 2017), a novel method was theoretically proposed to monitor the particle–membrane interactions in real-time by detecting the increased ion currents through the induced holes (Luan et al. 2017). MD simulations, sometimes together with experimental validations, have also been carried out to assess possible destructive effects on membrane bilayers for other two-dimensional (2D) nanomaterials (e.g., BN [Li et al. 2018; Xie et al. 2021], C_2N [Liu et al. 2018; Zhang et al. 2019], black phosphorus [Ma et al. 2021] and graphyne [Gu et al. 2017a]). Some distinctive findings were revealed, including the temperature-dependent membrane extraction effect of BN and the robust compatibility of C_2N with lipid membrane (Liu et al. 2018; Zhang et al. 2019). Gao and coworkers (Li et al. 2013) investigated the interactions of graphene and few-layer graphene sheets with cell membranes using combined MD simulations and experimental techniques. Different from other studies, they suggested that the penetration of graphene into cell membrane is initiated at corners or asperities of the sheets, thus avoiding a high energy barrier for the translocation of graphene through cell membranes. In addition to simulation investigations on 2D nanomaterials, there are also extensive MD studies exploring the nano-membrane interactions involving other nanomaterials, such as fullerene (Qiao et al. 2007), carbon nanotubes (Shen et al. 2020), Au nanoparticles (Gupta and Rai 2017) and silica nanospheres (Delle Piane et al. 2018). Hydrophobicity (Tu et al. 2013), surface charge (Tang et al. 2020), shape and size (Gupta and Rai 2017; Gupta et al. 2020) of nanomaterials are the most crucial factors that can effectively regulate the membrane permeability or compatibility, which might serve as fundamental guidance for either reducing the toxic effect or enhancing the therapeutic effects of related nanomedicines.

Simulation on the Nano-Nucleic Acids Interface Interactions

Nucleic acids, including DNA and RNA, serve as the primary information-carrying molecules in cells with an especially important function in directing the biosynthesis of proteins. Nanomaterials of certain sizes or with specific surface coatings can translocate through the cell membrane and directly interact with nucleic acids, which may cause adverse effects to the cell (Singh et al. 2009). By sorbing nucleic acids, nanomaterials can act as nanocarriers (Demirer et al. 2019). Therefore, investigations on the nano-nucleic acids interactions by computational modeling are of great importance for understanding the underlying molecular mechanisms of biological effects of nanomaterials as well as for developing applications for nanomaterials in DNA technology.

Double-stranded deoxyribonucleic acid (dsDNA) is considered the most stable DNA form based on the classical Watson–Crick model, which is often used to detect its interaction with nanomaterials. Zhou and coworkers (Gu et al. 2017b) studied the binding of dsDNA to a 2D carbon nitride nanomaterial, C_2N. They found that dsDNA bound to the C_2N monolayer by orienting the axis of dsDNA in a perpendicular

direction to the C_2N monolayer, regardless of the initial configurations. The two interfacial nucleotide bases located at the terminal position of dsDNA induced partial π−π stacking interactions with the C_2N surface, which stabilized the vertical binding mode. Moreover, the migration of dsDNA on the C_2N surface was restricted after the binding, with the dsDNA maintaining an upright position. This restricted binding was dominantly mediated by the high free energy barriers of nucleic bases moving on the C_2N surface. The same research group also found that the interfacial water modulated the interface interactions between dsDNA and different Pd crystal surfaces (Pd(100) and Pd(111)). Interestingly, dsDNA displayed "flat" conformation on the Pd(111) surface, whereas on Pd(100) surface the dsDNA exhibited "upright" conformation, positioning vertically or slightly tilted on the nanosheet, suggesting a stronger binding affinity of dsDNA to Pd(111) than to Pd(100). This can be attributed to the different densities and arrangements of water molecules in the FSS of the two Pd surfaces. In the FSS of Pd(100), the formed water hydrogen-bond network is compact and can resist the embedding of the nucleic acid bases. However, the water hydrogen-bond network in the FSS of Pd(111) is less compact, which allows direct penetration of nucleic acid bases to the surface metal atoms, thereby showing stronger binding. These theoretical predictions were subsequently validated by gel electrophoresis experiments.

Simulation of Interactions Between Nanomaterials and Organic Pollutants

Investigating the interactions between nanomaterials and organic pollutants is important for understanding the environmental fate of the two (emerging) pollutant classes and evaluating their environmental risks. Traditional experimental and modern simulation methods can be used for examining these interactions. Simulations with DFT or MD methods can not only serve as an alternative to the experimental methods but also provide more details at the atomic level than experiments. In addition, QSARs have been used for predicting the interactions between nanomaterials and organic pollutants. Herein, we introduce some examples of simulations and predictions of the interactions between carbon nanomaterials and organic pollutants.

Wang et al. (2017a, b) computed adsorption energies for 38 organic compounds onto graphene in both gaseous and aqueous phases using the DFT method. Based on the results, two polyparameter linear free energy relationships (pp-LFERs) were established for predicting the adsorption energies of organic compounds towards graphene, and the contributions of different interactions to the overall adsorption were estimated based on the pp-LFERs models. The results indicated that the dispersion and electrostatic interactions played dominant roles in the gaseous adsorption to graphene, and the dispersion and hydrophobic interactions had significant influences on the aqueous adsorption of organic compounds to graphene. Besides, the adsorption energies on single-walled carbon nanotubes (SWNTs) with different curvatures and

electronic properties showed that the curvature had a more significant influence on adsorption. Graphene was shown to have a stronger adsorption capability than SWNTs.

In order to investigate the influence of functional groups on adsorption, Wang et al. (2018) simulated the adsorption of 43 aromatic compounds onto graphene and graphene oxides with different functional groups (hydroxyl, epoxy and carbonyl) via MD method. It was demonstrated that graphene exhibits stronger adsorption capability than graphene oxides. The hydroxyl and carbonyl groups for graphene oxides can form hydrogen bonds with the organic compounds. Moreover, two theoretical linear solvation energy relationship (TLSER) models were established for predicting the adsorption equilibrium coefficients onto graphene and graphene oxide nanosheets.

These prediction models are only applicable to the adsorption onto carbon nanomaterials. There is still a research gap in predicting the adsorption of organic compounds for many other types of nanomaterials. Besides, many factors, e.g., ions in the solution, the functional groups or surface defects of nanomaterials, can also influence the adsorption. Novel models which take these different factors into account need to be established for predicting the adsorption of various environmental pollutants onto nanomaterials multi-dimensionally in the future.

Conclusions and Implications

We have briefly introduced the two most commonly used theoretical approaches (quantum mechanical methods and force field methods) and reviewed some of the recent advances in the simulation works on the interface interactions of nanomaterials (e.g., carbon-based nanomaterials, metallofullerenol $Gd@C_{82}(OH)_{22}$ and its derivatives, MoS_2 nanosheets, or noble metals Pt and Pd nanocrystal surfaces) with various biological building blocks (e.g., proteins, membranes, or nucleic acid) and organic pollutants. These theoretical simulations on nano-bio interface interactions either successfully predicted/designed a specific biological function(s) of nanomaterials or explained the molecular mechanisms underlying a specific biological effect of nanomaterials. On the other hand, these works also informed that a variety of physicochemical variables of nanomaterials including elemental composition, shape, size, surface charge distribution, functional group, defect, and interface water can have a crucial influence on the nano-bio interactions. Still, a comprehensive understanding of the role of these variables in modulating the nano-bio interactions remains at its early stage and warrants further theoretical and experimental endeavors. Overall, future work will be of great significance for de novo design of nanomedicines/nanodevices with better biocompatibility.

Perspectives

In this chapter, we have briefly introduced computational toxicology for studying nanomaterials and emerging contaminants and reviewed some of the recent advances in computational models developed for predicting toxicological properties or the adverse effects of nanomaterials and emerging contaminants. The applied models have shed light on the factors which are responsible for toxic interactions of emerging contaminants, including nanomaterials, with cells. In silico approaches used as alternatives to animal models are helping to address the ethical, economic, and time constraints of traditional toxicology while also advancing mechanistic understanding. However, there are still many challenges in predictive toxicology.

In Silico Studies for Predicting in Vivo Toxicity

As chemical risk assessment has continued to evolve in response to the shifting toxicity testing paradigm and the introduction of new regulations, the use of mammalian test animals for in vivo experiments in toxicology has faced criticism. In vivo studies generally provide more comprehensive and human-relevant data than in vitro studies, however, they are costly and time-consuming especially when a large number of substances need to be evaluated. There is clearly not enough time or resources to perform traditional animal studies on the high number of nanomaterials and emerging contaminants. Cost-effective in vitro approaches have significant potential for preliminary toxicity screening. One of the general limitations of in vitro test systems is that they are restricted to one or a few different cell types and, thus, cannot represent the biological responses in the whole organism. Furthermore, these systems are usually derived from cancer cell lines which can result in different outcomes when compared to in vivo tests. Ultimately, the goal of the predictive approach would be to develop a series of toxicity assays that can limit the demand for in vivo studies, both from a cost as well as an animal use perspective. However, currently very few approaches for using in vitro data to predict in vivo effects exist. Prioritization schemes that limit unnecessary in vivo testing are urgently needed. The complexity and the heterogeneity of available in vivo data on potential risks of nanomaterials and emerging contaminants, in addition to the interdependency of relevant influential attributes, makes it challenging to develop a generalization of their toxicity behavior. The lack of systematic approaches to investigate these risks further adds uncertainties and variability to the body of literature and limits the generalizability of existing studies. Rigorous approaches for assembling published evidence on in vivo toxicity of nanomaterials and emerging contaminants need to be developed and undiscovered relationships that were not targeted in the original publications need to be unraveled. Quantitative in vitro to in vivo extrapolation methods have the potential to fill this gap. They hold the promise of delivering urgently needed prioritizations for toxicity testing.

Nano-Specific Features for Predicting Nano-Toxicity

Some studies have included nano-specific descriptors in their final model, while others have included theoretically calculated descriptors as input variables, either separately or in combination with physicochemical characteristics. It is worth noting that the new nanomaterial-relevant revisions of the Annexes of the European Union's chemical legislation require that, quoting Clausen and Hansen (Clausen and Hansen 2018), "Thou shalt not use molecular structural similarities alone as a justification for grouping different nanoforms". This decree signifies the importance of incorporating nano-specific features in a model.

Databases Facilitation and Multisource Data Extraction

A lot of data has been published regarding the potentially harmful effects of a wide range of nanomaterials and emerging contaminants. However, it is difficult to collect and compare the data acquired by independent laboratories because of differences in experimental parameters and techniques. Especially for nanomaterials, differences in nanomaterial dispersion techniques, cytotoxicity assays, cell lines, and the ENM properties themselves could affect the results of toxicity assessments. To overcome this obstacle, standard protocols have been proposed. For instance, a standard protocol for the preparation and characterization of ENMs was developed by Deloid et al. (2017) to ensure consistent and reliable results in in vitro toxicity experiments of the same ENMs at different times and/or laboratories. These protocols can be applied in the future to minimize the variation in replicated experiments and to enable comparisons with other laboratories using the same protocol.

Co-exposure of Nanomaterials and Chemicals

Most ENMs are highly reactive, even those conventionally considered to be inert (e.g., TiO_2 and Au nanoparticles). Environmental transformation can remarkably alter their physicochemical properties, and consequently, their fate, transport and biological effects. Understanding how the properties of ENMs may change once released into the environment is urgently needed to better assess their toxic effects in the environment. It is encouraged that future environmental health and safety (EHS) studies focus on the types of materials that will more likely be found in the environment (e.g., nanocomposite and debris) and take into account the chemical and biological transformations of ENMs. Besides, nanoparticles interacting with proteins, membranes, cells, DNA and organelles establish a series of nanoparticle/biological interfaces

that depend on colloidal forces as well as dynamic bio-physiochemical interactions. These interactions lead to the formation of protein coronas, particle wrapping, intracellular uptake and biocatalytic processes that could have biocompatible or adverse outcomes. On the other hand, biomolecules may induce phase transformations, free energy releases, restructuring and dissolution at the nanomaterial surface. Probing these various interfaces allows the development of predictive relationships between structure and activity that are determined by nanomaterial properties such as size, shape, surface chemistry, roughness and surface coatings. This knowledge is important from the perspective of the safe use of nanomaterials.

References

Andujar P, Simon-Deckers A, Galateau-Salle F et al (2014) Role of metal oxide nanoparticles in histopathological changes observed in the lung of welders. Part Fibre Toxicol 11:23. https://doi.org/10.1186/1743-8977-11-23

Ban Z, Zhou QX, Sun AQ et al (2018) Screening priority factors determining and predicting the reproductive toxicity of various nanoparticles. Environ Sci Technol 52(17):9666–9676. https://doi.org/10.1021/acs.est.8b02757

Basant N, Gupta S (2017) Multi-target QSTR modeling for simultaneous prediction of multiple toxicity endpoints of nano-metal oxides. Nanotoxicology 11(3):339–350. https://doi.org/10.1080/17435390.2017.1302612

Cao JK, Pan Y, Jiang YT et al (2020) Computer-aided nanotoxicology: risk assessment of metal oxide nanoparticles via nano-QSAR. Green Chem 22(11):3512–3521. https://doi.org/10.1039/d0gc00933d

Chen SH, Kang SG, Luo JD et al (2018) Charging nanoparticles: increased binding of Gd@$C_{82}(OH)_{22}$ derivatives to human MMP-9. Nanoscale 10(12):5667–5677. https://doi.org/10.1039/c8nr00127h

Chen YJ, Cheng FX, Sun L et al (2014) Computational models to predict endocrine-disrupting chemical binding with androgen or oestrogen receptors. Ecotoxicol Environ Saf 110:280–287. https://doi.org/10.1016/j.ecoenv.2014.08.026

Chierici M, Giulini M, Bussola N et al (2018) Machine learning models for predicting endocrine disruption potential of environmental chemicals. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 36(4):237–251. https://doi.org/10.1080/10590501.2018.1537155

Cho W-S, Duffin R, Poland CA et al (2010) Metal oxide nanoparticles induce unique inflammatory footprints in the lung: important implications for nanoparticle testing. Environ Health Perspect 118(12):1699–1706. https://doi.org/10.1289/ehp.1002201

Chong Y, Ge CC, Yang ZX et al (2015) Reduced cytotoxicity of graphene nanosheets mediated by blood-protein coating. ACS Nano 9(6):5713–5724. https://doi.org/10.1021/nn5066606

Christensen FM, Johnston HJ, Stone V et al (2010) Nano-silver—feasibility and challenges for human health risk assessment based on open literature. Nanotoxicology 4(3):284–295. https://doi.org/10.3109/17435391003690549

Clausen LPW, Hansen SF (2018) The ten decrees of nanomaterials regulations. Nat Nanotechnol 13(9):766–768. https://doi.org/10.1038/s41565-018-0256-2

Cui L, Wang X, Sun BB et al (2019) Predictive metabolomic signatures for safety assessment of metal oxide nanoparticles. ACS Nano 13(11):13065–13082. https://doi.org/10.1021/acsnano.9b05793

Dasch J, D'Arcy J (2008) Physical and chemical characterization of airborne particles from welding operations in automotive plants. J Occup Environ Hyg 5(7):444–454. https://doi.org/10.1080/15459620802122720

Delle Piane M, Potthoff S, Brinker CJ et al (2018) Molecular dynamics simulations of the silica–cell membrane interaction: insights on biomineralization and nanotoxicity. J Phys Chem C 122(37):21330–21343. https://doi.org/10.1021/acs.jpcc.8b04537

DeLoid GM, Cohen JM, Pyrgiotakis G et al (2017) Preparation, characterization, and in vitro dosimetry of dispersed, engineered nanomaterials. Nat Protoc 12(2):355–371. https://doi.org/10.1038/nprot.2016.172

Demirer GS, Zhang H, Matos JL et al (2019) High aspect ratio nanomaterials enable delivery of functional genetic material without DNA integration in mature plants. Nat Nanotechnol 14(5):456–464. https://doi.org/10.1038/s41565-019-0382-5

Diamanti-Kandarakis E, Bourguignon J-P, Giudice LC et al (2009) Endocrine-disrupting chemicals: an endocrine society scientific statement. Endocr Rev 30(4):293–342. https://doi.org/10.1210/er.2009-0002

Ding HM, Ma YQ (2015) Theoretical and computational investigations of nanoparticle–biomembrane interactions in cellular delivery. Small 11(9–10):1055–1071. https://doi.org/10.1002/smll.201401943

Duan GX, Kang SG, Tian X et al (2015) Protein corona mitigates the cytotoxicity of graphene oxide by reducing its physical interaction with cell membrane. Nanoscale 7(37):15214–15224. https://doi.org/10.1039/c5nr01839k

Duan GX, Zhang YZ, Luan BQ et al (2017) Graphene-induced pore formation on cell membranes. Sci Rep 7:42767. https://doi.org/10.1038/srep42767

Flint S, Markle T, Thompson S et al (2012) Bisphenol a exposure, effects, and policy: a wildlife perspective. J Environ Manage 104:19–34. https://doi.org/10.1016/j.jenvman.2012.03.021

Gernand JM, Casman EA (2014) A meta-analysis of carbon nanotube pulmonary toxicity studies-how physical dimensions and impurities affect the toxicity of carbon nanotubes. Risk Anal 34(3):583–597. https://doi.org/10.1111/risa.12109

Gomes AR, Justino C, Rocha-Santos T et al (2017) Review of the ecotoxicological effects of emerging contaminants to soil biota. J Environ Sci Health A Tox Hazard Subst Environ Eng 52(10):992–1007. https://doi.org/10.1080/10934529.2017.1328946

Grisoni F, Consonni V, Ballabio D (2019) Machine learning consensus to predict the binding to the androgen receptor within the compara project. J Chem Inf Model 59(5):1839–1848. https://doi.org/10.1021/acs.jcim.8b00794

Gu ZL, Plant LD, Meng XY et al (2018) Exploring the nanotoxicology of MoS_2: a study on the interaction of MoS_2 nanoflakes and k^+ channels. ACS Nano 12(1):705–717. https://doi.org/10.1021/acsnano.7b07871

Gu ZL, Song W, Chen SH et al (2019a) Defect-assisted protein HP35 denaturation on graphene. Nanoscale 11(41):19362–19369. https://doi.org/10.1039/c9nr01143a

Gu ZL, Yang ZX, Kang SG et al (2016) Robust denaturation of villin headpiece by MoS_2 nanosheet: potential molecular origin of the nanotoxicity. Sci Rep 6:28252. https://doi.org/10.1038/srep28252

Gu ZL, Yang ZX, Luan BQ et al (2017a) Membrane insertion and phospholipids extraction by graphyne nanosheets. J Phys Chem C 121(4):2444–2450. https://doi.org/10.1021/acs.jpcc.6b10548

Gu ZL, Yang ZX, Wang LL et al (2015) The role of basic residues in the adsorption of blood proteins onto the graphene surface. Sci Rep 5:10873. https://doi.org/10.1038/srep10873

Gu ZL, Zhao L, Ge CC et al (2019b) Facet-regulated adhesion of double-stranded DNA on palladium surfaces. Nanoscale 11(4):1827–1836. https://doi.org/10.1039/c8nr06203j

Gu ZL, Zhao L, Liu ST et al (2017b) Orientational binding of DNA guided by the C_2N template. ACS Nano 11(3):3198–3206. https://doi.org/10.1021/acsnano.7b00236

Gupta R, Badhe Y, Mitragotri S et al (2020) Permeation of nanoparticles across the intestinal lipid membrane: dependence on shape and surface chemistry studied through molecular simulations. Nanoscale 12(11):6318–6333. https://doi.org/10.1039/c9nr09947f

Gupta R, Rai B (2017) Effect of size and surface charge of gold nanoparticles on their skin permeability: a molecular dynamics study. Sci Rep 7:45292. https://doi.org/10.1038/srep45292

He Z, Li JY, Chen SH et al (2019) Surface inhomogeneity of graphene oxide influences dissociation of a beta(16–21) peptide assembly. J Phys Chem B 123(43):9098–9103. https://doi.org/10.1021/acs.jpcb.9b07359

Hong HX, Thakkar S, Chen MJ et al (2017) Development of decision forest models for prediction of drug-induced liver injury in humans using a large set of FDA-approved drugs. Sci Rep 7:17311. https://doi.org/10.1038/s41598-017-17701-7

Hou TY, Weng CF, Leong MK (2018) Insight analysis of promiscuous estrogen receptor alpha-ligand binding by a novel machine learning scheme. Chem Res Toxicol 31(8):799–813. https://doi.org/10.1021/acs.chemrestox.8b00130

Huang Y, Li XH, Xu SJ et al (2020) Quantitative structure-activity relationship models for predicting inflammatory potential of metal oxide nanoparticles. Environ Health Perspect 128(6):67010. https://doi.org/10.1289/EHP6508

Idakwo G, Thangapandian S, Luttrell J et al (2019) Deep learning-based structure-activity relationship modeling for multi-category toxicity classification: a case study of 10K Tox21 chemicals with high-throughput cell-based androgen receptor bioassay data. Front Physiol 10:1044. https://doi.org/10.3389/fphys.2019.01044

Jin LM, Yu HY, Geng LM et al (2019) In silico study for inhibiting thyroid hormone sulfotransferase activity by halogenated phenolic chemicals. Ecotoxicol Environ Saf 180:146–151. https://doi.org/10.1016/j.ecoenv.2019.05.014

Kang SG, Zhou GQ, Yang P et al (2012) Molecular mechanism of pancreatic tumor metastasis inhibition by Gd@$C_{82}(OH)_{22}$ and its implication for de novo design of nanomedicine. Proc Natl Acad Sci USA 109(38):15431–15436. https://doi.org/10.1073/pnas.1204600109

Koch W, Holthausen MC (2001) A chemist's guide to density functional theory. John Wiley & Sons, Weinheim

Kohn W (1999) Nobel lecture: electronic structure of matter—wave functions and density functionals. Rev Mod Phys 71(5):1253–1266. https://doi.org/10.1103/RevModPhys.71.1253

Leach AR (2001) Molecular modelling: principles and applications. Pearson Education, Harlow

Lei XL, Ma HS, Fang HP (2020) Length feature of ssDNA adsorption onto graphene oxide with both large unoxidized and oxidized regions. Nanoscale 12(12):6699–6707. https://doi.org/10.1039/c9nr10170e

Li BY, Bell DR, Gu ZL et al (2019) Protein WW domain denaturation on defective graphene reveals the significance of nanomaterial defects in nanotoxicity. Carbon 146:257–264. https://doi.org/10.1016/j.carbon.2019.01.107

Li BY, Li WF, Perez-Aguilar JM et al (2017) Mild binding of protein to C_2N monolayer reveals its suitable biocompatibility. Small 13(12):1603685. https://doi.org/10.1002/smll.201603685

Li F, Xie Q, Li XH et al (2010a) Hormone activity of hydroxylated polybrominated diphenyl ethers on human thyroid receptor-beta: in vitro and in silico investigations. Environ Health Perspect 118(5):602–606. https://doi.org/10.1289/ehp.0901457

Li JJ, Muralikrishnan S, Ng C-T et al (2010b) Nanoparticle-induced pulmonary toxicity. Exp Biol Med 235(9):1025–1033. https://doi.org/10.1258/ebm.2010.010021

Li RB, Ji ZX, Chang CH et al (2014) Surface interactions with compartmentalized cellular phosphates explain rare earth oxide nanoparticle hazard and provide opportunities for safer design. ACS Nano 8(2):1771–1783. https://doi.org/10.1021/nn406166n

Li YF, Yuan HY, von Dem Bussche A et al (2013) Graphene microsheets enter cells through spontaneous membrane penetration at edge asperities and corner sites. Proc Natl Acad Sci USA 110(30):12295–12300. https://doi.org/10.1073/pnas.1222276110

Li Z, Zhang YH, Chan C et al (2018) Temperature-dependent lipid extraction from membranes by boron nitride nanosheets. ACS Nano 12(3):2764–2772. https://doi.org/10.1021/acsnano.7b09095

Liu L, Zhang ST, Zhao L et al (2018) Superior compatibility of C_2N with human red blood cell membranes and the underlying mechanism. Small 14(52):1803509. https://doi.org/10.1002/smll.201803509

Liu ST, Meng XY, Perez-Aguilar JM et al (2016) An in silico study of TiO_2 nanoparticles interaction with twenty standard amino acids in aqueous solution. Sci Rep 6:37761. https://doi.org/10.1038/srep37761

Liu ST, Yin XH, Zhou H et al (2019) Different platinum crystal surfaces show very distinct protein denaturation capability. Nanoscale 11(41):19352–19361. https://doi.org/10.1039/c9nr03682b

Luan BQ, Huynh T, Zhao L et al (2015) Potential toxicity of graphene to cell functions via disrupting protein-protein interactions. ACS Nano 9(1):663–669. https://doi.org/10.1021/nn506011j

Luan BQ, Zhou S, Wang DQ et al (2017) Detecting interactions between nanomaterials and cell membranes by synthetic nanopores. ACS Nano 11(12):12615–12623. https://doi.org/10.1021/acsnano.7b07005

Luan BQ, Cheng SF (2020) Potential interference with microtubule assembly by graphene: a tug-of-war. Nanoscale 12(8):4968–4974. https://doi.org/10.1039/c9nr10234e

Lyche JL, Gutleb AC, Bergman A et al (2009) Reproductive and developmental toxicity of phthalates. J Toxicol Environ Health B Crit Rev 12(4):225–249. https://doi.org/10.1080/10937400903094091

Ma DF, Meng XY, Bell DR et al (2018) Inhibition of CYP2C8 by metallofullerenol $Gd@C_{82}(OH)_{22}$ through blocking substrate channels and substrate recognition sites. Carbon 127:667–675. https://doi.org/10.1016/j.carbon.2017.10.080

Ma XC, Lee SY, Fei XS et al (2020) Proteasome activity regulated by charged gold nanoclusters: implications for neurodegenerative diseases. Nano Today 35:100933. https://doi.org/10.1016/j.nantod.2020.100933

Ma XY, Zhu XH, Huang CX et al (2021) Molecular mechanisms underlying the role of the puckered surface in the biocompatibility of black phosphorus. Nanoscale 13(6):3790–3799. https://doi.org/10.1039/d0nr08480h

Manganelli S, Roncaglioni A, Mansouri K et al (2019) Development, validation and integration of in silico models to identify androgen active chemicals. Chemosphere 220:204–215. https://doi.org/10.1016/j.chemosphere.2018.12.131

Mansouri K, Abdelaziz A, Rybacka A et al (2016) Cerapp: collaborative estrogen receptor activity prediction project. Environ Health Perspect 124(7):1023–1033. https://doi.org/10.1289/ehp.1510267

Mansouri K, Kleinstreuer N, Abdelaziz AM et al (2020) Compara: collaborative modeling project for androgen receptor activity. Environ Health Perspect 128(2):027002. https://doi.org/10.1289/EHP5580

Mayo SL, Olafson BD, Goddard WA (1990) Dreiding: a generic force field for molecular simulations. J Phys Chem 94(26):8897–8909. https://doi.org/10.1021/j100389a010

Mehra S, Sahay S, Maji SK (2019) A-synuclein misfolding and aggregation: implications in parkinson's disease pathogenesis. Biochim Biophys Acta Proteins Proteom 1967(10):890–908. https://doi.org/10.1016/j.bbapap.2019.03.001

Meng H, Xing GM, Blanco E et al (2012) Gadolinium metallofullerenol nanoparticles inhibit cancer metastasis through matrix metalloproteinase inhibition: imprisoning instead of poisoning cancer cells. Nanomedicine 8(2):136–146. https://doi.org/10.1016/j.nano.2011.08.019

Mills NL, Donaldson K, Hadoke PW et al (2009) Adverse cardiovascular effects of air pollution. Nat Clin Pract Cardiovasc Med 6(1):36–44. https://doi.org/10.1038/ncpcardio1399

Mu YS, Wu FC, Zhao Q et al (2016) Predicting toxic potencies of metal oxide nanoparticles by means of nano-QSARs. Nanotoxicology 10(9):1207–1214. https://doi.org/10.1080/17435390.2016.1202352

Nash JA, Kwansa AL, Peerless JS et al (2017) Advances in molecular modeling of nanoparticle–nucleic acid interfaces. Bioconjugate Chem 28(1):3–10. https://doi.org/10.1021/acs.bioconjchem.6b00534

Nel AE, Mädler L, Velegol D et al (2009) Understanding biophysicochemical interactions at the nano–bio interface. Nat Mater 8(7):543–557. https://doi.org/10.1038/NMAT2442

Ng HW, Doughty SW, Luo H et al (2015) Development and validation of decision forest model for estrogen receptor binding prediction of chemicals using large data sets. Chem Res Toxicol 28(12):2343–2351. https://doi.org/10.1021/acs.chemrestox.5b00358

Oh E, Liu R, Nel A et al (2016) Meta-analysis of cellular toxicity for cadmium-containing quantum dots. Nat Nanotechnol 11(5):479–486. https://doi.org/10.1038/NNANO.2015.338

Peng YY, Wu ZR, Yang HB et al (2019) Insights into mechanisms and severity of drug-induced liver injury via computational systems toxicology approach. Toxicol Lett 312:22–33. https://doi.org/10.1016/j.toxlet.2019.05.005

Perilla JR, Goh BC, Cassidy CK et al (2015) Molecular dynamics simulations of large macromolecular complexes. Curr Opin Struct Biol 31:64–74. https://doi.org/10.1016/j.sbi.2015.03.007

Puzyn T, Rasulev B, Gajewicz A et al (2011) Using nano-QSAR to predict the cytotoxicity of metal oxide nanoparticles. Nat Nanotechnol 6(3):175–178. https://doi.org/10.1038/NNANO.2011.10

Qiao R, Roberts AP, Mount AS et al (2007) Translocation of C_{60} and its derivatives across a lipid bilayer. Nano Lett 7(3):614–619. https://doi.org/10.1021/nl062515f

Roothaan CCJ (1951) New developments in molecular orbital theory. Rev Mod Phys 23(2):69–89. https://doi.org/10.1103/RevModPhys.23.69

Russo DP, Zorn KM, Clark AM et al (2018) Comparing multiple machine learning algorithms and metrics for estrogen receptor binding prediction. Mol Pharm 15(10):4361–4370. https://doi.org/10.1021/acs.molpharmaceut.8b00546

Sakkiah S, Selvaraj C, Gong P et al (2017) Development of estrogen receptor beta binding prediction model using large sets of chemicals. Oncotarget 8(54):92989–93000. https://doi.org/10.18632/oncotarget.21723

Salomon-Ferrer R, Götz AW, Poole D et al (2013) Routine microsecond molecular dynamics simulations with AMBER on GPUs. 2. Explicit solvent particle mesh Ewald. J Chem Theory Comput 9(9):3878–3888. https://doi.org/10.1021/ct400314y

Selvaraj C, Sakkiah S, Tong WD et al (2018) Molecular dynamics simulations and applications in computational toxicology and nanotoxicology. Food Chem Toxicol 112:495–506. https://doi.org/10.1016/j.fct.2017.08.028

Shen C, Zou GJ, Guo WL et al (2020) Lipid coating and end functionalization govern the formation and stability of transmembrane carbon nanotube porins. Carbon 164:391–397. https://doi.org/10.1016/j.carbon.2020.04.011

Shen J, Xu L, Fang H et al (2013) EADB: an estrogenic activity database for assessing potential endocrine activity. Toxicol Sci 135(2):277–291. https://doi.org/10.1093/toxsci/kft164

Singh N, Manshian B, Jenkins GJS et al (2009) Nanogenotoxicology: the DNA damaging potential of engineered nanomaterials. Biomaterials 30(23–24):3891–3914. https://doi.org/10.1016/j.biomaterials.2009.04.009

Tang XF, Zhang ST, Zhou H et al (2020) The role of electrostatic potential polarization in the translocation of graphene quantum dots across membranes. Nanoscale 12(4):2732–2739. https://doi.org/10.1039/c9nr09258g

Tian X, Yang ZX, Duan GX et al (2017) Graphene oxide nanosheets retard cellular migration via disruption of actin cytoskeleton. Small 13(3):1602133. https://doi.org/10.1002/smll.201602133

Toropova AP, Toropov AA (2017) Nano-QSAR in cell biology: model of cell viability as a mathematical function of available eclectic data. J Theor Biol 416:113–118. https://doi.org/10.1016/j.jtbi.2017.01.012

Trinh TX, Choi JS, Jeon H et al (2018) Quasi-SMILES-based nano-quantitative structure-activity relationship model to predict the cytotoxicity of multiwalled carbon nanotubes to human lung cells. Chem Res Toxicol 31(3):183–190. https://doi.org/10.1021/acs.chemrestox.7b00303

Tsutsumi Y, Yoshioka Y (2011) Quantifying the biodistribution of nanoparticles. Nat Nanotechnol 6(12):755–755. https://doi.org/10.1038/nnano.2011.220

Tu YS, Lv M, Xiu P et al (2013) Destructive extraction of phospholipids from *Escherichia coli* membranes by graphene nanosheets. Nat Nanotechnol 8(8):594–601. https://doi.org/10.1038/NNANO.2013.125

Wang JM, Wolf RM, Caldwell JW et al (2004) Development and testing of a general amber force field. J Comput Chem 25(9):1157–1174. https://doi.org/10.1002/jcc.20035

Wang WY, Sedykh A, Sun HN et al (2017a) Predicting nano-bio interactions by integrating nanoparticle libraries and quantitative nanostructure activity relationship modeling. ACS Nano 11(12):12641–12649. https://doi.org/10.1021/acsnano.7b07093

Wang Y, Chen JW, Wei XX et al (2017b) Unveiling adsorption mechanisms of organic pollutants onto carbon nanomaterials by density functional theory computations and linear free energy relationship modeling. Environ Sci Technol 51(20):11820–11828. https://doi.org/10.1021/acs.est.7b02707

Wang Y, Comer J, Chen ZF et al (2018) Exploring adsorption of neutral aromatic pollutants onto graphene nanomaterials via molecular dynamics simulations and theoretical linear solvation energy relationships. Environ Sci-Nano 5(9):2117–2128. https://doi.org/10.1039/c8en00575c

Wang ZY, Chen JW, Hong HX (2021) Developing QSAR models with defined applicability domains on PPARγ binding affinity using large data sets and machine learning algorithms. Environ Sci Technol 55(10):6857–6866. https://doi.org/10.1021/acs.est.0c07040

Xie XJ, Hou ZY, Duan GX et al (2021) Boron nitride nanosheets elicit significant hemolytic activity via destruction of red blood cell membranes. Colloids Surf, B 203:111765. https://doi.org/10.1016/j.colsurfb.2021.111765

Yan XL, Zhang J, Russo DP et al (2020) Prediction of nano-bio interactions through convolutional neural network analysis of nanostructure images. ACS Sustain Chem Eng 8(51):19096–19104. https://doi.org/10.1021/acssuschemeng.0c07453

Yang K, Ma YQ (2010) Computer simulation of the translocation of nanoparticles with different shapes across a lipid bilayer. Nat Nanotechnol 5(8):579–583. https://doi.org/10.1038/NNANO.2010.141

Yang XH, Liu HH, Yang Q et al (2016) Predicting anti-androgenic activity of bisphenols using molecular docking and quantitative structure-activity relationships. Chemosphere 163:373–381. https://doi.org/10.1016/j.chemosphere.2016.08.062

Yang XH, Lyakurwa F, Xie HB et al (2017) Different binding mechanisms of neutral and anionic poly-/perfluorinated chemicals to human transthyretin revealed by in silico models. Chemosphere 182:574–583. https://doi.org/10.1016/j.chemosphere.2017.05.016

Yang ZX, Ge CC, Liu JJ et al (2015) Destruction of amyloid fibrils by graphene through penetration and extraction of peptides. Nanoscale 7(44):18725–18737. https://doi.org/10.1039/c5nr01172h

Yang ZX, Wang ZG, Tian XL et al (2012) Amino acid analogues bind to carbon nanotube via π–π interactions: comparison of molecular mechanical and quantum mechanical calculations. J Chem Phys 136(2):025103. https://doi.org/10.1063/1.3675486

Zhang Q, Yan L, Wu Y et al (2017) A ternary classification using machine learning methods of distinct estrogen receptor activities within a large collection of environmental chemicals. Sci Total Environ 580:1268–1275. https://doi.org/10.1016/j.scitotenv.2016.12.088

Zhang ST, Liu L, Duan GX et al (2019) Cytotoxicity of C_2N originating from oxidative stress instead of membrane stress. ACS Appl Mater Inter 11(38):34575–34585. https://doi.org/10.1021/acsami.9b06713

Zhou RH (2014) Molecular modeling at the atomic scale: methods and applications in quantitative biology. CRC Press, New York

Zhou RH, Gao HJ (2014) Cytotoxicity of graphene: recent advances and future perspective. Wiley Interdiscip Rev Nanomed Nanobiotechnol 6(5):452–474. https://doi.org/10.1002/wnan.1277

Zhou RH, Weikl T, Ma YQ (2020) Theoretical modeling of interactions at the bio-nano interface. Nanoscale 12(19):10426–10429. https://doi.org/10.1039/d0nr90092c

Chapter 15
Environmental Risk Assessment of Emerging Contaminants—The Case of Nanomaterials

Anders Baun and Khara Grieger

Abstract Risk assessment is a powerful tool to help evaluate potential environmental and health risks of novel materials. However, traditional risk assessment frameworks and methods often face significant challenges when evaluating novel materials due to uncertainties and data gaps. Engineered nanomaterials is one prominent example of new, advanced materials whereby scientists, researchers and decision-makers are still discussing best practices to modify and update risk assessment frameworks after nearly two decades of research. This chapter focuses on how early warning signs within the environmental risk assessment development process for nanomaterials were addressed with a focus on characterizing uncertainty. We shed light on how environmental risk assessment of nanomaterials transitioned from a state of "known unknowns" to data-driven inputs to conducting risk assessments. We also discuss ecotoxicological testing considerations, and in particular how methodological and technical challenges were addressed. Finally, we provide recommendations on how best to transfer identified best practices and knowledge to other emerging technologies and advanced materials.

Introduction—Environmental Risk Assessment of Nanomaterials and the Role of Uncertainty

The development of new materials, their widespread use in society and eventually their end-of-life management raises potential concerns over their environmental risks and safety (Hansen et al. 2013a). Key questions often include "Is this material an emerging contaminant?" and "Will this material pose new, hitherto unknown, risks

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to the environment and society?" These questions have proven to, in fact, be very complex to answer since multiple factors influence the environmental distribution, fate, effects, and ultimately the risks posed by any material. When we are dealing with novel materials, however, the complexity increases compared to conventional or well-known materials, as the scientific uncertainty can often be difficult to quantify and decision-makers are subsequently left to make choices that are not necessarily supported by scientific evidence.

While this opens up a whole range of theoretical and practical questions and considerations on how best to deal with novel materials, the introduction of engineered nanomaterials in consumer products and industrial applications provides a recent example of the development and application of a group of novel materials that has proved challenging to assess and formulate risk-based decisions. This chapter will therefore focus on the case of nanomaterials, and illustrates how early warning signs were addressed to move the field of nano-environmental fate and effects from a state of "known unknowns" to data-driven input to risk assessments. While this chapter will relate to nanomaterial risk assessment, it does not aim to evaluate the frameworks or tools to evaluate risks of nanomaterials nor the underlying or associated regulations. We conclude the chapter with several reflections on the field of nanomaterial risk assessment and provide recommendations on how best to transfer the acquired knowledge to other emerging technologies and advanced materials.

First, it is important to highlight that (quantitative) risk assessments are performed in order to evaluate risks and to support decision-making, rather than primarily serving as an academic exercise. Ideally, risk assessment should fully rely on scientific evidence (e.g., causal relationships between exposure and effect, such as dose–response assessments). However, this seldom occurs for novel environmental contaminants that have greater degrees of uncertainty, and therefore, more research is often needed to complete risk assessments and make decisions regarding the risks. This means that decision-making based on and/or assisted by risk assessments will often take place in the face of uncertainty, and the evaluation of uncertainty plays (or should play) a major role in any risk appraisal. This is widely acknowledged in the current regulatory practice and uncertainty analysis is for example an integrated part of the chemical safety assessment procedures issued by the European Chemicals Agency (ECHA 2012). Uncertainty is, however, a very dynamic parameter (or set of parameters), and only through time can the environmental risks of novel materials be more fully understood. The use and development of engineered nanoparticles in a variety of consumer products and other applications is no exception to this; although scientific knowledge has advanced and expanded significantly since the first early warning signs of adverse effects of nanoparticles on environmental organisms (Oberdörster 2004), significant uncertainty persists in understanding their environmental risks even after nearly two decades of research (Grieger et al. 2019).

Many different risk assessment frameworks and tools for nanomaterials exist today (Grieger et al. 2012; Hristozov et al. 2012; Oomen et al. 2018; Franken et al. 2020; Sorensen et al. 2019), and though they are different in their scope, applicability and resulting outcomes, they generally follow the traditional "risk assessment paradigm", i.e., that risk is a function of exposure and effects. Therefore, most procedures within

these nano-risk assessment frameworks and tools begin with information gathering and material characterization steps and then advance to effects (or "dose–response") assessment and exposure assessment. The outcome of these last two steps, which is most often scenario-based, is then aggregated into a final risk characterization step, which essentially concludes with the identification of risk or that no risk is expected (and/or more information may be needed). As mentioned above, this outcome of the risk assessment procedure is accompanied (or should be accompanied) by an uncertainty analysis that informs decision-makers of identified data gaps, limitations, and/or uncertainties relevant for the conclusion reached.

In 2007, the European Commission's Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) highlighted the following as main areas of uncertainty for identification of environmental risks of nanomaterials: environmental fate, behavior, and mobility; degradation, persistency, and bioaccumulation; and adverse effects to a variety of organisms (SCENIHR 2007). In other words, all steps of the environmental risk assessment framework of nanomaterials were considered to have serious data gaps and high degrees of uncertainty. Further data gaps were identified that centered around testing methods, equipment for testing and analyses, and the most appropriate metrics for expressing test results.

In addition to the SCENIHR 2007 report, Grieger et al. (2009) analyzed and characterized the types, levels, and nature of different uncertainties within the field of environmental risks of nanomaterials in a systematic characterization of the "known unknowns" of nanomaterial safety. This analysis was conducted by applying the Walker and Harremoës framework (Walker et al. 2003) to 31 peer-reviewed scientific papers and reports published between 2004 and 2008 on potential environmental, health, and safety (EHS) risks of nanomaterials. Overall, this work provided valuable insight on the data gaps and uncertainties as identified by scientific experts, governmental agencies, regulatory bodies, and national/international organizations in the early phase of nanomaterial risk identification. In the analysis that mapped the main areas of uncertainty and data gaps according to the reviewed papers and reports, Grieger et al. (2009) found that testing considerations, characterization of nanomaterials, effects assessment, and exposure assessment all were associated with significant uncertainty (Fig. 15.1). These findings were further supported by other reviews of the EHS data of nanomaterials with regards to uncertainty and knowledge gaps of the environmental risks of nanomaterials (*e.g.*, Maynard 2006; USEPA 2007; DEFRA 2007; OECD 2007, Baun et al. 2008). The Grieger et al. (2009) analysis showed that within the general locations of nanomaterial characterization, effects assessment, exposure assessment, testing considerations, and other areas (Fig. 15.1), a number of sub-locations of uncertainty stood out in terms of their frequency of being mentioned in the reviewed materials. The most frequently cited sub-locations of uncertainty across all sub-locations were: (1) lack of reference materials and standardization, (2) characterizing the environmental fate and behavior of nanomaterials, and (3) determining environmental effects and/or ecotoxicity.

In addition to mapping the main areas of uncertainty related to nanomaterial environmental risk assessment, Grieger et al. (2009) also analyzed the level and

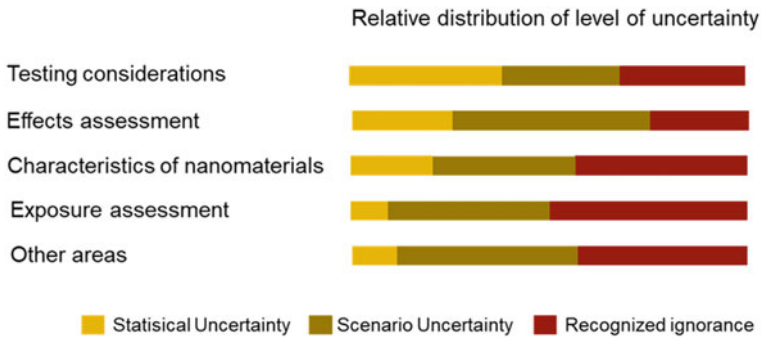

Fig. 15.1 Illustration of the relative distribution of the levels of uncertainty related to environmental, health and safety risks of nanomaterials among the five risk assessment areas where uncertainty was most predominant (redrawn from Grieger et al. 2009)

nature of these uncertainties. In accordance with the Walker and Harremoës framework, the level of uncertainty ranges from "ignorance" (i.e., unknown-unknowns) to "deterministic knowledge" (i.e., no uncertainty)—neither of which applies to nanomaterial risk assessment. Between these two extremes, other levels of uncertainty include "recognized ignorance" (i.e., known-unknowns), "scenario uncertainty" (i.e., known outcomes, unknown probabilities), and "statistical uncertainty" (i.e., known outcomes, known probabilities). While recognized ignorance is self-explanatory but impossible to quantify, the two other levels deserve a bit of explanation. In brief, scenario uncertainty relates to, e.g., scientific experiments where the outcomes are known (or can be expected) but dependent on a specific scenario, and therefore probabilities of the outcomes are unknown. In contrast to this, statistical uncertainty describes the uncertainty normally addressed in scientific studies, in that the possible outcomes, e.g., of an experiment, are known and the probabilities of these outcomes are also known or can be quantified using statistical models. While statistical uncertainty can be reduced by increased experimentation, scenario uncertainty can be reduced by more empirical research. Understanding and describing the level of uncertainty is therefore useful when evaluating whether more empirical research may help reduce uncertainties, while conducting more research can move the level of uncertainty from scenario uncertainty towards statistical uncertainty—a common approach for scientists modeling and quantifying uncertainty. In their analysis, Grieger et al. (2009) found that the level of uncertainty across all locations was between scenario uncertainty and recognized ignorance (Fig. 15.1). This showed that the general level of knowledge in 2006–2009 was at a relatively early stage of development. Finally, the nature of uncertainty was evaluated to be mainly epistemic, indicating that further research could be expected to reduce most of the uncertainties within the field.

Leveraging these key findings on the uncertainties of nano-EHS risks, the following sections take a closer look at how knowledge and data were acquired in the field of nanomaterial environmental risk assessment, including aspects of nano-effects and exposure assessments, since 2009. Using the Walker and Harremoës

framework and the Grieger et al. (2009) application of their framework, we see that the scientific community made advancements that moved from "scenario uncertainty" towards "statistical uncertainty" in the reduction of nano-risk assessment uncertainties, particularly in ecotoxicity testing. Testing considerations will therefore be given a specific emphasis in the following sections, as this was the area of uncertainty that was most frequently cited in the systematic review of "known unknowns" of nano-EHS risks in Grieger et al. (2009). It should also be mentioned that since risk is a function of exposure and effects, it is equally important to reflect on the fate, effect, and exposure assessment when discussing risks of novel materials. These topics are, however, not included in this chapter, although a number of reviews have been published in these fields including Peijnenburg et al. (2015), Baun et al. (2017), and Nowack (2017).

We also note that in the following sections on ecotoxicity testing of nanomaterials and implications for nano-environmental risk assessment, the terms "relevance" and "reliability" are used often. These terms have specific meanings when used in a regulatory context (Box 15.1). Taken together, relevance and reliability form the cornerstones in defining test results as being deemed "adequate for regulatory purposes". Further, only data that are adequate for regulatory purposes can be used in risk assessment by regulatory bodies, and therefore new/updated methods for nanomaterials must be both reliable and relevant to have an impact on risk assessments (OECD 2005).

Box 15.1 The Organisation for Economic Co-operation and Development (OECD) definitions of regulatory reliability and relevance (OECD 2005)

"Reliability is defined as the extent of reproducibility of results from a test within and among laboratories over time, when performed using the same standardised protocol. The relevance of a test method describes the relationship between the test and the effect in the target species, and whether the test method is meaningful and useful for a defined purpose, with the limitations identified. In brief, it is the extent to which the test method correctly measures or predicts the (biological) effect of interest, as appropriate."

Ecotoxicity Testing of Nanomaterials—Developments and Implications for Risk Assessment

The number of studies regarding the ecotoxicological effects of nanomaterials has increased rapidly since the first paper was published in this field in 2004 (Oberdörster 2004). For example, in the project ENRHES (Engineered Nanoparticles—Review of Health and Environmental Safety), a comprehensive literature study revealed that 89 nano-ecotoxicity studies had been published from 2004 to 2009 (Stone et al. 2010a). Five years later, the NanoE-Tox database included 1,518 nano-ecotoxicity studies in

2015 (Juganson et al. 2015), and only six years after that in 2021, a search in literature databases resulted in more than 6,000 hits, e.g., 6,156 papers listed in Web of Science (search term "nano*" AND "ecotox"; May 2021). These findings correspond to a 69-fold increase in nano-ecotoxicity papers over an 11-year time span.

Although the number of scientific papers has increased quite dramatically over the past 10–15 years, the regulatory adequacy of the performed studies has been questioned by several authors (Hartmann et al. 2017; Hjorth et al. 2017). This is a critical aspect when evaluating the performed nano-ecotoxicity studies in the peer-reviewed literature, especially for decision-making purposes. Several authors have also expressed concerns about the regulatory adequacy of nano-ecotoxicity tests even if the tests were carried out in accordance with the existing OECD test guidelines (OECD TGs). In fact, the questions regarding the need for adapting the OECD TGs, or to provide test-specific guidance, have been raised multiple times since the early days of nanotoxicology. In response to the concerns raised, OECD launched a Working Party for Manufactured Nanomaterials in 2006. The work of this international cooperation culminated in 2020 with the publication of the OECD Guidance Document 317 on aquatic and sediment toxicological testing of nanomaterials (OECD 2020; Petersen et al. 2021). In the context of improving the regulatory adequacy of ecotoxicity tests for nanomaterials, this work is highly relevant and urgently needed (Hjorth et al. 2017; Hansen et al. 2017). The guidance is targeted at improving the reliability and relevance of test results obtained in experiments following the OECD TGs, e.g., for fulfilling information requirements in REACH (Nielsen et al. 2021). In addition, the OECD 317 guidance is expected to improve the general quality of scientific studies by providing urgently needed recommendations for "best principles" for ecotoxicity studies of nanomaterials (Hon et al. 2019).

While it is beyond the scope of this chapter to give a full account of all the considerations that have shaped the current guidance document for nanomaterial aquatic toxicity testing, we will provide a glimpse into the numerous testing considerations that have arisen during the past 15 years in the following sections. In particular, we elaborate on the need for nanomaterial characterization for exposure assessment, nanoparticle-specific testing considerations, the search for nano-specific effects, and finally the regulatory use of ecotoxicological data generated in standardized tests of nanomaterials.

Nanomaterial Characterization in Exposure Assessment

The physical and chemical (termed "physicochemical") characterization of nanomaterials before, during and after testing has been a major focus in improving the reliability of ecotoxicity test results. This is because the physicochemical parameters of nanomaterials (e.g., size, size distribution, shape, and charge) are considered to play critical roles in not only understanding potential exposures to, e.g., ecological organisms, but also how these exposures impact effects, and therefore risks. Since 2010, the field of nanomaterial characterization has significantly improved,

but the number of relevant physicochemical characterization parameters and the importance of each one is still a topic debated in the scientific literature. It is nevertheless clear that in order to ensure proper, scientifically justified results from nano-ecotoxicity tests, exposure needs to be appropriately characterized, which relies on the characterization of nanomaterials across a range of physicochemical parameters. For regular, bulk-scale chemicals, the results of ecotoxicity tests are often evaluated using dose (concentration)-response curves, where the concentrations of the chemicals and therefore exposures are known. However, for nanomaterials, other physicochemical parameters than mass-based concentrations may be more relevant to describe exposures and risks, such as nanomaterial size, size distribution, shape, charge, zeta potential, or dissolution rate (Drasler et al. 2017).

As an example, particle size determination has been of high priority in many studies due to the expectation that the biological effects of nanomaterials may be related to particle size and numbers of particles. In the media used for ecotoxicity testing, it is not trivial to determine the particle size distributions – a process most often performed by dynamic light scattering (DLS), which provides indirect measures of hydrodynamic diameters based on scattered light. The potential release of metal ions from some nanomaterials (e.g., Ag, CuO, and ZnO) is also a topic that has received a lot of attention over the past decade since the metal ions in many cases have been found to account for most of the toxicity observed (Notter et al. 2014). Therefore today, it is required that dissolution is accounted for in ecotoxicity tests involving metal-based nanomaterials (OECD 2020). Further, and similar to all transformation processes taking place during testing, it is the dissolution kinetics related to aquatic media that need to be documented rather than the dissolution constant. In fact, several studies have shown that the quantification of dissolution kinetics, as well as nanomaterial losses before, during and after incubation, was crucial for determining the actual exposure concentrations of nanomaterials in ecotoxicity tests (Sørensen and Baun 2015; Sekine et al. 2015; Cupi et al. 2015).

Throughout the development of the nano-ecotoxicology field, a number of physicochemical parameters have been suggested to be of importance for characterizing nanomaterials during testing (Stone et al. 2010b). Even today there is no full scientific understanding of which parameters govern the ecotoxicity of nanomaterials (Hjorth et al. 2017; Bondarenko et al. 2016; Hund-Rinke et al. 2015, 2016; Drasler et al. 2017). As mentioned before, the recent OECD Guidance Document No. 317 provides recommendations for regulatory-oriented testing, while from a scientific standpoint, the current recommendation is that as much characterization data as possible should be reported for ecotoxicity tests of nanomaterials. Hjorth et al. (2017) phrased this as: "a move from the traditional focus on controlling exposure, (as recommended) in TGs applied to dissolved chemicals, toward a focus on describing exposure through a range of different techniques (Wickson et al. 2014; Sørensen et al. 2016)".

Finally, it should be emphasized that most published studies on nano-ecotoxicology have focused on nanoparticle characterization in the stages before ecotoxicity testing. However, it has been increasingly recognized and deemed essential to quantify the actual exposure during testing to increase both the scientific value and the regulatory adequacy of nano-ecotoxicological studies (Hartmann et al.

2017). The lack of nanoparticle characterization during testing may be problematic for the interpretation of individual test results as well as for comparing studies, even if the same nanomaterials were tested using the same test method. In other words, it can be difficult to draw general conclusions even from test results using standard methods without full nanomaterial characterization. This, in turn, poses challenges for the development of validated in silico methods for predicting the ecotoxicological potential of nanomaterials based on physicochemical material properties.

Challenges in Nanomaterial Effect Assessments—Particles Are Not Dissolved Chemicals

Overall, standardized ecotoxicological tests have been challenged by the fact that nanomaterials do not behave like dissolved chemicals in aqueous suspensions. For example, it may be very problematic to maintain stable suspensions during the testing period, and even with extensive characterization, new and unexpected phenomena may be encountered as described in the previous section.

To illustrate these challenges, we will take a closer look at the algal growth rate inhibition test. This test is one of the three mandatory ecotoxicological tests to be carried out for classification, labeling, and risk assessment purposes for chemicals and nanomaterials in the European Union (EU). The procedure for the algal test is described in OECD TG201 (OECD 2011), as well as in the somewhat stricter standard of the International Organization for Standardization (ISO 2012). When nanomaterials are tested in algal growth rate inhibition tests, the nanomaterials may scatter light and therefore decrease the amount of light reaching the algal cells, thereby inhibiting or reducing the growth rate (i.e., "shading"), rather than contributing to or leading to any ecotoxicological effect on the algal cells. Shading has, in fact, been identified as a potential confounding factor of algal testing of nanomaterials since the very first publications in the area (Hund-Rinke and Simon 2006) and continues today (Hjorth et al. 2017; Nguyen et al. 2020). Although practical solutions exist to determine whether shading occurs (Fig. 15.2), it is still an open question as to what degree it influences the outcome of standard testing. Shading caused by nanomaterials in algal tests is a prominent example of a nano-specific influence that could be interpreted as an effect, but may also be a result of how tests are performed. Therefore, extrapolation of such effects from the algal growth rate inhibition tests to other organisms or the ecosystem will often not be valid due to this confounding effect of shading (Skjolding et al. 2016; Hjorth et al. 2017).

Further, the potential shading effects of nanomaterials have often been mentioned as a possible cause of the effects observed in algal toxicity tests (Handy et al. 2012; Petersen et al. 2015; Sørensen et al. 2016). A number of scientific studies on disclosing shading effects of nanomaterials have been conducted, predominantly by separating algal cells from the nanomaterial suspension (Fig. 15.2) to eliminate any direct toxicity, and illuminating the algae through the nanomaterial suspension

Fig. 15.2 Illustration of a testing setup for algal toxicity tests (**a**) and two setups to distinguish between physical and chemical effects of nanomaterials (**b** and **c**). In **b**, shading effects may be investigated using a double-vial setup, where algal cells are contained in the smaller inner-vial, surrounded by the nanoparticle suspension in the larger outer-vial. In **c**, the so-called "sandwich" setup reveals whether physical shading occurs in the algal test. Here one 6-well plate is filled with algal suspension without nanomaterials (c1) and another plate (c2) is filled with nanomaterial suspension without algae added. Plate c2 is placed on top of c1 and the combined "sandwich" setup is illuminated from above. For both modified setups (**b** and **c**), a decline in growth rate will be caused by physical shading caused by the tested nanomaterial (Modified from Sørensen et al. 2015). In addition to measuring growth rate (or biomass) in algal tests, changes in the algal pigment composition have also been a direct measure to quantify the effects of nanomaterial shading (Hjorth et al. 2016). This approach relies on the ability of algae to rapidly adapt their pigment composition in response to changing light conditions and therefore serves as an effective endpoint to quantify shading effects of nanomaterials (Hjorth et al. 2016)

(a so-called "sandwich" test) to determine if growth rate inhibition occurs as a result of nanomaterials obstructing the light available to the algae (Aruoja et al. 2009; Hartmann et al. 2010; Hund-Rinke and Simon 2006; van Hoecke et al. 2009). These studies have generally rejected this type of indirect shading as a cause of growth inhibition. In contrast, Sørensen et al. (2016) identified substantial growth rate inhibition when separating algae and platinum nanoparticles (PtNPs) in a double-vial setup.

Both the "sandwich" and separation setup shown in Fig. 15.2 aim to quantify whether shading from nanomaterials in suspension contributes to growth reduction. However, shading from nanomaterials attached directly to the algae (sometimes referred to as "cellular shading") is not considered by these approaches. Cellular shading may be highly important, as several studies have demonstrated nanomaterial attachment to algal cells (Aruoja et al. 2009; van Hoecke et al. 2009, Hartmann et al. 2013; Sørensen et al. 2016; Pang et al. 2020; Abdolahpur Monikh 2021). In general, it is believed that algal cells can overcome temporary shading and that this may not cause population effects due to growth rate reductions. Adhesion of nanomaterials to algal cells can however result in permanent shading but can also cause other physical effects like the limitation of nutrient availability.

The issues mentioned above for the algal test illustrate how each of the standardized ecotoxicity tests used for risk assessment purposes faces specific challenges when applied to particle suspensions rather than the dissolved chemicals that they were developed for (Skjolding et al. 2016). The standardized tests with fish and

crustaceans are both challenged by keeping nanomaterial suspensions stable during incubation but also by a particle-specific change in exposure pathway by active or passive intake, either through ingestion or attachment on gills. This is fundamentally different from the exposure through molecular diffusion that is dominating in tests of dissolved chemicals.

As described above, it is not an easy task to document that the exposure to nanomaterials is controlled during an experiment. This is further complicated by the presence of organisms since their presence and interaction with nanomaterial suspension will interfere with the characterization techniques. For example, using DLS to determine the "in situ" size distribution of the suspension at the end of an algal test is hampered by the fact that samples extracted do not only contain algae but also that the algae have excreted exudates during incubation. Figure 15.3 shows two examples of the changes that mono-dispersed suspensions of gold and titanium dioxide nanoparticles undergo during 48 h of testing in a standardized algal toxicity test (from Hartmann et al. 2013). It is evident that the size distribution of particles in the medium is affected, but also that significant interaction with organisms also takes place.

The presence of exudates will not only influence the nanoparticle characterization (Fig. 15.3) but may also affect the toxicity of the nanomaterials. Exudates as well as naturally occurring organic matter (NOM) may form a coating on the nanomaterial surface. This is often referred to as an eco-corona. The influence of NOM on the ecotoxicity of nanomaterials has been a topic of many ecotoxicity studies (e.g., Arvidsson et al. 2020), while fewer studies have focused on eco-coronas composed of organism exudates (Docter et al. 2015; Nasser and Lynch 2016). Knowledge in this area is developing and shows that biomolecule coronas are established rapidly (Hjorth et al. 2017). This type of interaction is likely to occur for all nanomaterials but perhaps to different degrees depending on the composition and surface properties of nanoparticles. This aspect is of high importance for environmental risk assessment since the occurrence of pristine nanomaterials, once released into the environment, is unlikely.

In a recent meta-analysis of the influence of NOM on the aquatic ecotoxicity of nanomaterials, Arvidsson et al. (2020) examined 66 studies of metal and metal oxide nanomaterials. It was found that 84% of these studies showed a reduction in nanomaterial ecotoxicity in the presence of NOM. No strong correlation between the 50% effective, inhibitory or lethal concentrations (XC_{50} values) and concentrations of NOM occurred, but it was found that the toxicity decreased 1–10 times when NOM was present during testing (Arvidsson et al. 2020). This led the authors to suggest that XC_{50} values from experiments without NOM present may be used in environmental risk assessments of nanomaterials as reasonably conservative estimates of XC_{50} values with NOM present.

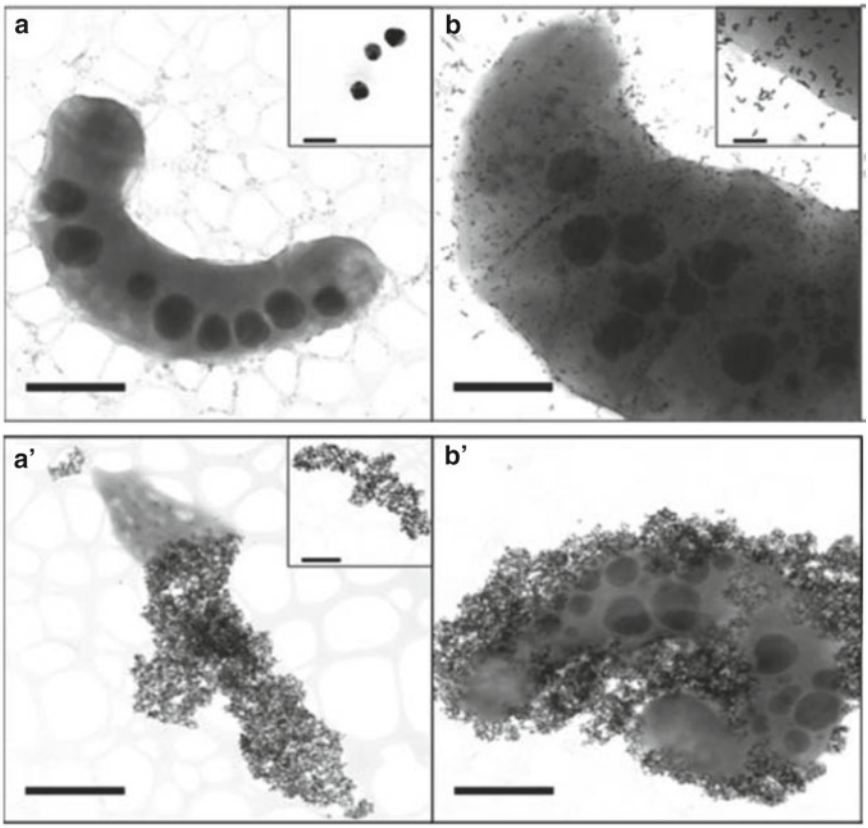

Fig. 15.3 Transmission electron microscopy (TEM) images of algal cells exposed to 1.9 mg/L gold nanoparticles (upper panel) and 35 mg/L titanium dioxide nanoparticles (lower panel). Scale bars are 2 μm. In the upper panel, TEM images were taken at the start of the test (**a**) and after 24 h of testing (**b**). Insert shows, **a**: individual gold nanoparticles (scale bar is 40 nm), **b**: attached gold nanoparticles at the edge of an algal cell (scale bar is 300 nm). In the lower panels, TEM images were taken after 24 h (**a**') and after 48 h (**b**') of testing. Modified from Hartmann et al. (2013)

Nanomaterial-Specific Effects and Modes of Action

While the discipline of nanoecotoxicology has developed significantly during the past decade, the exact toxic mechanisms or modes of action of nanomaterials remain unclear. This may be influenced by the technical challenges in testing, as discussed briefly above and as outlined by Skjolding et al. (2016) who highlighted that several factors must be accounted for to disclose whether a "nano-specific effect" occurs. As shown in Fig. 15.4, the different responses and potential confounding factors depend on the intrinsic and extrinsic properties of the nanomaterials. The response types relate to the dissolution in aqueous media, intake and discrete localization within the test organisms, as well as physical effects on test organisms (Fig. 15.4). As illustrated

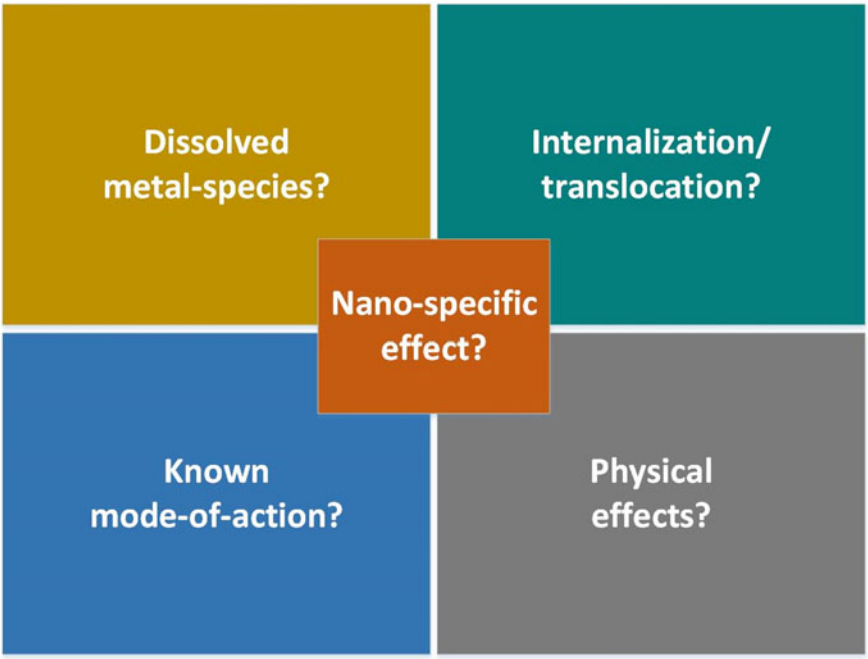

Fig. 15.4 Responses that occur concomitantly in ecotoxicity tests of nanomaterials which influence or dominate the outcome of the tests: Effects related to the dissolved fraction (top left), effects of internalization and translocation of nanomaterials due to their small size (top right), physical effects of the nanoparticles (bottom right), and the nanoparticle effects via a proposed mode of action related to, e.g., the generation of reactive oxygen species (bottom left). Based on Skjolding et al. (2016)

for the algal test above, the test setup may not allow for a differentiation of response type and since these occur simultaneously and (very) dynamically, it is often difficult to disclose an actual mode of action (Hjorth et al. 2017).

A multitude of studies has searched for modes of action or toxic mechanisms across a range of nanomaterials. For example, Lynch et al. (2014) summarized the possible mechanisms as follows:

- Dissolution, whereby the observed effects are caused by toxic ions;
- Nanomaterial surface effects, which lead to effects on the conformation of biomolecules;
- Nanomaterial structure effects, such as photochemical and redox properties resulting from bandgap or crystalline forms of nanomaterials;
- Nanomaterials as vectors, whereby nanomaterials may act as vectors to transport other toxic chemicals to sensitive targets (e.g., Trojan horse effects).

As mentioned above, metals and metal oxide nanomaterials (e.g., CuO, ZnO or Ag) are prone to dissolution. Further, many studies have focused on the role of the nanomaterial versus the released ions in explaining nano-ecotoxicity. For metals and

Table 15.1 Overview of use of standardized test methods in nanomaterial risk assessment

Use of testing data	Aim of testing	
	Classification	Environmental protection
Risk assessment step	Hazard identification Hazard ranking	Hazard assessment
Focus in relation to regulatory use	Reliability	Relevance
Experimental focus	Controlled experiments	Environmental realism
Results evaluation	Concentration–response curves	Assessment factors Species sensitivity distributions
Outcome - values	LC_{50}, EC_{50}, NOEC	PNEC
Nature of outcome	Relative	Absolute

metal oxide nanomaterials, the ability to generate reactive oxygen species (ROS, such as superoxide, hydroxyl radicals, and hydrogen peroxide) and induce oxidative stress is still the only distinct modes of action documented for nanomaterials in aquatic organisms (Ivask et al. 2014; Juganson et al. 2015; von Moos and Slaveykova 2014). However, even if this mode of action is suspected, it may be technically difficult to prove in tests, since the formation of extra- or intracellular ROS can trigger a cascade of cellular events (von Moos and Slaveykova 2014). Therefore, care has to be taken before conclusions can be drawn, even on this known mechanism of toxicity.

Finally, it is important to underline that while the ecotoxicity tests used for risk assessment do not allow an assessment of the mechanism causing the toxicity, this is in alignment with current regulatory approaches for the hazard assessment of conventional chemicals. The understanding of the potential mode of action of nanomaterials in environmental organisms is a question of high scientific relevance. This may in turn influence the design and endpoints of ecotoxicity testing for regulatory purposes, as shown by the recommendations of the 2020 OECD guidance document for aquatic toxicity testing of manufactured nanomaterials (OECD 2020). All this is tightly linked to the use and role of ecotoxicity data in risk assessment (Table 15.1) as will be further described in the following section.

Use of Ecotoxicity Data in Nanomaterial Risk Assessment

In risk assessment of "regular" (i.e., conventional) chemicals, ecotoxicity data are used in two distinct ways: 1) for "classification" purposes, i.e., to classify, label and determine the toxicity (T) criterion in a PBT assessment (PBT – persistence, bioaccumulation, toxicity), and 2) for "protection" purposes, i.e., the derivation of predicted no-effect concentrations (PNECs). Table 15.1 lists a number of differences in the purpose of ecotoxicity testing for these two uses of the data generated, also applicable to risk assessment of nanomaterials.

This approach is in agreement with the classical distinction in ecotoxicology between "anticipatory laboratory ecotoxicity testing" aimed at hazard identification and "assessment testing" aimed at environmental impact evaluation (Calow 1997; Hjorth 2016). As such, ecotoxicity testing using guidelines should support regulatory hazard ranking and labeling, whereas field testing should ideally inform decisions on environmental quality standards aimed at protecting the environment. For this reason, guideline tests focus on controlled experiments and inter-laboratory transferability (i.e., regulatory reliability), whereas environmental realism plays a much stronger role in the validity of field-scale tests.

In reality, for nanomaterials as well as for conventional chemicals, test results generated by following guideline recommendations for classification purposes as shown in Table 15.1 will also form the basis for evaluations of environmental protection. This has been termed as a "double use" of the data from guideline testing (Hjorth et al. 2017). The measures for regulatory adequacy, like relevance and reliability, will favor studies carried out according to internationally agreed-upon guidelines and standards (Hartmann et al. 2017). Therefore, there is a very strong focus on tests using the core set of organisms (i.e., fish, crustaceans, algae). The dataset generated with the original aim of ranking and classifying chemicals (i.e., a relative outcome) will then often be the only dataset available, and will therefore be used for defining environmental protection goals, i.e., an absolute outcome (Table 15.1).

For conventional chemicals, a precedent for this "double use" of ecotoxicity data has been established over the last 30 years. The use of the same test results at different stages in the risk assessment procedure relies on cut-off values and extrapolation methods that have been agreed upon by regulatory authorities and stakeholders, often following advice from expert groups (Syberg and Hansen 2015). For nanomaterials, the double use of guideline testing data for both purposes, as shown in Table 15.1, remains to be critically evaluated, though the appropriateness of extrapolation from guideline test data for PNEC determination has been questioned by several authors (Lützhøft et al. 2015; Baun et al. 2009). This critique has been made on the basis of the fundamental difference between dissolved chemicals and particles with regard to their behavior and effects in laboratory studies, compared to real-world behaviors and effects that may occur in complex environmental matrices.

The current procedures for establishing protective values have been transferred directly from dissolved chemicals to nanomaterials, relying mainly on so-called assessment factors, and in some cases, species sensitivity distributions (SSD). Both methods rely on the use of extrapolation approaches from laboratory studies to the protection of ecosystem functions. These approaches are founded on the basic toxicological notion that a higher concentration will lead to greater effects. Thus, monotonous concentration–response curves and stable suspensions during testing are inherent requirements for the ecotoxicity data to be valid for risk assessment purposes. As described above, this prerequisite is often severely challenged when nanomaterials are tested in standardized tests. In concentration–response experiments, changes in nanoparticle behavior may furthermore be nanoparticle concentration-dependent (e.g., stronger aggregation at high concentrations of nanoparticles) and this may alter

the bioavailability of the particles and not necessarily result in monotonous concentration–response curves. For PNEC estimations by the assessment factor approach, this may be a problem for the validity of the extrapolation from standardized tests, since actual environmental effects may occur at lower concentrations than those used in the standardized tests, although this remains to be systematically investigated.

The other approach for PNEC determination, i.e., using SSDs, has only been used to a limited extent for nanomaterials (Sørensen et al. 2020). This field of application has significantly increased since the first nano-SSD was developed in 2013 by Gottschalk et al. (2013). For example, Chen et al. (2018) constructed SSDs considering nanomaterial structural characteristics, such as coating, size, shape, and experimental conditions for Ag, CeO_2, CuO, TiO_2 and ZnO nanoparticles. To account for the differences in the relevance and reliability of ecotoxicological data across studies, Semenzin et al. (2015) developed a nano-species sensitivity weighted distribution (n-SSWD). This approach was compared to a conventional SSD model as well as to a probabilistic SDD model for nanomaterials by Sørensen et al. (2020). In this model comparison, only studies regarding two reference materials, NM-300 K (silver) and NM-105 (titanium dioxide), were included and all data were evaluated by the nano-specific "nanoCRED" reliability criteria (Hartmann et al. 2016). While it was found that the conventional SSD generally yielded the most conservative but least precise output, the estimated hazardous concentrations for 5% of species (HC_5 values) of all models were within a narrow concentration range (Sørensen et al. 2020). Interestingly, the majority of studies were evaluated as being reliable from the regulatory perspective, although it was found that the degree of nano-specific characterization varied greatly (Sørensen et al. 2020). Generally, this study showed that for a large, well-curated data set, the output relevant for PNEC estimation was not very sensitive towards the choice of SSD model, but also that regulatory adequacy was improved by taking nano-specific considerations into account. This is important for the further development of nanomaterial risk assessment approaches, since PNEC values generated from SSD were not as dependent on extrapolation factors as PNEC values estimated by the assessment factor approach.

Overall, this section emphasizes that nanoecotoxicology tests serve different purposes, and different tests are needed to fulfill different regulatory needs in regard to risk assessment (Table 15.1). For hazard identification purposes, an ideal test would allow for controlled exposure conditions which, in combination with thorough nanomaterial characterization, would allow for reliable and reproducible benchmarking. For hazard assessment purposes, testing should ideally be carried out at environmentally realistic concentrations and under realistic conditions (Table 15.1). This type of testing will inherently be challenged in the current definition of regulatory reliability, but their relevance for the regulatory question at hand will be higher than that for the currently used approaches. For this, new tests/test designs and ecotoxicological endpoints are most likely needed, and extrapolation methods should be scrutinized in a systematic way (Lützhøft et al. 2015; Hjorth 2016; Aitken et al. 2011; Palmqvist et al. 2015; Syberg and Hansen 2015). Lastly, it is important to underline that the data with little regulatory relevance should not be confused with "bad or flawed data". This is because scientific studies without a regulatory focus or regulatory compliance

have a very high value in themselves and are still needed to further develop the field of nanoecotoxicology (Hjorth et al. 2016; Wickson et al. 2014).

Reflections from Nanomaterial Toxicity Testing and Perspectives for Risk Assessment of Emerging Contaminants

In the preceding sections, some of the fundamental uncertainties related to performing risk assessment on novel materials have been described and exemplified by reviewing the development of regulatory-relevant ecotoxicity testing for nanomaterials over the past 15 years. Through this reflection, it becomes clear that the knowledge base has expanded significantly over this time period, and for several areas, the level of uncertainty has moved from "recognized ignorance" to "scenario uncertainty" to "statistical uncertainty". More than 15 years later, the fields of nano-EHS and risk assessment have expanded significantly, with thousands of peer-reviewed articles published on the topic of nano-ecotoxicity alone, and therefore a thorough review of papers and reports on nano-EHS knowledge would require a substantial undertaking, that may benefit from new advancements in data mining and automatic text analysis.

Also, through a reflection of the field of nano-ecotoxicology, it has become clear that it takes time and dedicated research efforts to reach a level where specific testing guidance can be given regarding relevant and reliable data generation for risk assessment of novel materials. In the case of ecotoxicity of nanomaterials, work in this field was initiated by the OECD in 2007 and ultimately finalized 13 years later through the publication of the previously-mentioned guidance document (OECD 2020). While this may seem like a long time period to develop this guidance, it is in fact faster than what has been observed previously for other emerging contaminants (Syberg and Hansen 2015). During this time, the production and application of nanomaterials have also increased significantly (Hansen et al. 2020), and the reliance on risk assessment for decision-making for nanomaterials has faced numerous challenges. This is partly due to the complexities and uncertainties described in this chapter but also due to underlying challenges for risk assessment frameworks for decision making (Grieger et al. 2009, 2019).

For novel materials and emerging environmental contaminants, this opens the question whether we must wait another 13 years to assess each new case, or whether we have learned from the case of nanomaterials, that a more proactive approach to risk assessment can be implemented. It is important to reflect on what we have learned from the nano-risk analysis that could be applicable to other fields that also are characterized by having sparse data and having a level of uncertainty that ranges between "recognized ignorance" and "scenario uncertainty."

Grieger et al. (2019) provided a perspective on this issue and concluded that the preceding 15 years of experience with nanomaterial risk analysis should be used to address potential risk issues of other emerging technologies or contaminants since

the pace of innovation is surpassing the pace of risk identification and quantification. The authors suggested a number of best practices that may be applicable to other emerging and disruptive technologies (e.g., synthetic biology, 3-D printing, climate engineering technologies) (Box 15.2, Grieger et al. 2019).

Box 15.2 Five best practices for risk analysis of emerging technologies based on experiences from nanomaterial risk analysis (summary based on Grieger et al. 2019)

1. Promote Research Tailored for Regulatory Decision-Making
Nano-risk research has largely been directed towards understanding the science rather than meeting decision-making and regulatory needs. While it is possible to link evolving nano-safety data to decision and policy-relevant needs using "bottom-up" strategies, the initiation of strategic, purposeful regulatory-relevant science programs (*i.e.*, using "top-down" strategies) at the start of major risk-based efforts for emerging technologies could help target research more effectively towards regulatory decision-making

2. Set Realistic Time, Cost, and Complexity Estimates to Develop Risk Analysis
Similar to nano-risk analysis, the process of identifying risks, adapting or developing assessment protocols and procedures, and testing, validating, and harmonizing risk assessment methods for other emerging technologies are also likely be complex, time-consuming, and expensive. This may especially be the case if this process is based on the traditional approach of relying on experimental evidence and knowledge-based assessments for risk evaluations. It may help prepare and align stakeholder expectations early on to have realistic estimates of the time, costs, and degrees of complexities involved to derive concrete conclusions regarding risks. These estimates may help prepare industry, policymakers, and other decision-makers prioritize research efforts and funding programs directed at near-term methods, policy or decision-making while the underlying safety science is developed

3. Develop Proactive Strategies to Deal with Uncertainties in Risk Analysis and Decision-Making
Scientific uncertainty has been one of the main obstacles in nanomaterial risk analysis. In general, standard approaches to handle uncertainties in risk assessment may not be well-suited for emerging technologies that are often characterized by having deep and extensive uncertainties in terms of potential risk pathways and consequences. Rather, risk assessment efforts for nanomaterials and other emerging technologies may benefit from including or being complemented by separate uncertainty assessments that identify and describe different scientific uncertainties and communicate how they may impact overall risk assessments and evaluations. Dynamic risk evaluation and management processes may be useful for dealing with emerging technological risks, as they allow for adaptive responses to quickly evolving scenarios or in light of new information. Adaptive and responsible risk governance frameworks that specifically account for uncertainty in risk evaluations, incorporate diverse stakeholder perspectives, and include procedural robustness may also be useful to proactively deal with uncertainty in risk analysis and decision-support

(continued)

(continued)

4. Develop Mechanisms to Share Risk Data while Protecting Privacy, Confidentiality, and Proprietary Information
Concrete conclusions regarding the potential risks of nanomaterials have been hampered by challenges related to how nano-risk data have been managed and harmonized, along with issues of privacy, confidentiality, and intellectual property. Having more harmonized, multi-scale, and decision-directed approaches may help avoid some challenges related to data harmonization and integration. Future risk assessment and management efforts could rely on robust communication mechanisms between researchers and, with appropriate funding, integrate risk research efforts with respect to curation functionality, infrastructure, and communication processes at multiple levels of granularity from the onset

5. Critically Evaluate and Select Robust, Fit-For-Purpose Tools for Risk Analysis
While pursuing and deriving risk assessment for nanomaterials and other emerging technologies is clearly worthwhile in many cases (*e.g.*, following "traditional science" processes), it may also be lengthy and time-consuming, and there may be other risk evaluation approaches that could be more applicable for a given decision. A process that critically evaluates diverse evaluation frameworks and approaches followed by a transparent selection process for an emerging technology could be beneficial early-on to ensure that the most fit-for-purpose risk analysis framework is selected for utilization, further exploration, or to ultimately produce outcomes that meet decision-makers' needs

While the perspectives and best practices put forward by Grieger et al. (2019) target a more general level for evaluating risks of other new or novel technologies, other articles have identified "early" risk indicators for nanomaterials (Subramanian et al. 2016; Arvidsson et al. 2018). In fact, as early as in 2008, Hansen et al. (2008) analyzed the introduction of nanomaterials and associated risks according to the "Late lessons for Early Warnings" presented by the European Environment Agency in 2001 (EEA 2001) and updated in 2013 (Hansen et al. 2013a). The same year, five early warning signs for harmful properties of nanomaterials were suggested by Hansen et al. (2013b), including novelty, persistency, bioaccumulation, the potential for being readily dispersed in the environment, and potential for causing irreversible action (e.g., toxicity). Hansen et al. (2013b) assessed these early warning signs using a set of five well-known nanomaterials, but more importantly for the field of emerging risk areas, they also discussed how these warning signs could be used by stakeholders in an effort to develop safe(r) nanomaterials and to communicate what is risk and uncertainties from a precautionary angle. The authors suggested that regulators could directly use the five early warning signs for precautionary action, ranging from a ban to the implementation of risk research. Also later in 2013, the early warning signs were incorporated into the hazard and exposure ranking tool, NanoRiskCat, which was aimed to support companies and regulators in their first-tier risk assessment and communication process regarding the known hazard and exposure potentials of consumer products containing nanomaterials (Hansen 2013c). The NanoRiskCat tool has been applied to all the 5,157 products claimed to contain nanomaterials in

the NanoDataBase (nanodb.dk, visited 4 June 2021), and it also formed the basis for the suggestion for a new regulatory framework for nanomaterials called ReactNow (Hansen 2017).

The example given above illustrates that there are indeed many key findings and best practices we have learned from the field of nanomaterial risk assessment that may be transferred to other emerging risk issues. It should also be recognized that uncertainty and incomplete understanding will continue to be inherent parts of any risk analysis of novel chemicals, materials, or technologies. The nanomaterial risk analysis field has also shown that there are ways to deal with these issues, and decision-support can be provided even under high (and different) levels of uncertainty by drawing on past experiences, rather than to (continue to) expect and/or wait for full scientific evidence.

References

Abdolahpur-Monikh F, Chupani L, Arenas-Lago D et al (2021) Particle number-based trophic transfer of gold nanomaterials in an aquatic food chain. Nat Commun 12:899

Aitken RA, Bassan A, Friedrichs S et al (2011) Specific advice on exposure assessment and hazard/risk characterisation for nanomaterials under REACH (RIP-oN 3) – Final Project Report. European Commission, Brussels, Belgium

Aruoja V, Dubourguier H-C, Kasemets K et al (2009) Toxicity of nanoparticles of CuO, ZnO and TiO2 to microalgae Pseudokirchneriella subcapitata. Sci Total Environ 407:1461–1468

Arvidsson R, Furberg A, Baun A et al (2018) Proxy measures for simplified environmental assessment of manufactured nanomaterials. Environ Sci Technol 52:13670–13680

Arvidsson R, Hansen SF, Baun A (2020) Influence of natural organic matter on the aquatic ecotoxicity of engineered nanoparticles: recommendations for environmental risk assessment. NanoImpact 20:100263

Baun A, Hartmann NB, Grieger K et al (2008) Ecotoxicity of engineered nanoparticles to aquatic invertebrates: a brief review and recommendations for future toxicity testing. Ecotoxicology 17:387–395

Baun A, Hartmann NB, Grieger KD et al (2009) Setting the limits for engineered nanoparticles in European surface waters—Are current approaches appropriate. J Environ Monit 11:1774–1781

Baun A, Sayre P, Steinhäuser KG et al (2017) Regulatory relevant and reliable data for the environmental fate of manufactured nanomaterials. NanoImpact 8:1–10

Bondarenko OM, Heinlaan M, Sihtmae M et al (2016) Multilaboratory evaluation of 15 bioassays for (eco)toxicity screening and hazard ranking of engineered nanomaterials: FP7 project NANOVALID. Nanotoxicology 10:1229–1242

Calow P (1997) General principles and overview. In: Calow P (ed) Handbook of ecotoxicology. Blackwell Scientific Publications, Oxford, England

Chen G, Peijnenburg WJGM, Xiao Y et al (2018) Developing species sensitivity distributions for metallic nanomaterials considering the characteristics of nanomaterials, experimental conditions, and different types of endpoints. Food Chem Toxicol 112:563–570

Cupi D, Hartmann NB, Baun A (2015) The influence of natural organic matter and aging on suspension stability in guideline toxicity testing of ZnO, TiO_2, and Ag nanoparticles with Daphnia magna. Environ Toxicol Chem 34:497–506

DEFRA (2007) Characterising the potential risks posed by engineered nanoparticles; Second U.K. Government research report; Department for Environment, Food and Rural Affairs: London, UK

Docter D, Westmeier D, Markiewicz M et al (2015) The nanoparticle biomolecule corona: lessons learned—Challenge accepted? Chem Soc Rev 44:6094–6121
Drasler B, Sayre P, Steinhauser K et al (2017) In vitro approaches to assess the hazard of nanomaterials. NanoImpact 8:99–116
ECHA (2012) Guidance on information requirements and chemical safety assessment Chapter R.19: Uncertainty analysis. European Chemicals Agency, Helsinki, Finland
EEA (2001) Late lessons from early warnings: the precautionary principle 1896–2000, European Environmental Agency, Copenhagen, Denmark
Franken R, Heringa MB, Oosterwijk T et al (2020) Ranking of human risk assessment models for manufactured nanomaterials along the Cooper stage-gate innovation funnel using stakeholder criteria. NanoImpact 17:100191
Gottschalk F, Kost E, Nowack B (2013) Engineered nanomaterials in water and soils: a risk quantification based on probabilistic exposure and effect modeling. Environ Toxicol Chem 32:1278–1287
Grieger K, Hansen SF, Baun A (2009) The known unknowns of nanomaterials: describing and characterizing uncertainty within environmental, health and safety risks. Nanotoxicology 3:1–12
Grieger KD, Linkov I, Hansen SF et al (2012) Environmental risk analysis for nanomaterials: review and evaluation of frameworks. Nanotoxicology 6:196–212
Grieger K, Jones JL, Hansen SF et al (2019) What are the key best practices from nanomaterial risk analysis that may be relevant for other emerging technologies? Nat Nanotechnol 14:998–1001
Handy RD, Cornelis G, Fernandes TF et al (2012) Ecotoxicity test methods for engineered nanomaterials: practical experiences and recommendations from the bench. Environ Toxicol Chem 31:15–31
Hansen SF, Maynard A, Baun A et al (2008) Late lessons from early warnings for nanotechnology Commentary. Nat Nanotechnol 3:444–447
Hansen SF, Maynard A, Baun A et al (2013a) Nanotechnology—Early lessons from early warnings. Chapter 22 in "Late Lessons from Early Warnings vol. II". European Environment Agency, European Commission, Copenhagen, Denmark
Hansen SF, Nielsen K, Knudsen N et al (2013b) Operationalization and application of "early warning signs" to screen nanomaterials for harmful properties. Environ Sci Process Impacts 15:190–203
Hansen SF, Jensen KA, Baun A (2013c) NanoRiskCat—A conceptual tool for categorization and communication of exposure potentials and hazards of nanomaterials in consumer products. J Nanoparticle Res 16: 2195
Hansen SF (2017) React now regarding nanomaterial regulation. Nat Nanotechnol 12:714–716
Hansen SF, Hjorth R, Skjolding LM et al (2017) A critical analysis of the environmental Dossiers in the OECD sponsorship programme for manufactured nanomaterials. Environ Sci Nano 4:282–291
Hansen SF, Hansen OFH, Nielsen MB (2020) Advances and challenges towards consumerization of nanomaterials. Nat Nanotechnol 15:964–965
Hartmann NB, Von der Kammer F, Hofmann T et al (2010) Algal testing of titanium dioxide nanoparticles-testing considerations, inhibitory effects and modification of cadmium bioavailability. Toxicology 269:190–197
Hartmann NB, Engelbreckt C, Zhang J et al (2013) The challenges of testing insoluble metal and metal oxide nanoparticles in algal bioassays: titanium dioxide and gold nanoparticles as case studies. Nanotoxicology 7:1082–1094
Hartmann NB, Ågerstrand M, Lützhøft HCH et al (2017) NanoCred: a transparent framework to assess the regulatory adequacy of ecotoxicity data for nanomaterials—Relevance and reliability revisited. NanoImpact 6:81–88
Hjorth R (2016) Environmental risk assessment and management of engineered nanomaterials—The role of ecotoxicity testing (PhD Thesis) Technical University of Denmark, Kgs. Lyngby, Denmark
Hjorth R, Sørensen SN, Olsson ME et al (2016) A certain shade of green: can algal pigments reveal shading effects of nanoparticles? Integr Environ Assess Manag 12:200–202

Hjorth R, Skjolding LM, Sørensen SN et al (2017) Regulatory adequacy of aquatic ecotoxicity testing of nanomaterials. NanoImpact 8:28–37
Leong HS, Butler KS, Brinker CJ et al (2019) On the issue of transparency and reproducibility in nanomedicine. Nat Nanotechnol 14:626–635
Hristozov DR, Gottardo S, Critto A et al (2012) Risk assessment of engineered nanomaterials: a review of available data and approaches from a regulatory perspective. Nanotoxicology 6:880–898
Hund-Rinke K, Baun A, Cupi D et al (2016) Regulatory ecotoxicity testing of nanomaterials—Proposed modifications of OECD test guidelines based on laboratory experience with silver and titanium dioxide nanoparticles. Nanotoxicology 10:1442–14447
Hund-Rinke K, Herrchen M, Schlich K et al (2015) Test strategy for assessing the risks of nanomaterials in the environment considering general regulatory procedures. Environ Sci Eur 27:1–12
Hund-Rinke K, Simon M (2006) Ecotoxic effect of photocatalytic active nanoparticles TiO_2 on algae and daphnids. Environ Sci Pollut Res 13:225–232
ISO (2012) Water quality - Fresh water algal growth inhibition test with unicellular green algae. ISO 8692:2012. International Organization for Standardization, Geneva, Switzerland
Ivask A, Juganson K, Bondarenko O et al (2014) Mechanisms of toxic action of Ag, ZnO and CuO nanoparticles to selected ecotoxicological test organisms and mammalian cells in vitro: a comparative review. Nanotoxicology 8:7–71
Juganson K, Ivask A, Blinova I et al (2015) NanoE-Tox: new and in-depth database concerning ecotoxicity of nanomaterials. Beilstein J Nanotechnol 6:1788–1804
Lützhøft HCH, Hartmann NB, Brinch A et al (2015) Environmental effects of engineered nanomaterials: estimations of predicted no-effect concentrations (PNECs). Danish Environmental Protection Agency, Copenhagen, Denmark
Lynch I, Weiss C, Valsami-Jones E (2014) A strategy for grouping of nanomaterials based on key physico-chemical descriptors as a basis for safer-by-design NMs. Nano Today 9:266–270
Nasser F, Lynch I (2016) Secreted protein eco-corona mediates uptake and impacts of polystyrene nanoparticles on Daphnia magna. J Proteomics 137:45–51
Maynard A (2006) Nanotechnology: a research strategy for addressing risk, project on emerging nanotechnologies, Report No. PEN 3; Woodrow Wilson International Center for Scholars: Washington, DC
Nielsen MB, Baun A, Mackevica A et al (2021) European nanomaterial regulation—Methodological challenges for registration and safety assessment. Environ Sci Nano 8:731–747
Nguyen MK, Moon J-Y, Lee Y-C (2020) Microalgal ecotoxicity of nanoparticles: an updated review. Ecotoxicol Environ Saf 201:110781
Notter DA, Mitrano DM, Nowack B (2014) Are nanosized or dissolved metals more toxic in the environment? A meta-analysis. Environ Toxicol Chem 33:2733–2739
Nowack B (2017) Evaluation of environmental exposure models for engineered nanomaterials in a regulatory context. Nanoimpact 8:38–47
Oberdörster E (2004) Manufactured nanomaterials (fullerenes, C(60)) induce oxidative stress in the brain of Juvenile Largemouth Bass. Environ Health Perspect 112:1058–1062
OECD (2005) Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment [ENV/JM/MONO(2005)14], Organisation for Economic Co-operation and Development, Paris, France
OECD (2007) Current Developments/Activities on the Safety of Manufactured Nanomaterials/Nanotechnologies, Tour de table at the 2nd meeting of the working party on manufactured nanomaterials, Berlin, Germany, April 25–27, 2007; Report No. ENV/JM/MONO(2007)16. Organisation for Economic Co-operation and Development, Paris, France
OECD (2011) Test No. 201: freshwater alga and cyanobacteria, growth inhibition test. Organisation for Economic Co-operation and Development, Paris, France
OECD (2020) Guidance document on aquatic and sediment toxicological testing of nanomaterials. Series on Testing and Assessment No. 317. ENV/JM/MONO(2020)8. Organisation for Economic Co-operation and Development, Paris, France

Oomen AG, Steinhäuser KG, Bleeker EAJ et al (2018) Risk assessment frameworks for nanomaterials: scope, link to regulations, applicability, and outline for future directions in view of needed increase in efficiency. NanoImpact 9:1–13

Pang C, Skjolding LM, Selck H et al (2020) A "point-of-entry" bioaccumulation study of nanoscale copper phthalocyanine in aquatic organisms. Environ Sci Nano 8:554–564

Palmqvist A, Baker L, Forbes VE et al (2015) Nanomaterial environmental risk assessment. Integr Environ Assess Manag 11:519

Peijnenburg WJGM, Baalousha M, Chen J et al (2015) A review of the properties and processes determining the fate of engineered nanomaterials in the aquatic environment. Crit Rev Environ Sci Technol 45:2084–2134

Petersen EJ, Goss GG, von der Kammer F et al (2021) New guidance brings clarity to environmental hazard and behaviour testing of nanomaterials. Nat Nanotechnol 16:482–483

Petersen EJ, Diamond SA, Kennedy AJ et al (2015) Adapting OECD aquatic toxicity tests for use with manufactured nanomaterials: key issues and consensus recommendations. Environ Sci Technol 49:9532–9547

SCENIHR (2007) The appropriateness of the risk assessment methodology in accordance with the technical guidance documents for new and existing substances for assessing the risks of nanomaterials; European Commission Health and Consumer Protection Directorate-General, Directorate C—Public Health and Risk Assessment, C7—Risk Assessment: Scientific Committee on Emerging and Newly Identified Health Risks Brussels, Belgium

SCENIHR (2009) Risk assessment of products of nanotechnologies; European Commission Health and Consumer Protection Directorate-General, Directorate C—Public Health and Risk Assessment, C7—Risk Assessment: Scientific Committee on Emerging and Newly Identified Health Risks Brussels, Belgium

Semenzin E, Lanzellotto E, Hristozov D et al (2015) Species sensitivity weighted distribution for ecological risk assessment of engineered nanomaterials: the n-TiO_2 case study. Environ Toxicol Chem 34:2644–2659

Sekine R, Khurana K, Vasilev K et al (2015) Quantifying the adsorption of ionic silver and functionalized nanoparticles during ecotoxicity testing: test container effects and recommendations. Nanotoxicology 9:1005–1012

Skjolding LM, Sørensen SN, Hartmann NB et al (2016) A critical review of aquatic ecotoxicity testing of nanoparticles—The quest for disclosing nanoparticle effects. Angew Chem Int Ed 55:15224–15239

Stone V, Hankin S, Aitken R et al (2010a) Engineered nanoparticles: Review of health and environmental safety (ENRHES) (RPRT), Engineered nanoparticles. European Commission, Brussels, Belgium

Stone V, Nowack B, Baun A et al (2010b) Nanomaterials for environmental studies: classification, reference material issues, and strategies for physico-chemical characterisation. Sci Total Environ 408:1745–1754

Subramanian V, Semenzin E, Hristozov D et al (2016) Sustainable nanotechnology decision support system: bridging risk management, sustainable innovation and risk governance. J Nanopart Res 18:1–13

Syberg K, Hansen SF (2015) Environmental risk assessment of chemicals and nanomaterials—The best foundation for regulatory decision-making? Sci Total Environ 541:784–794

Sørensen SN, Wigger H, Zabeo A et al (2020) Comparison of species sensitivity distribution modeling approaches for environmental risk assessment of nanomaterials—A case study for silver and titanium dioxide representative materials. Aquatic Toxicol 225:105543

Sørensen SN, Baun A (2015) Controlling silver nanoparticle exposure in algal toxicity testing—A matter of timing. Nanotoxicology 9:201–209

Sørensen SN, Hjorth R, Giron Delgado C et al (2015) Nanoparticle ecotoxicity—Physical and/or chemical effects? Integr Environ Assess Manag 11:722–724

Sørensen SN, Engelbrekt C, Lützhøft HCH et al (2016) A multi-method approach for disclosing algal toxicity of platinum nanoparticles. Environ Sci Technol 50:10635–10643

Sørensen SN, Baun A, Burkard M et al (2019) Evaluating environmental risk assessment models for nanomaterials according to requirements along the product innovation stage-gate process. Environmental Sci Nano 6:505–518

US EPA (2007) Nanotechnology White Paper, Report No. EPA 100/B-07/001; Science Policy Council, United States Environmental Protection Agency: Washington, DC

Van Hoecke K, Quik JT, Mankiewicz-Boczek J et al (2009) Fate and effects of CeO_2 nanoparticles in aquatic ecotoxicity tests. Environ Sci Technol 43:4537–4546

Von Moos N, Slaveykova VI (2014) Oxidative stress induced by inorganic nanoparticles in bacteria and aquatic microalgae–state of the art and knowledge gaps. Nanotoxicology 8:605–630

Walker W, Harremoes P, Rotmans J et al (2003) Defining uncertainty: a conceptual basis for uncertainty management in model-based decision support. J Integr Asses 4:5–17

Wickson F, Hartmann NB, Hjorth R et al (2014) Balancing scientific tensions. Nat Nanotechnol 9:870