The Handbook of Environmental Chemistry 103
Series Editors: Damià Barceló · Andrey G. Kostianoy

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Nicola Montemurro
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Interaction and Fate of Pharmaceuticals in Soil-Crop Systems

The Impact of Reclaimed Wastewater

Springer

The Handbook of Environmental Chemistry

Volume 103

Founding Editor: Otto Hutzinger

Series Editors: Damià Barceló • Andrey G. Kostianoy

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Interaction and Fate of Pharmaceuticals in Soil-Crop Systems

The Impact of Reclaimed Wastewater

Volume Editors: Sandra Pérez Solsona · Nicola Montemurro · Serge Chiron · Damià Barceló

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Series Preface

With remarkable vision, Prof. Otto Hutzinger initiated *The Handbook of Environmental Chemistry* in 1980 and became the founding Editor-in-Chief. At that time, environmental chemistry was an emerging field, aiming at a complete description of the Earth's environment, encompassing the physical, chemical, biological, and geological transformations of chemical substances occurring on a local as well as a global scale. Environmental chemistry was intended to provide an account of the impact of man's activities on the natural environment by describing observed changes.

While a considerable amount of knowledge has been accumulated over the last four decades, as reflected in the more than 150 volumes of *The Handbook of Environmental Chemistry*, there are still many scientific and policy challenges ahead due to the complexity and interdisciplinary nature of the field. The series will therefore continue to provide compilations of current knowledge. Contributions are written by leading experts with practical experience in their fields. *The Handbook of Environmental Chemistry* grows with the increases in our scientific understanding, and provides a valuable source not only for scientists but also for environmental managers and decision-makers. Today, the series covers a broad range of environmental topics from a chemical perspective, including methodological advances in environmental analytical chemistry.

In recent years, there has been a growing tendency to include subject matter of societal relevance in the broad view of environmental chemistry. Topics include life cycle analysis, environmental management, sustainable development, and socio-economic, legal and even political problems, among others. While these topics are of great importance for the development and acceptance of *The Handbook of Environmental Chemistry*, the publisher and Editors-in-Chief have decided to keep the handbook essentially a source of information on "hard sciences" with a particular emphasis on chemistry, but also covering biology, geology, hydrology and engineering as applied to environmental sciences.

The volumes of the series are written at an advanced level, addressing the needs of both researchers and graduate students, as well as of people outside the field of

"pure" chemistry, including those in industry, business, government, research establishments, and public interest groups. It would be very satisfying to see these volumes used as a basis for graduate courses in environmental chemistry. With its high standards of scientific quality and clarity, *The Handbook of Environmental Chemistry* provides a solid basis from which scientists can share their knowledge on the different aspects of environmental problems, presenting a wide spectrum of viewpoints and approaches.

The Handbook of Environmental Chemistry is available both in print and online via www.springerlink.com/content/110354/. Articles are published online as soon as they have been approved for publication. Authors, Volume Editors and Editors-in-Chief are rewarded by the broad acceptance of *The Handbook of Environmental Chemistry* by the scientific community, from whom suggestions for new topics to the Editors-in-Chief are always very welcome.

Damià Barceló
Andrey G. Kostianoy
Series Editors

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Part I
Introduction

The Journey of Human Drugs from Their Design at the Bench to Their Fate in Crops

Nicola Montemurro, Juan Manuel Peña-Herrera, Antoni Ginebreda, Peter Eichhorn, and Sandra Pérez

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Abstract The topic of this book is dedicated to the analysis, fate, metabolism, effects, and remediation of pharmaceutically active compounds in water-soil-biota systems. While the majority of readers are likely to already have a broad understanding of potential entry points, flows, transformation pathways, and temporary and permanent sinks of drugs in the environment, the objectives of this first chapter are fourfold: (a) to provide a concise overview of the journey a drug takes from its inception at the laboratory bench to the desk of the reviewer at the regulatory agency; (b) to understand the biological and physiological processes a drug undergoes from administration to humans – or to the animal in case of veterinary medicines – to their excretion and ultimately discharge into wastes; (c) to describe the physico-chemical space small-molecule drugs reside in as this characteristic largely governs their later environmental fate; (d) to review their presence, fate, and metabolism in crops and

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plants determined using innovative analytical methods; as well as (e) to evaluate the effects and remediation of drugs in crops and biota.

Keywords ADME, Analytical methods, Crops, Distribution, Drug development, Drug discovery, Earthworms, Fate, Humans, Metabolism, Soil, Wetlands

1 Introduction

According to the definition of the US Food and Drug Administration (FDA), an active ingredient is "any component that provides pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body of man or animals."[1] Within the realm of the pharmaceutical industry and the regulatory agencies, the terms "active ingredient," "pharmaceutically active substance," and "drug" are used interchangeably with the latter term commonly preferred for the sake of simplicity. Here, for the remainder of this introductory chapter, the term drug is used, although many publications in the field of environmental sciences tend to differentiate between pharmaceutically active compounds (PhACs) on the one hand and drugs in the sense of illegal or illicit ones on the other hand.

Regarding the above definition, most drugs elicit their pharmacological effect in the target organism through interactions with specific macromolecular entities which are involved in physiological processes or signaling cascades. The understanding of these biological processes at a molecular level allows to design drugs for the selective modulation of their activity. In view of the broad variety of potential pharmacological targets comprising enzymes, transmembrane receptors, ion channels, transport proteins, nuclear receptors, protein-protein interfaces, RNA, and DNA [1], the chemical structures of drug molecules are highly diverse, and after the dominating role of small-molecule drugs for most of the existence of modern drug research, novel therapeutic modalities are becoming ever more important, including monoclonal antibodies, proteins, peptides, and antisense oligonucleotides [2]. Within the context of environmental studies, though, the focus has been on low molecular weight compounds (<800–1,000 Da) of well-defined structure that are accessible through classical organic synthesis or can be isolated, and if needed chemically modified, with relative ease from naturally occurring microorganisms (e.g., macrolide antibiotics).

Irrespective of the drug class to aim at, engaging into the business of drug discovery and development is characterized by a lengthy and tedious process, tremendous investment, high risk of failure, and uncertainty about the return on

[1] https://www.fda.gov/drugs/drug-approvals-and-databases/drugsfda-glossary-terms

investment. The multi-faceted challenges encountered in drug development are closely associated with the fact that the entire path toward marketing authorization is highly regulated by the respective agencies (the FDA in the United States, the European Medicines Agency at the level of the European Union, and the Pharmaceuticals and Medical Devices Agency in Japan). It is the task of these regulatory bodies to evaluate the efficacy and safety of novel drug and may ultimately grant marketing authorization for drug applications.

Taking into consideration that it takes tens of thousands of compounds to be screened for affinity toward the pharmacological target, about 10 years from the first hint of disease-relevant activity in an animal model to the solid proof of clinical safety and efficacy in a large patient population, and an estimated 1,000,000,000 USD until submission of the dossier to the regulatory agency, it is clear that such an undertaking requires an exceptional combination of financial resources, scientific expertise, and, once the drug product has been approved, a well-implemented commercial network that reaches out to patients and prescribers. This explains why nowadays – after 25 years of major mergers and acquisitions in the sector – a few global players among the pharmaceutical companies dominate the market, the largest one being the United States with a value of 340 billion USD.

2 Drug Discovery and Development

The drug discovery and development process can be roughly divided into three phases: the discovery phase (Fig. 1) usually starts with the hypothesis that alteration of a physiologic pathway may lead to changes in a pathologic condition. For example, inhibition of the enzyme cyclooxygenase (COX) was identified to reduce the conversion of polyunsaturated fatty acids into prostaglandins, which are key factors in the development of inflammation (this discovery eventually resulted in the family of widely used non-steroidal anti-inflammatory drugs). Following the decision to pursue a target, chemical libraries with thousands of compounds are screened for their affinity toward the receptor (e.g., COX). Modification of the resulting hits by conventional organic synthesis then aims at improving the binding properties. It is the medicinal chemists who attempt to understand the impact of specific structural changes on the potency of the compounds. This reiterative process leads to the discovery of highly potent, and ideally selective, substances. In parallel to the improvements in potency, the chemical design addresses the aspects of target exposure in order ensure efficient delivery of the drug to the site of action at concentrations sufficiently high to trigger the desired biological response (see Sect. 4 on pharmacokinetic principles). Next, the in vivo efficacy is tested in animal models mimicking the human disease, for example, carrageenan-induced edema of the rat paw. All these efforts made in a research program make up about 3 to 4 years of the overall process and ultimately aim at identifying a candidate for preclinical development. Apart from demonstrated in vitro potency, target exposure, and in vivo efficiency, additional criteria for selecting appropriate compounds are high

Fig. 1 Drug discovery and development process of small-molecule drugs

selectivity for the target and absence of liabilities of the cardiovascular system and the central nervous system.

The objective of the preclinical development stage (Fig. 1) is to conduct a series of toxicity studies in rodent and non-rodent species that support the first clinical trials with healthy human volunteers. In these studies, the animals are dosed by acute or sub-chronic treatment to achieve systemic concentrations by far exceeding those predicted for clinical settings with humans, which allows to identify target organs of toxicity and to anticipate potential undesirable effects in humans. By comparing exposure scenarios between the so-called no observed adverse effect (NOAEL) levels in the test species with the estimated exposure in humans at the therapeutically active dose, the safety margins of the compound are calculated. These safety assessments go alongside the development of a pharmaceutical formulation that ensures the physical, chemical, and microbiological stability of the active ingredient in the drug product. In addition, strict requirements apply as to controlling and documenting the manufacturing process of the active and the formation and identification of possible by-products in the chemical synthesis. Finally, to move the

compound forward into clinical testing, approval of the national or international regulatory agency is sought for by submitting the application for a new investigational drug. Provided that the mandatory studies have been executed according to the relevant guidelines and that the outcomes of the toxicological evaluations allow to discard potential concerns about the risk of the drug substance on health of the human subjects, the third phase can be initiated.

The clinical development stage (Fig. 1) comprises three sequential phases and commonly stretches over a period of 6–8 years. The major objectives of Phase I trial are to recruit a small number of healthy volunteers (usually adult males of young age) for assessing safety, tolerability, and pharmacokinetics of the new drug. By gradually increasing the dose from sub-therapeutic levels, potential adverse effects are recorded, and a maximum tolerated dose can be identified. Despite all the encouraging findings collected during discovery and preclinical development, statistically speaking, about half of all Phase I trials conclude with the premature termination of the project, be it for lack of safety and tolerability and due to suboptimal pharmacokinetics properties. Compounds surviving the scrutiny of the first clinical assays enter Phase II trials which is the first time that a patient population with the target indication is treated with the new drug at therapeutic doses. At this point, it all comes down to obtaining the proof of concept in studies of limited duration and a relatively small population size (several tens to a few hundreds): is the drug efficacious at the given dose with an acceptable safety profile? This question is of particular importance for novel mechanisms of action, which have not yet been validated in the clinics. In case of satisfactory findings, the drug enters Phase III trials to evaluate efficacy and safety in a large number of patients (several hundreds to several thousands) over an extended treatment period.

Despite all the understanding of the underlying mechanism of a disease and the arsenal of "(bio)chemical weapons" to modulate them, and despite sufficiently high safety margins estimated from a large number of toxicological studies in animal species, the discouraging reality in the twenty-first century is that, on average, a mere 10% of all drugs entering into clinical trials are eventually granted marketing authorization by the regulatory agencies as the ultimate authority for evaluating the risk-benefit ratio of new drugs [3, 4].

Notwithstanding this rather modest success rate, numerous are the cases where breakthroughs in treatment options of chronic diseases have led to outstanding commercial triumphs. Two examples shall serve here to illustrate the economic impact of very successful drugs: the monoclonal antibody adalimumab (AbbVie's Humira), which is prescribed for the treatment of several autoimmune diseases, was in 2018 the best-selling drug worldwide generating revenues of 20.4 billion USD. Another record is hold by the cholesterol-lowering atorvastatin (Pfizer's Lipitor) that generated sales figures on the amount of 150 billion USD from the time of approval in 1997 until patent expiration, and therefore loss of market exclusivity, in 2011.

3 Physico-chemical Space of Small-Molecule Drugs

With the exclusive coverage of small-molecule drugs in this book, the focus of this section is on the 3,000+ substances with marketing approval. The physico-chemical space they occupy can be defined by three simple, and experimentally readily accessible, properties, namely, molecular weight (MW), lipophilicity, and ion class. While the molecular weight is derived directly from the elemental formula, lipophilicity is commonly measured as the partition coefficient of the compound between n-octanol and water (yielding logP for neutral species or the pH-dependent logD for charged species). The ion class reflects the presence of functional groups in the chemical structure that are ionizable at physiological pH, e.g., the carboxylic acid-bearing anti-inflammatory drug diclofenac belongs the group of acids, whereas the aliphatic amine in the antidepressant sertraline acts as a basic center amenable to protonation. The modern cookbook of the medicinal chemist is replete of synthetic approaches to design virtually any organic molecule; but what are his/her criteria in the search for the right spot in the three-dimensional space? The answer lies first and foremost at the molecular level in the binding of the molecule to the receptor whose physiological function he/she set out to modulate in the belief that this ultimately translates into the desired pharmacological response in humans. For this binding to take place, the drug molecule has to establish a number of specific interactions with the structural building blocks of the receptor, typically in the form of hydrogen bonding, van der Waals interactions, and hydrophobic interactions. In case of protein targets, the drug molecule interacts through its functional groups with the structurally diverse amino acids constituting the protein. The number and strength of interactions of the molecule with the site of the receptor essential for its physiological function, e.g., the site of the enzyme where its natural ligand binds to, then determine the thermodynamic association constant, i.e., its potency toward the target. Tuning these interactions for high affinity toward the selected pharmacological target while minimizing off-target selectivity is the ultimate goal of the synthetic efforts. When no previous knowledge on the chemical environment of function-altering site is available in the public domain, screening of chemical libraries is a commonly applied approach with subsequent optimization of hit compounds in a trial-and-error mode in order to establish structure-activity relationships. On the other hand, if the three-dimensional structure of the receptor has been elucidated, e.g., through X-ray crystallography, and competitor compounds are available, virtual docking methods can help identify ways to improve the binding of the ligand to the receptor. Such strategy ultimately yields structurally very similar drugs, which may exhibit differences in one or several aspects relating to potency, pharmacokinetic properties, and overall safety and tolerability profiles.

Next to the aforementioned binding criteria, a further key attribute intimately linked to the physico-chemical properties of the compound is the ability to reach the target site in amounts sufficient for coverage. For example, atorvastatin contained in an orally dosed tablet undergoes a sequence of events to reach the site of action: liberation of the active, dissolution, absorption in the intestine, transfer into the

bloodstream, and transport to the liver where it finally reaches the enzyme HMG-CoA reductase anchored in the membrane of the endoplasmic reticulum. For this to happen, an oral small-molecule drug – and in fact, the large majority of marketed drugs are given by the oral route for ease of administration and patient compliance – needs to combine two important features: aqueous solubility and membrane permeability. Unfortunately, these two properties work in opposite directions insofar as solubility increases with decreasing logP (and MW) while the crossing of biological membrane composed of lipid bilayers is facilitated with increasing logP. Consequently, optimization of compound properties during the drug discovery phase always has a close eye on the lipophilicity. Moving too far away from the drug-like space increases the risk of acquiring suboptimal pharmacokinetic properties. For instance, very polar drugs ($\log P < 0$) suffer from poor absorption, while very lipophilic ones ($\log P > 5$) are poorly soluble in the aqueous environment of the gastrointestinal tract and are likely to display poor absorption behavior. As a general rule, a logP in the range of 1–3 is considered a good compromise for oral small-molecule drugs.

As far as the molecular weight is concerned, for an efficient interaction of a drug molecule with its target receptor, as thus its potency, specific structural elements and functional groups are necessary, which in the end determine the size of the molecule. With increasing molecular weight, however, organic drug-like molecules tend to gain in lipophilicity, which compromises water solubility [5]. To render large molecules more soluble, medicinal chemists can introduce functional groups or motifs that enhance their polarity, be it by adding polar functionalities (hydroxyl or amino group) or by substituting lipophilic building blocks through closely related more polar motifs of similar size and shape (phenyl ring to N-heterocycle switch). Again, such modifications need to be considered with caution because too much polarity in a large molecule, computed as topological polar surface area (TPSA), is detrimental to its ability of passively diffusing through biological membranes. Taken together, finding the right balance in compound structure and properties to satisfy the requirements in potency, membrane permeability, and water solubility eventually defines the chemical space: the majority of oral drugs have a logD 0–4 and a molecular weight of 200–500 Da [6].

Regarding the ion class [7], selection of one type or the other may be dictated by the preference of the receptor for ligands with specific functional groups. For instance, the serotonin re-uptake inhibitor sertraline competes with the endogenous ligand for its binding site on the neuron. Hence, designing a drug with a basic center to displace the likewise basic natural binding partner of the 5-HT receptor is a way of building in affinity toward the target. It is worth stressing that ion class has a direct impact on the three important inherent compound properties: lipophilicity, water solubility, and membrane permeability. Functional groups that are ionized at physiological pH to a significant extent – mostly basic amines with basicity constants (pK_b) above 8 and carboxylic acid-bearing compounds with acidity constants (pK_a) typically below 4.5 – cause a reduction of the logP, i.e., the logD as a more physiologically relevant measure of lipophilicity is shifted to lower values. While this benefits their water solubility (with the exception of the acidic environment of

the stomach (pH 2–3) where carboxylic acids are mostly present in their undissociated form), it comes at the expense of passive permeability. The latter is particularly prominent for anionic drugs owing to repulsive interactions with the negatively charged head groups of the phospholipids constituting the lipid bilayer of cell membranes.

4 Absorption, Distribution, Metabolism, and Excretion (ADME)

The multi-parametric optimization of drug properties extends well beyond target affinity, solubility, and permeability (not even considering the experimental evaluation of in vivo efficacy in disease model, off-target selectivity, or safety pharmacology!) with pharmacokinetic properties being a pivotal decision criterion for defining compound quality. Although small-molecule drugs can be delivered locally and systemically by different routes, including inhalation, subcutaneous and intramuscular injection, or dermal application, oral administration represents the single most important delivery option. Once swallowed, the formulation reaches the highly acidic environment of the stomach where release and dissolution of the active ingredient usually take place. Following gastric emptying into the duodenum (pH 5.5–7), the concentration gradient between the drug in the lumen and the enterocytes as the epithelial cells lining the inner surface of the intestine drives the absorption. Importantly, the fraction of dose absorbed in the intestine is largely governed by the two aforementioned compound properties; for the compound to be efficiently absorbed, it needs to be present in dissolved state and exhibit good cellular permeability. When these two requirements are not properly addressed during chemical design, the active is discharged from the body in altered form through feces and thereby contributes directly to the drug burden in municipal sewage.

After uptake into the enterocyte, the drug gets into the blood of the portal vein and flows to the liver (weighing about 1.5 kg in an adult male) as the port of entry to systemic circulation. Next to the physical barrier of the intestinal wall, this organ represents the second barrier to the drug molecule on its way to the site of action (unless it is the liver itself as in case of the family of statins acting on HMG-CoA reductase). As a mechanism of natural protection from foreign substances of no apparent beneficial value, the human liver has evolved over the course of evolution to produce specific enzymes capable of metabolizing unwanted compounds. Synthetic drugs, but also potentially harmful drugs of natural origins, are recognized and subject to metabolic reactions (see chapter “Metabolism of Pharmaceuticals in Plants and their Associated Microbiota” for a more detailed description of drug metabolism) which convert the substrate into more polar and thereby more readily excretable metabolites. This presystemic elimination is referred to as first-pass metabolism and is in most instances an undesired process that needs to be strictly

controlled whenever high blood concentrations are necessary to achieve the desired pharmacological effect.

Once in systemic circulation, provided that the drug is sufficiently membrane permeable, it is distributed to all organs and tissues until an equilibrium is achieved where the rate of transfer into tissues equals the rate of back-diffusion into blood. The extent of this distribution is dependent on the partition coefficient (K_p) between tissue/organ and blood which in turn is a function of the affinity of the compound for plasma proteins on the one hand and non-specific binding to tissue components on the other. The pharmacokinetic parameter that quantitatively describes this property of a drug is the volume of distribution, V_d (in units of L/kg), with a high value indicating extensive tissue distribution [8]. Apart from lipophilicity, the compound property with the largest effect on V_d is the ion class: the low V_d of acids arises from strong binding to albumin as the most abundant plasma protein (4–5%) with numerous basic amino acids available for electrostatic interactions [9]. A further contributing factor to the low V_d of acidic drugs is the repulsion by phospholipid-based membranes of tissue cells. Basic compounds, in turn, display a pronounced affinity for tissues (pH range: 7.1–7.4) because they are, in least in part, protonated under these settings which allows for strong interaction with the negatively charged phospholipids. At a macroscopic level, this translates into a high partition coefficient with total tissue concentrations exceeding those measured in blood [10]. When interpreting partition coefficients, however, it is crucial to understand that a high K_p is not indicative of tissue accumulation but the consequence of non-specific binding to tissue components; in other words, total tissue concentrations provide very limited information and do not allow to infer the actual unbound concentration potentially triggering off-target pharmacological responses. This fundamental concept helps explain the high tissue-specific K_p´s of sertraline reported for vertebrates exposed to this highly lipophilic base (logP: 5.15).

The two major eliminatory processes contributing to the removal of drug from the bloodstream are renal and biliary excretion of the unchanged parent compound and hepatic metabolism. As a simple rule, polar drugs of small size (logD < 0) are excreted with relative ease into urine through glomerular filtration at the nephron, whereas large molecules with high TPSA are amenable to biliary excretion, which drains the compound through the bile duct into the intestine for excretion alongside feces [11, 12]. The major elimination mechanism for the majority of marketed drugs, however, is enzyme-mediated biotransformation giving rise to drug metabolites [13].

Given the diversity and substrate selectivity of drug-metabolizing enzymes, particularly in the liver as the predominant site of drug metabolism, a molecule can undergo a variety of reactions generating metabolites with an inherent chance of being excreted into human wastes. With the ultimate goal of maximizing human exposure at the lowest possible dose, identification of the site of metabolism within a discovery compound and chemical design to improve the metabolic stability in the next generation of compounds is one of the principal tasks of dedicated ADME scientists who work closely together with medicinal chemists. The fact that commonly tens to hundreds of compounds need to be screened in oral drug discovery

programs in the search for chemical structures with low affinity for drug-metabolizing enzymes – while maintaining satisfactory properties in all other disciplines – reflects the impact of metabolic clearance in the optimization cycle during all stages until nomination of a candidate for preclinical development. If renal and biliary excretion are negligible pathways, it is the intrinsic metabolic clearance of a compound that has a large influence on the size of the human efficacious dose and eventually on the amount of intact drug reaching the sewage plant. Suboptimal drugs require not only a high dose, but they also suffer from the need for frequent dosing in order to ensure sufficient coverage of the molecular target over the dosing interval. When applying today's standards for a successful drug with respect to dose size and administration frequency, paracetamol with its thrice daily dosing of up to 1,000 mg each clearly ranks at the bottom of the favorite drugs' list and automatically becomes a drug with high amounts in untreated sewage, either in form of intact parent or as metabolites.

It is a legitimate question to ask whether novel small-molecule drugs can be designed to be "environmentally friendly," i.e., combining low therapeutic doses with high biodegradability in the activated sludge treatment of the wastewater treatment plant. The former aspect is undoubtedly addressed in research programs at the pharmaceutical industry for a number of reasons: lower cost of goods, competitive dose sizes and dosing intervals, and reduced risk of adverse effects and drug-drug interactions. The latter, however, is – to the disappointment of environmental scientists – not among the compound optimization criteria. Designing compounds with structural elements susceptible to rapid and efficient microbial degradation will be counterproductive. Too large are the similarities between xenobiotic-recognizing enzymes (hydrolytic, oxidative, and conjugative ones) between the human body and microbial communities.

5 Environmental Regulatory Perspective in the European Union

Drugs that have been absorbed to reach systemic circulation are metabolized and subsequently excreted through the bodily wastes as a mixture of parent compound and the metabolites generated in the target organism. Finally, the complex mixture is discharged through the sewage system and often, but not always, treated in wastewater plants, before the final effluents are released into the environment, where further biotic and abiotic processes including sorption, photolysis, hydrolysis, and biodegradation. Whereas excretion is the predominant input of drugs into the environment, other pathways like inappropriate disposal, industrial spills, or manure spread (particularly for veterinary drugs) should not be overlooked. From the environmental point of view, the following facts of concern are worth considering:

1. Drug consumption is increasing owing to demographic growth, aging population, and improved access to medication. The process is ubiquitous on space and continuous on time and extends to some thousands of available drugs.
2. Depending on each specific compound and characteristics of the wastewater treatment, their elimination may be not complete. Although specific tertiary treatment steps may be added, an additional technology increases the economic cost of the waste treatment.
3. Even though many drugs are degraded, their continuous input into the environment makes them to behave as "pseudo-persistent."
4. Drugs are, by design, biologically active compounds, targeted to specific organisms, and having modes of action. Possible side effects on other unintentionally non-targeted exposed organisms living in the receiving ecosystem cannot be ruled out.
5. In addition to long-term ecotoxicity effects, there is a human health concerns about antimicrobials (antibiotics, antifungals), whose occurrence in the environment has been proved to promote the development of resistance genes (antimicrobial resistance genes, ARG).
6. The "cocktail" of many drugs, metabolites, and transformation products may have unexpected and unpredictable mixture interaction effects (i.e., synergistic, antagonistic, etc.) on environmental wildlife.
7. Last but not least, the anthropogenic water cycle includes the safe supply of drinking water to the population. The occurrence of drugs residues in drinking water may pose a risk in human health; therefore their presence must be minimized.

Altogether, it is recognized that the occurrence of drugs in the environment is not devoid of risk and there is a need for incorporating environmental safety aspects into existing regulatory frameworks. In this regard, two broad areas of legislation may be highlighted, namely, (1) the registration process of drugs and (2) the occurrence of drugs in the environment and more specifically in the aquatic environment. Here we will briefly review the current status of both aspects of drug legislation in the European Union.

(a) Regulation of Drugs in the Aquatic Environment

The preservation of the aquatic environment, either surface (marine and fresh) or groundwater in Europe is essentially regulated by the Water Framework Directive (WFD) (Directive 2000/60/EC) [14] and derived pieces of legislation. In that context, to achieve the good status of European water bodies, both the good ecological and chemical status requirements must be fulfilled. For the latter, environmental levels must be in compliance with the environmental quality standards (EQS) of the so called priority substances, whose EQS were set up in the WFD daughter directives (Directive 2008/105/EC amended by Directive 2013/39/EU) [15, 16]. Currently, the list of priority substances constituted 45 chemical species. However, the "list of priority substances" is subjected to periodic revision, meaning that it is open to the incorporation of new candidate substances. To do so Directive

Table 1 Pharmaceutical substances included in the "watch list" according to Decision (EU) 2015/495 and Decision (EU) 2018/840

Substance	CAS	Therapy class	Remark
17-Alpha-ethinylestradiol (EE2)	57-63-6	Synthetic hormone	Included in Decision (EU) 2015/495 [18]
17-Beta-estradiol (E2)	50-28-2	Hormone	Included in Decision (EU) 2015/495 [18]
Estrone (E1)	53-16-7	Hormone	Included in Decision (EU) 2015/495 [18]
Erythromycin	114-07-8	Macrolide antibiotic	Included in Decision (EU) 2018/840 [19]
Azithromycin	83905-01-5	Macrolide antibiotic	Included in Decision (EU) 2018/840 [19]
Clarithromycin	81103-11-9	Macrolide antibiotic	Included in Decision (EU) 2018/840 [19]
Amoxicillin	26787-78-0	Penicillin antibiotic	Included in Decision (EU) 2018/840 [19]
Ciprofloxacin	85721-33-1	Quinolone antibiotic	Included in Decision (EU) 2018/840 [19]
Diclofenac	15307-86-5	NSAID	Included in Decision (EU) 2015/495 [18] Withdrawn in Decision (EU) 2018/840 [19]

2013/39/EU (Article 8b) foresees establishing a "watch list" of new substances of concern for which new monitoring data need to be gathered (sic) "for the purpose of supporting future prioritization exercises in accordance with article 16(2) of the WFD." Hence, the inclusion of a substance in the "watch list" might be regarded as a necessary (but not sufficient) condition to its final incorporation into the list of priority substances. The European Commission, on the other hand, in art. 8c of the aforementioned Directive 2013/39/EU entitled "Specific provisions for pharmaceutical substances", undertakes to carry out a specific study on the risks posed by medicinal products in the environment and developing within the next 2 years a strategic approach to pollution of water by pharmaceutical substances. The final outcomes of such approach are available in the corresponding document issued by the European Commission [17].

Up to now the list of priority substances does not include any pharmaceutical substance. Contrastingly, several of them have been already added in the "watch list," in their two successive versions (Table 1). The compounds included are three estrogenic natural or synthetic hormones and five antibiotics belonging to three different families (three macrolides, one penicillin, and one quinolone). It is worth mentioning the case of the NSAID diclofenac that appeared in the first watch list but was finally withdrawn from the second one. A new "watch list" is currently under preparation led by the JRC (Ispra, Italy), but at the time of writing this book chapter, only a preliminary draft is available [20]. Notably, this document incorporates new pharmaceuticals as proposed candidate substances, namely, the antifungals

clotrimazole, fluconazole, and miconazole, the antibiotics sulfamethoxazole and trimethoprim, the proton pump inhibitors omeprazole and its metabolite 4-hydroxy omeprazole sulfide (OM14), the synthetic hormone norethisterone, and the antidepressant venlafaxine.

(b) Environmental Aspects in the Registration of Pharmaceuticals

The legal basis for the registration, production, distribution, and use of medicinal products in the EU member states is governed by the Directive 2001/83/EC. In accordance with their provisions, any medical product should get an official authorization previous to its marketing, which is issued by the European Medicines Agency (EMA) after the proper completion of a registry process. According to Article 8(3) of the aforesaid directive, applicants must submit a dossier containing the necessary information to ensure the safety and therapeutic and clinical efficacy of the pharmaceutical product. As part of this authorization file, information regarding the potential risks of the drug to the environment is required. Therefore, companies wishing to register a new drug have to provide an Environmental Risk Assessment (ERA) [21, 22]. The norm has as well some exceptions (i.e., magistral formulas, research products, radionuclides, blood derivatives, or natural constituents like electrolytes, carbohydrates, lipids, amino acids, peptides, proteins, etc.). Medicinal products consisting of genetically modified organisms have specific requirements as well.

To facilitate and harmonize the ERA procedures, the EMA has elaborated the corresponding guidelines, which have been conveniently updated [23].

According to the EMA guideline 2019 [23], the overall process is depicted in Fig. 1 and includes a risk assessment and a specific hazard assessment. The risk assessment is focused on the environmental occurrence (exposure) and ecotoxic potential effects of the product on the exposed organisms. For some specific biological effects and substances (i.e., endocrine disruptors, antibiotics, etc.), additional aspects have to be considered. In turn, the hazard assessment refers to intrinsic properties of the products considered harmful for the living organisms exposed regardless of the concentration and specifically to the persistence, bioaccumulation, and toxicity (in short, PBT) characteristics. For full details the interested readers are addressed to the above referred EMA guideline [23].

Briefly, the procedure includes two phases (Fig. 2). In general, Phase I consists of a decision tree mostly addressed to differentiate among products that require a further assessment (Phase II) or those that not. This is done on the basis of the predicted environmental concentration (PEC) in surface water of the product estimated from its predicted use. If PEC $\geq$ 0.01 μg/L, the product enters Phase II; otherwise the process is finished. Phase II is a tiered process, starting with the study of physico-chemical properties, environmental fate and ecotoxicity, and a Predicted No Effect Concentration (PNEC). Among other aspects like potential risk to the groundwater and the soil, or the possibility of secondary poisoning in across the trophic chain, Tier A examines the risk ratio PEC/PNEC in surface water, and if it exceeds 1, a Tier B with PNEC refinement is performed. The PBT hazard assessment, carried out (if necessary) in parallel to the risk assessment, aims at evaluating

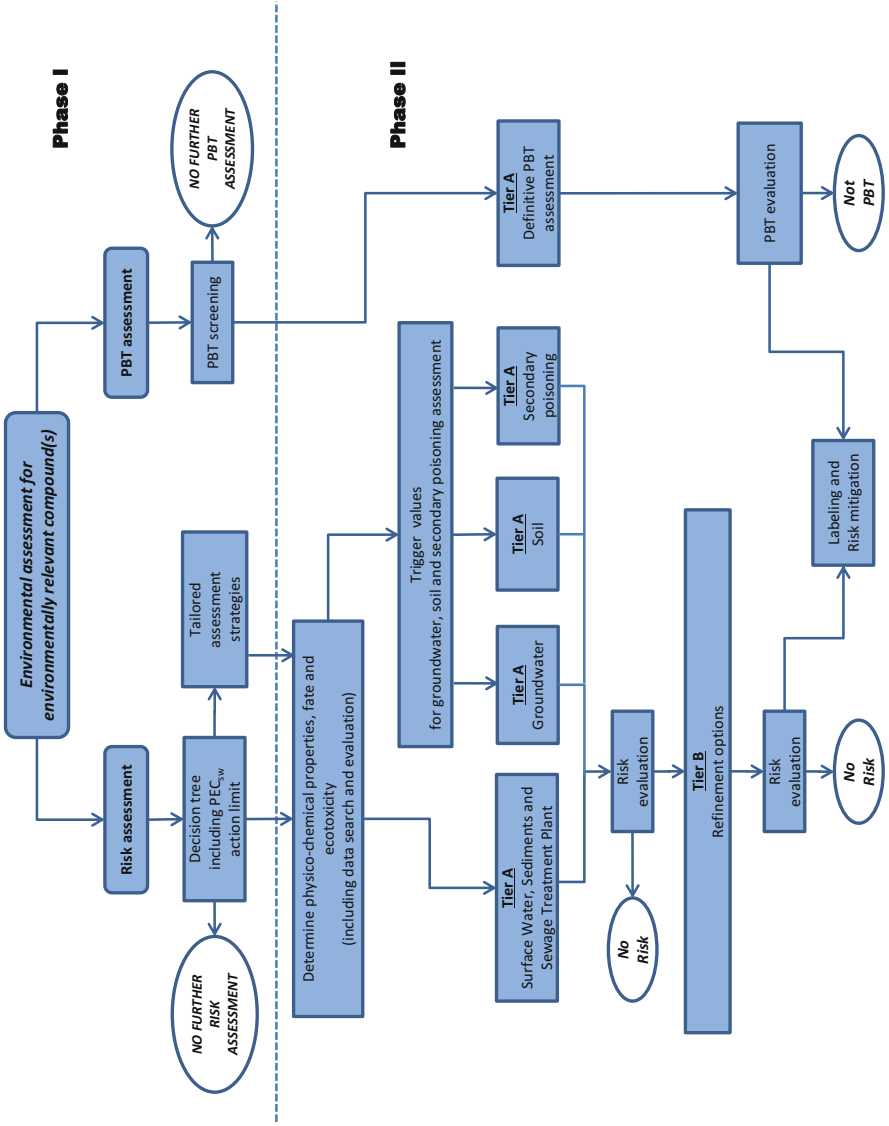

Fig. 2 Flow chart of the environmental risk-assessment process prescribed by the European Medicines Agency (EMA) to be used in the registration of new medicines (adapted from EMA guideline, [23])

the potential long-term effects of the product in the environment, regardless of its environmental exposure concentration. Depending on the results obtained in the Phase I (screening phase), a more detailed and definitive assessment is performed in Phase II.

To conclude, and despite recognizing that the implementation of the ERA procedures has constituted a relevant progress to prevent the environmental undesirable effects of pharmaceuticals, some limiting aspects are worth to be mentioned:

- Even though that the presentation of an ERA is mandatory in the registration of a drug, the final authorization or refusal does not depend on the ERA itself.
- ERAs are compulsory for new drugs, but not for those authorized before the approval of the Directive 2001/83/EC.
- ERAs are conducted with pharmaceutical products rather than with drugs.

6 Presence of Pharmaceuticals in Wastewater

The presence of pharmaceuticals in treated wastewater, surface water, and other freshwater resources is a common phenomenon at a global level that has been documented for almost four decades [24–32]. As seen before, drugs and metabolites are excreted and reach wastewater treatment plants (WWTPs) [30, 33–48]. Drugs for human use are the main source in wastewater, whereas hospital wastes are the second largest source. Discharges from drug manufacturers are of minor relevance [32].

The persistence of pharmaceutical compounds in wastewater (or pseudo-persistence) is mainly due their continuous release into the WWTPs. Although the total amount is affected by the continuous degradation wastewater treatment processes, their continuous inputs in small quantities due to multiple sources, cause many pharmaceutical products remain in the aquatic environment for long periods of time [49–53]. For example, some pharmaceuticals such as carbamazepine, clofibric acid, diclofenac, naproxen, sulfamethoxazole, and lamotrigine can pass the treatment in WWTPs. Once in the aquatic environment, naproxen and sulfamethoxazole can resist up to 1 year in nature being biologically active, while clofibric acid can be maintained in its original form for several years [32].

The most frequently detected families of pharmaceuticals in wastewater worldwide are antibiotics, analgesics, blood lipid regulators, cardiovascular drugs, and antidepressants. For example, while the highest amount of antibiotics was detected in Asia, the highest amount of painkillers was detected in Europe, and the highest concentration of antidepressants was measured in North America [24, 54]. Currently, of the approximately 1,500 pharmaceutical ingredients most used and studied in total (which represent only a limited portion of the total) [55], nearly 560 different compounds have been effectively detected globally in wastewater [24]. Figure 3 shows the total concentrations of several families of drugs detected in European WWTPs since 2010. Analgesics/anti-inflammatory drugs, β-blocker agents, drugs

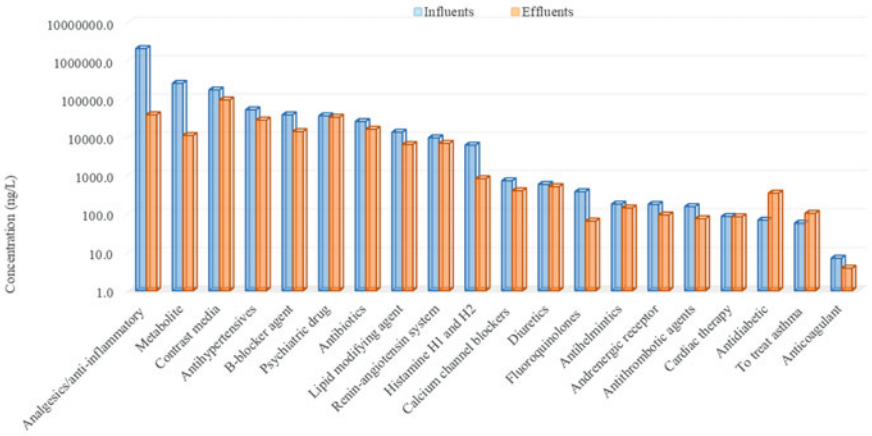

Fig. 3 Total concentration of families of pharmaceuticals reported in influents and effluents studies in European wastewater treatment plants. Sources: [31, 56–62]

used for lipid control, and psychiatric drugs are the top four families present in raw sewage, with a total concentration of up to 1.5 mg/L in the case of analgesic/anti-inflammatory drugs and 1 mg/L for β-blockers. The highest concentration of an individual drug was reported for acetaminophen in Portugal with a concentration of 0.6 mg/L [61].

Removal efficiency is class-dependent with contrast agents, analgesic/anti-inflammatory drugs, psychiatric drugs, and antihypertensives being the four most detected therapeutic classes (Fig. 3). Contrast agents were detected with total concentrations as high as up to 0.094 mg/L, and the analgesic/anti-inflammatory drugs were presented at 0.037 mg/L. The highest concentrations, reported for iopromide (contrast agent), valsartan (antihypertensive), and gabapentin (psychiatric drug), were reported in Portugal and Germany at 0.085, 0.015, and 0.012 mg/L [59, 62].

WWTPs typically employ primary and secondary treatment systems, with aerobic and anaerobic treatment [32, 63]; nevertheless, those processes are able to reduce partially the pharmaceuticals concentration from the wastewater, because WWTPs are not designed for complete removal of this type of contaminants in water. Advanced wastewater treatments such as photocatalysis, sonolysis, or advances oxidation processes can be implemented to reduce considerably the load of pharmaceuticals in wastewater effluents, but investments into installation of full scale at elevated operational costs hamper their widespread implementation [32, 63].

7 Pharmaceuticals in Crops Irrigated with Treated Wastewater

With freshwater becoming increasingly scarce, the water crisis is turning into a political, economic and social issue. Hence, the growing pressure on water resources forces to look for alternative sources of freshwater. In this sense, the reliance on extraction of freshwater, surface water, and groundwater can be diminished by using reclaimed wastewater. The reuse of wastewater is a fundamental requirement for the management of water resources, although globally there is a lack of legislation on the minimum requirements for water quality and monitoring for water reclamation. In view of the high content of nutrients such as phosphorus and nitrogen, treated wastewater lends itself readily for irrigation purposes in agriculture. Furthermore, by reducing the need for additional mineral fertilizer applications, water reclamation contributes to the promotion of the circular economy by recovering nutrients and saving money. The use of wastewater in agriculture for irrigation and fertilization purposes has ancient origins that are lost over time [64]. Currently, Israel uses around 50% of its treated wastewater for its agricultural sector. However, before authorizing the use of recycled water in agriculture on a global scale, it would be advisable to develop minimum requirements to reduce the possible human and environmental risk resulting from this type of practice, as well as the uncontrolled introduction of exogenous contaminants into the agricultural ecosystem [65]. Today there are concerns remaining about the safety of irrigation with treated wastewater from conventional wastewater treatments plants because it contains various contaminants among them pharmaceuticals and their metabolites. Soil irrigated with treated wastewater will therefore be exposed to these xenobiotics. Besides, crops and soil fauna such as earthworms are sensitive to environmental pollutants contributing directly or indirectly to their degradation. Not surprisingly, the use of treated wastewater represents the main source of drugs in arable land, in agreement with the agricultural water management systems [66]. Therefore, in real crops, pharmaceuticals and their metabolites can be retained in the soil, metabolized in earthworms, directly taken up by crops, or translocated from soil to plant tissues above the ground.

8 Uptake, Distribution, and Metabolism of PhACs in Crops

Studies on the absorption, translocation, and metabolism of PhACs are still very scarce or limited to studies under controlled or laboratory conditions, while studies in the natural environment or field conditions are minimal. Experiments in greenhouse or laboratory conditions such as hydroponic systems are usually performed with a period of controlled light, relative humidity, temperature, etc.

The uptake of PhACs by crops is related to growth conditions, soil properties, plant biology, exposure medium, and compound properties. The properties of

PhACs, among them lipophilicity and molecular diameter, strongly influence their ability to passively pass through the membranes of plant cells. Higher lipophilicity may allow faster diffusion between lipid bilayers but may impede translocation in cell wall or the cytosol. However, the majority of PhACs for human or veterinary use are polar or ionizable compounds. Plants absorb water and the mineral salts dissolved in it from the soil, through the root. A hydraulic mechanism displaces water from the roots to the leaves in response to an energy difference in water potential from a region where the water potential is higher to one in which it is lower by dragging inorganic elements and organic molecules in its movement. If polar and ionizable compounds such as PhACs are present in the soil pore water, these are captured by the roots and absorbed by the plant. Once absorbed, the water devoid of nutrients retained by the cells is released into the atmosphere in the form of water vapor (transpiration). Most of the water absorbed by the roots is lost by transpiration through the leaves and returned to the atmosphere. It is estimated that only 1–5% of all the water absorbed is retained by the plant, while the rest is emitted from the leaves. This suction force of the leaves reaches values of tens of atmospheres. Water and small solutes and consequently polar PhACs in ionized form can move from the soil pore water to the vascularized tissue of the roots through three paths:

- *Transmembrane pathway*, from cell to cell (crossing twice the plasmatic membrane of each single cell, in and out)
- *Symplastic pathway*, after having crossed once the plasmalemma (through cells via plasmodesmata)
- *Apoplastic pathway* (along the cell walls through the intercellular space) up to the endoderm where it must necessarily cross the plasma membrane

In the symplastic and transmembrane way, water and salts pass through the cytoplasmic membranes of the root hairs and penetrate into the symplast. In the apoplastic pathway, water and salts pass through the apoplast without ever crossing a plasmatic membrane.

In order to enter the stele, the water and minerals must pass into the symplast because the passage through the apoplast is prevented by the walls of the endoderm (Casparian strip). The endoderm acts as a hydrophobic barrier preventing any substance from reaching the conductive tissue without crossing a membrane and preventing the reflux of water and salts from the stele. After passing the endoderm, the water and minerals will be translocated in the symplastic continuum and finally reach the leaves.

The ions actively absorbed by the rhizodermic cells follow the symplastic pathway spread from cell to cell through plasmodesmata after having passed the Casparian strip. At the xylematic parenchyma level, they are actively transferred into the tracheas or tracheids. Ions transported together with the water via the apoplastic way are stopped at the lipophilic endodermis and selected by the cytoplasma membrane of the endodermis cells.

According to a recent study, the symplast pathway could allow the passage of the small PAhCs absorbed with the flow of water through the Casparian strip toward the xylem, while the large PAhCs would enter the root through the apoplastic path, and

therefore blocked by the Casparian strip [67]. Hence, polar and ionizable compounds absorbed exclusively by the apoplastic pathway cannot cross the Casparian strip and will not reach the vascular tissue. Consequently, they remain confined at the root level and accumulate there [68]. Furthermore, given the negative charge of the plant cell wall, positively charged compounds will also be hindered from entering through the cell wall matrix. Transport through the cell membrane could only be allowed through the passage through non-selective channels which would allow to bypass the Casparian strip. It has also been suggested that the absorption of basic compounds would occur through processes mediated actively by carrier proteins due to similarities of natural compounds absorbed by these pathways [68]. Polar compounds that are able to pass the Casparian strip or enter the root through passive diffusion into the symplastic path or by active absorption can move through the roots and reach and accumulate in the aerial parts of the plant. Once in the xylem, transpiration guides these compounds from the roots to the shoots and leaves with the water flow. However, it seems that many PhACs tend to accumulate mainly in the roots and in the green parts, shoots and leaves, rather than in the fruits, and that translocation occurs mainly via xylem [69–71].

The biological characteristics of the plants, the physical-chemical properties of the PhACs (molecular weight, Kow and pKa), the ionic nature of the PhACs, and the characteristics of the soil are all factors that influence absorption and translocation of PhACs in roots and aerial parts of the plant. PhACs with log Kow between 1 and 4 can easily be translocated in the different compartments of the plant [72]. In a recent review based mainly on hydroponic studies, it is assumed that anionic PhACs preferably accumulate in the roots, while neutral and cationic PhACs preferentially move in the green parts of the plant or even into the fruits [73].

Soil also plays a key role in the absorption and distribution of drugs in cropping systems. In fact, the soil is the first bulk receptor of organic contaminants when agricultural fields are irrigated with wastewater [74]. The concentration of PhACs in the water of the soil pores, and then in the availability of PhACs for the absorption of plants, depends on the physico-chemical characteristics of the soil, in particular from soil texture. The negative charge of organo-mineral colloids is the resultant of the sum of the permanent negative charge of the clay minerals and the pH-dependent charge of the humic matrices. High percentages of silt and clay make soil less fertile and reduce the availability of ionizable compounds due to the presence of a greater number of negative charges due mainly to clays. A strong electrostatic bond of PhACs to soil particles generally reduces availability for plants, especially for those chemicals with strong hydrophobicity or positive charge.

Once absorbed by plant cells, contaminants can accumulate as they are or undergo metabolic processes of plants, which have similarities to those of the mammalian detox system since many enzymes responsible for cell detoxification, including cytochromes P450 (CYP450s), present high similarity. The processes that the plant cell puts in place to reduce the toxicity of these exogenous compounds begin with Phase I metabolism processes (oxidation, reduction, and hydrolysis) and then move on to Phase II processes where they are conjugated with a polar molecule such as sugars or amino acids, or glutathione [68]. Compared to mammalian cells, a

part of these two phases, the plant cell can implement a further phase III detoxification mechanism constituted by the enzymes present in the vacuole [75].

Among the numerous studies carried out, it appears that the psychoactive drugs are compounds that most accumulate, translocate, and metabolize inside the plant. In particular, the carbamazepine is among the most studied model compounds, since its presence in wastewater is pseudo-constant due to its recalcitrance to degradation. Hence, numerous studies report the presence of carbamazepine and its metabolites in different agricultural crops [76–78].

9 Presence of Drugs in Earthworms

The presence and effects of drugs following the use of wastewater in agriculture has also been observed in soil invertebrates [79–85]. Of all the terrestrial invertebrates that live in the area explored by the roots, earthworms are the most abundant species in terms of biomass (80% of the soil biota). This causes earthworms to be considered key organisms of the soil-root-plant system since, by constantly digging through the soil, earthworms recycle nutrients and create the conditions for good soil aeration and drainage, leading to a fertile environment [85]. Unfortunately, in addition to making the soil more fertile, earthworms with their movements redistribute organic contaminants from the deepest areas to the root area, consequently increasing the availability of these compounds for plants. Being in close contact with the soil matrix, earthworms are exposed to a variety of anthropogenic organic pollutants including pharmaceutical products. They are therefore indispensable organisms for assessing soil contamination by these substances and would also help us better understand the entry of contaminants into food chains, since they occupy the lowest level in the trophic web.

However, the number of studies to evaluate the effects of pharmaceutical products on soil invertebrates is currently quite limited, and most of the research is largely focused on laboratory tests. In part this could also be due to the lack of reliable multi-residual analytical methods, since the extraction and analysis of these compounds from earthworm tissues is quite complicated [85].

10 Drugs in Constructed Wetlands

Drugs can be also depurated from wastewater by plants in constructed wetlands (CW). They are a low-cost alternative wastewater treatment technology mainly used for the treatment of urban or agro-livestock wastewater from small rural towns. Consisting of flooded vegetated beds designed to imitate the well-known water purification capacity of natural wetlands, CWs are a nature-based solution for wastewater management. Furthermore, in recent years, CWs have proven to be an excellent advanced (tertiary) treatment system since they reduce most of the

pathogens and other components such as nutrients (very effective denitrification processes) and metals. In addition, CWs represent a potential low-cost solution for the removal of contamination from emerging organic contaminants, including pharmaceutical products in wastewater effluents [63]. In fact, in these natural environments, a multitude of physical, chemical, and biological processes occur simultaneously, such as adsorption (soil or sediments), photolysis, volatilization, absorption and accumulation in plants, exudation, and microbial degradation [86, 87]. CWs allow to treat a high load of wastewater with large quantities of organic substances and PhACs representing a great potential of use in low-income countries and in rural areas [88]. The removal efficiency of PhACs in wetlands may be influenced by numerous design parameters in addition to the presence and type of vegetation as well as the type of substrate. In addition to the design and operating factors (area, bed depth, hydraulic loading speed, organic loading speed, and hydraulic retention time), other variables that could influence the removal efficiency of the CW are the physical-chemical parameters (dissolved oxygen, temperature, and pH), the amount of sunlight, the type and composition of microbiota, the age of the wetland, and the seasonality of the high microbial biomass. The geographic variables and the temperature together with the type of vegetation affect the microbial activity and evapotranspiration and consequently the mobility and the degradation of organic compounds [89]. In fact, seasonality affects the intensity and the cycle of light, consequently influencing the biological cycle of the plants, microbes, and their activities. Generally, constructed wetlands consist of a single species, e.g., dense reed plantations with heights that can reach 2 or 3 meters. These reeds are mainly formed by marsh straw or rushes (*Phragmites australis* or *Juncus effusus*), in areas with lower water, while in waters of greater depth, mainly by cattails (*Typha latifolia* or *Typha angustifolia*), may be utilized. Plants absorb pollutants through their roots which can be translocated to non-immersed parts, such as stems and leaves where they can be accumulated, translocated, metabolized, or degraded by the plant itself or in cooperation with the endophytic microorganisms inhabiting plant tissues. In fact, the rhizosphere constitutes the most active reaction zone of the submerged plants of the wetlands, where physical-chemical and biological processes occur induced by the interaction of plants, microorganisms, substrate, and pollutants [90, 91]. In most cases, greatest biodegradation of pharmaceutical products occurs there [92]. Although CWs are a green and economic alternative to wastewater treatment especially in rural or economically disadvantaged areas, the biggest problem is the removal and disposal of the large vegetable biomass produced. A non-careful management of this biomass, in fact, could recirculate large amounts of toxic organic substances accumulated in it into the environment.

11 Analysis of Drugs and Their Metabolites

Currently, the scarcity of sensitive multi-residue analytical methods represents the bottleneck to the comprehensive screening of wastewater contaminants. Although hundreds of analytical methods for the quantitative determination of drugs and their metabolites in wastewater and surface water have been published over the years, the number of robust and reliable methods for their determination in plant tissues and soil is still quite few. Several analytical methods have been developed to extract drugs from plant tissues using a wide array of techniques. Some methods have been developed to extract wastewater-borne pollutants from plant tissues using traditional approaches with large amounts of solvents such as solid-liquid extraction [76, 93], accelerated solvent extraction [94], and ultrasound-based extraction [77, 81, 95–98]. However, the aforementioned methods are not environmentally sustainable. In recent years, several analytical methods have proposed the use of a rapid, easy, cheap, effective, robust, and safe method, QuEChERS protocols, for the determination of pharmaceutical products in lettuce or other vegetable products [76, 94, 99–103]. This extraction method is widely used in the analysis of pesticides in fruits and vegetables. The determination of pharmaceuticals and their metabolites by means of liquid chromatography coupled with mass spectrometry (LC-MS) is challenging because of their low concentrations, but it is the method of choice because it is the unique method capable of detecting such amounts of drugs after their extraction and preconcentration.

Book Description

In the following chapters, the long journey of drugs from the first synthesis (chapter "The Journey of Human Drugs from Their Design at the Bench to Their Fate in Crops") to the whereabouts in crops and ultimately their analysis is described beginning with the identification of the sources of drugs in water (chapter "Sources of Pharmaceuticals in Water"). Their main routes of entry into the environment are alongside wastewater and sewage sludge. Both can be considered valuable resources rather than waste products in accordance with circular economy rules (chapter "Environmental, Economic, and Ethical Assessment of the Treated Wastewater and Sewage Sludge Valorization in Agriculture"). The amount of essential nutrients supplied to the soil through wastewater irrigation and amendment of digested sewage sludge must be carefully considered. Furthermore, these practices imply the conscious yet undesired introduction of known or unknown trace contaminants such as human drugs into agricultural soils, whose impact (chapter "Wastewater Reuse in Agriculture: Effects on Soil-Plant System Properties") on soil quality needs to be evaluated. Drugs originating from reclaimed wastewater or biosolids enter crops through plant roots and may accumulate to different degrees in various plant compartments. The uptake and translocation are dependent on multiple parameters, namely, physico-chemical properties of the compounds, plant physiology, and environmental factors (chapter "Uptake and Translocation of Pharmaceuticals in Plants: Principles and Data Analyses"). Drugs remaining in the soil following land application of wastewater, sewage sludge, and manures (chapter "Soil Sorption and

Degradation Studies of Pharmaceutical Compounds Present in Recycled Wastewaters Based on Enantiomeric Fractionation") can affect plants and lower animals from our agroecosystems which have similar receptor and metabolic enzymatic systems (chapter "Uptake and effects of pharmaceuticals in the Soil-Plant-Earthworm System"). However, plants have evolved very sophisticated detoxification systems including a complementary battery of enzymes that are capable of transforming xenobiotic compounds to yield chemically diverse metabolites (chapter "Metabolism of Pharmaceuticals in Plants and their Associated Microbiota"). Many of them are formed in analogy to the liver in mammalian systems, but a number of plant-specific metabolic reactions have also been identified (chapter "Metabolism of Pharmaceuticals in Plants and their Associated Microbiota"). The presence of drugs residues in soil can compromise the abundance, diversity, and activity of the soil microbial community which is one of the key players in a range of soil ecosystem services (chapter "Impact of PhACs on Soil Microorganisms"). Moreover, drug accumulation in agricultural soils may pose a serious threat to non-target organisms and natural resources (chapter "Biomarkers in Earthworms"). As the accumulation of drugs in soil can constitute a potential risk for soil quality and food security, several engineered remediation methodologies have been developed for their removal from contaminated soils. Unfortunately, these techniques are often economically prohibitive and may cause adverse side effects in the environment. Microbes, soil fauna (e.g., earthworms), and their interactions exert a strong control in the organic matter decomposition and nutrient cycling of soil. By taking advantages of these naturally occurring processes, the use of earthworms has been proposed to clean biosolids and manure and to reduce the bioavailability of pharmaceuticals to plants (chapter "Vermiremediation of Pharmaceutical-Contaminated Soils and Organic Amendments"). Another remediation technique is constructed wetland which is one of the most commonly applied natural solutions relying on plants for wastewater purification (chapter "Constructed Wetlands and Phytoremediation as a Tool for Pharmaceutical Removal"). In these environmentally friendly and cost-efficient systems, drugs are adsorbed and metabolized in soil and can also be taken up and metabolized in plants. To understand the whereabouts of drugs in the environment once there have been emitted from the various sources, sensitive analytical methodologies are required for their detection and quantification as well as for the identification of metabolites in soil and plants (chapters "Development of Methods for the Determination of PhACs in Soil/Earthworm/Crop System Irrigated with Reclaimed Water" and "Analytical Approaches for the Determination and Identification of Drug Metabolites in Plants After Uptake").

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Sources of Pharmaceuticals in Water

Roberto Parra-Saldivar, Carlos Castillo-Zacarías, Muhammad Bilal, Hafiz M. N. Iqbal, and Damiá Barceló

Contents

Abstract This chapter focuses on the increasing environmental apprehensions and persistence of numerous organic contaminants so-called emerging contaminants (ECs), including biologically active elements from pharmaceutical source industries. Several types of diverse pharmaceutical-related compounds are being detected in environmental matrices and wastewater treatment units. Owing to this broader occurrence, transformation, and detection of pharmaceutical-related compounds in water matrices, people and legislative authorities are now more concerned about potential sources and ecological consequences of ECs. This is mainly because the free movement of ECs in water matrices is posing noteworthy adverse effects on human, aquatic animals, and naturally occurring plants, even at minimal

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concentrations. So far, several detection and treatment processes have been proposed and exploited against numerous pharmaceutical-related ECs. The useful and side effects of pharmaceutical-related compounds have been extensively inspected. Owing to this substantial research gap, the sources and environmental persistence of pharmaceutical-related ECs and their direct/indirect adverse effects have now been the topic of intensive studies. From the surface water perspective, wastewater treatment plants (WWTPs) are the major source of pharmaceutical-related ECs. The current chapter spotlights the widespread occurrence, numerous sources, and transportation fate of pharmaceutical-related ECs in water matrices.

Keywords Aquatic environment, Biological risks, Emerging contaminants, Hazardous compounds, Pharmaceuticals, Sources, Toxicity, Transmission fate, Wastewater treatment

1 Introduction: Problem Statement and Opportunities

In recent years, the term "emerging contaminants (ECs)" has raised the ecological concerns and public attention to the presence of toxic entities in the aquatic environment. Such harmful entities, with particular reference to the occurrence of pharmaceutical-related ECs, are mainly introduced in our water matrices through various industrial and domestic practices. A constant rise in the global population and urbanization and their associated increase in the consumption of pharmaceuticals have redirected the researches' attention. In addition, wide-ranging water contamination by pharmaceutical compounds has become a growing worldwide concern [1, 2]. The concentrations of persistent organic pollutants, such as pharmaceuticals and their metabolites, are continuously rising in the natural environment due to human activities [3–6]. The most practiced pharmaceuticals include analgesics/anti-inflammatories and synthetic antibiotic compounds. Some of them are β-lactams (amoxicillin and penicillin), cardiovascular pharmaceuticals (β-blockers/diuretics), estrogens and hormonal compounds (estriol, estradiol, estrone, and 17α-ethinylestradiol), and antiepileptic drugs (carbamazepine) [7].

The major source of pharmaceutical-related ECs is the treated/untreated effluents of WWTPs. WWTPs are not designed to eliminate environmental pollutants completely, and thus they can percolate through WWTPs and incorporated into the aquatic systems (streams and rivers) [8]. For example, the contents of diclofenac (an anti-inflammatory drug) and carbamazepine reached 0.99 and 0.95 μg/L, respectively, in WWTP effluents [9]. Particularly, a detectable level of diclofenac has been identified in drinking, surface, and groundwaters in the range of ng/L to μg/L in Sweden, Spain, Switzerland, and the Baltic region [10–12]. Apart from this, other pharmaceuticals, including tramadol, carbamazepine, ibuprofen, oxazepam, and naproxen, have also recently been detected in drinking water supplies in some

countries. These concentrations of pharmaceuticals can induce serious environmental threats such as congenital disorders, physical abnormalities, impairments of the endocrine and reproductive system, and feminization of some fish species [13, 14]. Due to the capability of micro-pollutants and pharmaceutically active compounds to cause adverse effects to the ecosystem and human health, they have attracted the principal research focus in recent days. Some notable adverse effects of numerous ECs are shown in Fig. 1 [14].

This chapter focuses on the widespread occurrence, and numerous sources, such as domestic, medical, agricultural, and industrial sectors that discharge pharmaceutical-related ECs into water matrices. The focus is also given to the fate of pharmaceutical-related ECs in the aquatic environment. The later part of the chapter discusses risk management issues to advance the existing knowledge further to improve the sewage WWTPs and increase public consciousness of the concentration of pharmaceutical-related ECs and biologically active residues in the water matrices.

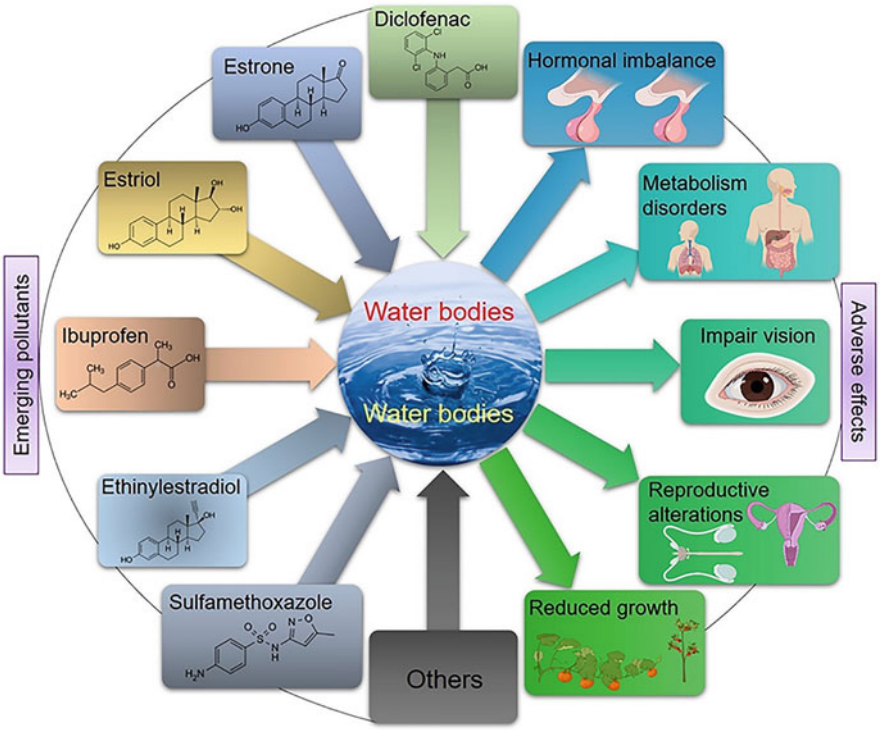

Fig. 1 A schematic illustration of considerable adverse effects of abundant ECs. Reprinted from Morsi et al. [14] Laccases and peroxidases: The smart, greener and futuristic biocatalytic tools to mitigate recalcitrant emerging pollutants. Science of The Total Environment, 714, 136572, © 2020 Elsevier B.V., with permission from Elsevier

2 Sources of Pharmaceuticals

Broadly speaking, the sources of pharmaceuticals can be categorized in two ways, i.e., (1) point-based pharmaceutical-related ECs and (2) diffuse-based pharmaceutical-related ECs. The former type is further considered as a single identifiable source that initiates from various location-based sources, such as domestic sewage sources, domestic solid waste, pharmaceutical-related industrial sector waste effluents, biomedical (hospital) wastes (effluents and solid wastes), and WWTPs. Such point-based sources are easy to identify and quantify from the specific location hotspots and can be calculated via mathematical modeling [7, 15]. Moreover, the wastewater effluent-based point sources are the main cause of environmental pollution and soil zone and water matrix contamination. Unlike point-based source pollution, the exact source location of the second category, i.e., diffuse-based source pollution, is hard to identify, which generally occurs over a broader geographical scale [15]. Some main examples of diffuse-based source pollution are the agricultural soil erosion/runoff, urban runoff, and unrecognized leakage of waste from wastewater treatment systems and plants [7, 16]. As it can be seen from Fig. 2, it shows the possible routes of antibiotics, one type of pharmaceuticals, that how such polluting agents are discharged from their sources and found their way to the receptor locations, such as ground and surface water bodies [1]. In addition to this, the receptor locations/hotspots, which are directly or indirectly influenced by the pharmaceutical-related

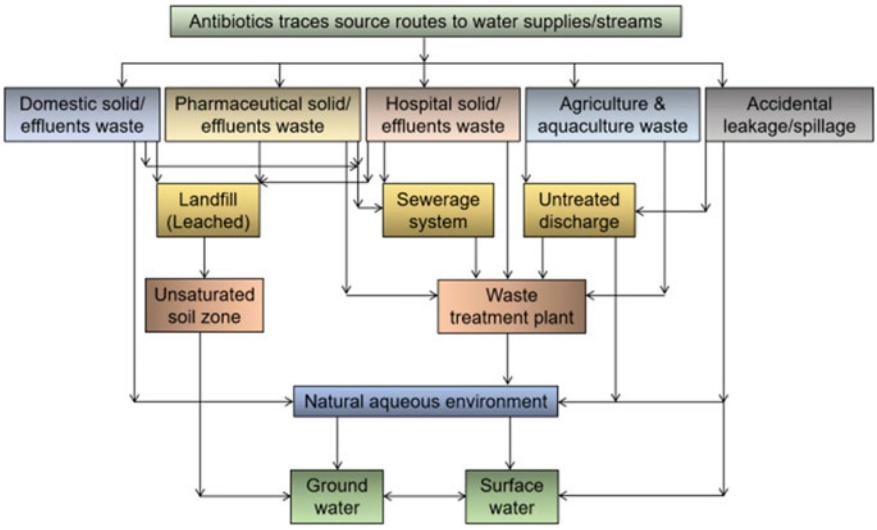

Fig. 2 Major point and nonpoint-based sources of antibiotics pollution and their possible transmission routes to groundwater and surface water bodies. Reprinted from Bilal et al. [1] Biocatalytic degradation/redefining "removal" fate of pharmaceutically active compounds and antibiotics in the aquatic environment. Science of The Total Environment, 691, 1190–1211, © 2019 Elsevier B.V., with permission from Elsevier

ECs, can be categorized into three main spots, i.e., (1) unsaturated soil zone, (2) groundwater, and (3) surface water. Plentiful aspects such as the type and class of antibiotics, concentration, unwarranted dosage, acquaintance time, persistence duration, removal pattern, reception hotspots, i.e., soil, water, or air. Moreover, the occurrences of multi-antibiotics along with other biologically active pollutants as a complex mixture pointedly affect their conceivable transmission into the aquatic environment. Consequently, a diverse spectrum of biologically active constituents of antibiotics has been found as micro-contaminants in soil and water matrices, in the past two decades [17, 18].

In addition, besides their broader occurrence, the concentration is disturbingly growing in an uncontrolled manner. The hefty usage of numerous antibiotics, regardless of types and classes, is being practiced around the globe in a controlled or uncontrolled fashion [1]. Main examples of heavily consumed antibiotics include active members from the class penicillins (under the category of amoxicillin, ampicillin, and dicloxacillin), active members from the class cephalosporins (under the category of cephalexin, cefaclor, cefotaxime, and ceftazidime), active members from the class macrolides (under the category of erythromycin, clarithromycin, and azithromycin), active members from the class quinolones (under the category of ciprofloxacin, levofloxacin, moxifloxacin, and ofloxacin), active members from the class sulfonamides (under the category of sulfasalazine and trimethoprim), active members from the class tetracyclines (under the category of minocycline, eravacycline, demeclocycline, and doxycycline), active members from the class glycopeptides (under the category of dalbavancin, oritavancin, telavancin, and vancomycin), active members from the class aminoglycosides (under the category of gentamicin, tobramycin, and amikacin), and active members from the class carbapenems (under the category of meropenem, doripenem, ertapenem, imipenem, and cilastatin) (Fig. 3) [1].

In a modern medicine practice, several types of antibiotics as mentioned above are among the most recurrently prescribed medications. According to one study, in the USA alone, out of 61 million US women with reproductive age, i.e., 15–44 years, around 99% used at least one contraceptive-based medicine, whereas other 60% regularly use contraceptive-based medicine [19–21]. More specifically, out of all those who used contraceptive-based medicine, approximately 72% practice nonpermanent methods, i.e., primarily hormonal methods (i.e., the pill, patch, implant, injectable, and vaginal ring) [21, 22]. Ultimately, upon excretion in the domestic sewage of poorly metabolized active residues of the used contraceptives find their way into the aquatic environment [17], even after passing through a partial or inadequate treatment at of the swage waste at the WWTPs. Similarly, other pharmaceuticals, such as ibuprofen, naproxen, acetaminophen, acetylsalicylic acid, and carbamazepine, have been considered high-use and/or overuse antibiotics in Canada [23]. Despite the excessive consumption of pharmaceuticals by humans, several other pharmaceutically active constituents, such as antibacterials, antifungals, and parasiticides, are tremendously employed in the aquaculture, veterinary, agriculture, and animal care settings. In the USA alone, about 92,500 and 196,400 kg antibacterials/year are used for aquaculture-based applications. Moreover, around 8.5 and 11.2 million kg antibacterials are employed in the agricultural setting, annually [24, 25]. Regardless of their usefulness in the respective sectors, the heavily

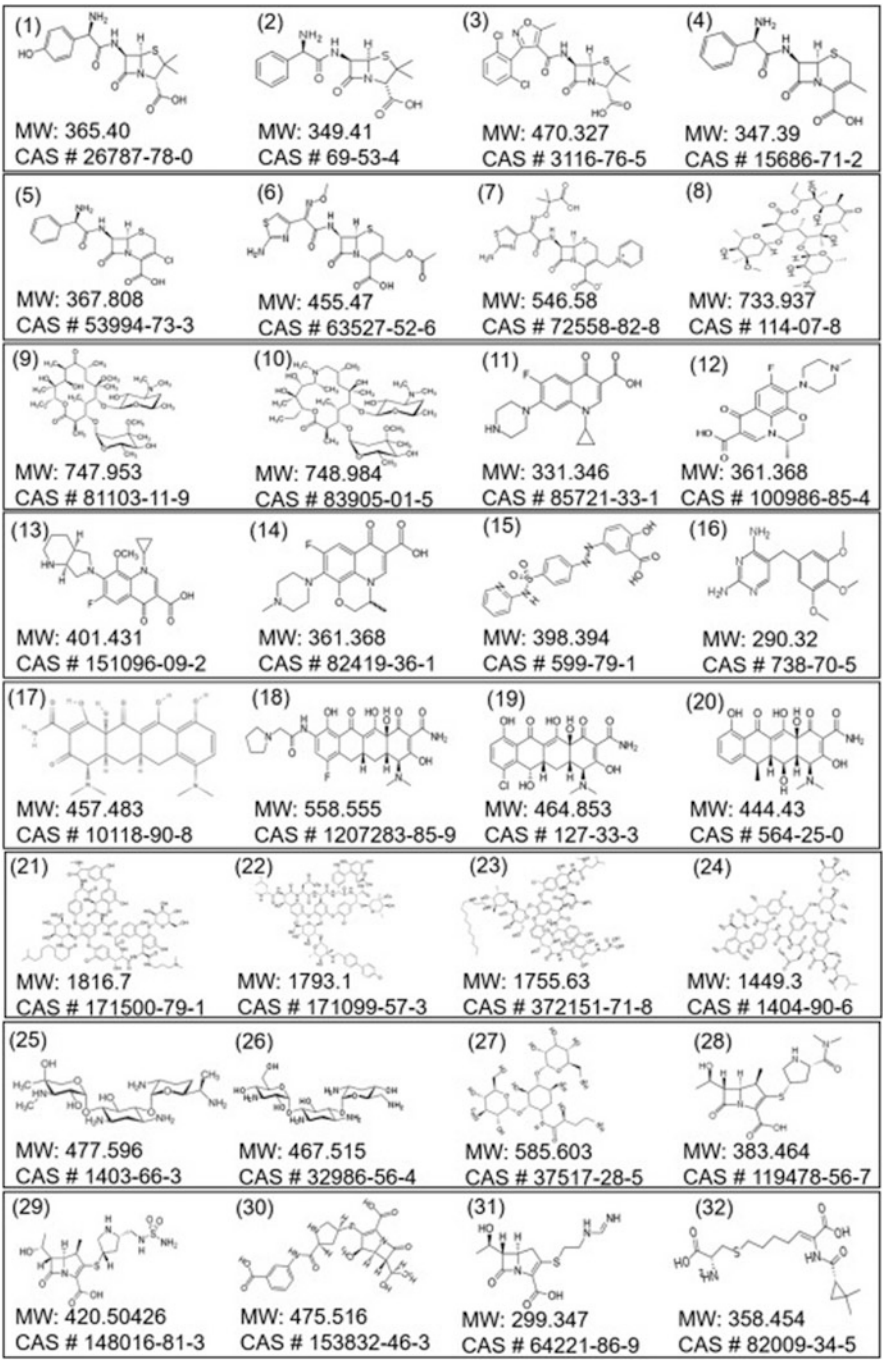

Fig. 3 Illustration of selected antibiotics. Structural, molecular, and CAS details for each represented antibiotic are also given accordingly. (1) Amoxicillin, (2) ampicillin, (3) dicloxacillin, (4) cephalexin, (5) cefaclor, (6) cefotaxime, (7) ceftazidime, (8) erythromycin, (9) clarithromycin, (10) azithromycin, (11) ciprofloxacin, (12) levofloxacin, (13) moxifloxacin, (14) ofloxacin,

exploited antibiotics end up their transmission fate to the soil, groundwater reservoirs, and surface waters, directly or indirectly, through runoff or drain-off [26]. Such a controlled or uncontrolled transmission of antibiotics residues massively stress the ecosystem that should be dealt with care for their effective mitigation prior to release into water matrices. Other potential sources of pharmaceuticals in our water bodies include the unrestrained spillage or improper dumping of expired drugs in the landfill site. Besides, drainage/sewage system and waste effluent streams are also the points of significant contamination [27, 28].

3 Case Studies of Point-Based Source Pollution

As mentioned earlier, WWTPs are considered one of the significant and imperative point-based sources of pharmaceutical-related ECs in water matrices [29–31]. The existing literature evidently shows that a diverse range of around 16 to 54 types of pharmaceuticals is found in wastewater effluents. For instance, He et al. [31] performed a scale-based approximation of pharmaceutical concentrations and associated environmental risk in the Japanese wastewater system. It was recorded that 36 pharmaceuticals, majority of them were antibiotics and analgesics, had high predicted environmental concentrations in influent with pranlukast, a receptor antagonist which has the highest concentration in wastewater influent at 257.0 μg/L. Moreover, among all tested pharmaceuticals, the occurrence concentrations of 26 were relatively higher than 1.0 μg/L, while the predicted environmental concentrations of 6 other pharmaceutical-related compounds were extremely higher than 10.0 μg/L. Such existence or occurrence of pharmaceuticals at extreme/higher level possibly attributes to excessive consumption rates by consumers and poor removal rates in WWTPs. From a consumers-based source view, partially or incompletely metabolized pharmaceutical excretion into the domestic sewage stream is the main cause of pharmaceuticals to the aquatic environment [32]. Among several reported pharmaceutical compounds, analgesics/anti-inflammatories (i.e., acetaminophen, salicylic acid, and salicylamide) are abundant in wastewater influent (>100 ng/L) in Japan [33, 34]. Likewise, the occurrence of pharmaceuticals in wastewater stream/influents in the USA, the UK, Spain, Italy, India, and China has been reported [35–40]. To avoid literature redundancy, Table 1 summarizes various studies that report the notable occurrence of pharmaceuticals in environmental matrices.

Fig. 3 (continued) (15) sulfasalazine, (16) trimethoprim, (17) minocycline, (18) eravacycline, (19) demeclocycline, (20) doxycycline, (21) dalbavancin, (22) oritavancin, (23) telavancin, (24) vancomycin, (25) gentamicin, (26) tobramycin, (27) amikacin, (28) meropenem, (29) doripenem, (30) ertapenem, (31) imipenem, and (32) cilastatin. MW: molecular weight (g/mol). See CAS # for further details. Reprinted from Bilal et al. [1] Biocatalytic degradation/redefining "removal" fate of pharmaceutically active compounds and antibiotics in the aquatic environment. Science of The Total Environment, 691, 1190–1211, © 2019 Elsevier B.V., with permission from Elsevier

Table 1 Various studies that report the notable occurrence of pharmaceuticals in the environmental matrices

Reference	Pharmaceuticals	Remarks/highlights
He et al. [31]	Acetaminophen, salicylic acid, salicylamide, ibuprofen, naproxen, ketoprofen, clarithromycin, trimethoprim, roxithromycin, azithromycin, sulfamethoxazole, sulpiride, thiamphenicol, atenolol, diphenhydramine, and pirenzepine	Thirty-six pharmaceuticals, majority of them were antibiotics and analgesics, had high predicted environmental concentrations in influent. Nine pharmaceuticals in the effluent showed high toxicity based on predicted environmental concentrations/predicted no effect concentration ratio
Felis et al. [41]	Aminoglycosides, β-lactams, glycopeptides, macrolides, fluoroquinolones, sulfonamides and trimethoprim, tetracyclines	Occurrence and environmental implications of antimicrobial pharmaceuticals in the aquatic environment WWTPs are indeed the main source responsible for the prevalence of these factors in the aquatic environment
Nantaba et al. [42]	Trimethoprim, azithromycin, sulfamethoxazole, diclofenac, ibuprofen, sulfamethazine, enoxacin, sulfacetamide, atenolol, oxytetracycline, metoprolol, tetracycline, erythromycin, roxithromycin, bezafibrate, ciprofloxacin, levofloxacin, norfloxacin, sparfloxacin, metronidazole, diazepam, acetaminophen, carbamazepine, and fluoxetine	Occurrence of pharmaceutical residues in Africa's largest freshwater lake Twenty-four pharmaceuticals were detected in water from Lake Victoria, Uganda
Su et al. [43]	Caffeine, carbamazepine, azithromycin, bezafibrate, metoprolol, sulfadiazine, sulfamethoxazole, clarithromycin, erythromycin, roxithromycin, and trimethoprim	Spatiotemporal distribution of 27 pharmaceuticals in the Chaobai River Agriculture area presented the highest pharmaceutical concentrations The acute toxic pressure in the river was mainly driven by caffeine
Stroski et al. [6]	Atenolol, carbamazepine, metoprolol, naproxen, sulfapyridine, sulfamethoxazole, and trimethoprim	Seven pharmaceuticals were detected in Canadian Arctic wastewater Abundances of pharmaceuticals varied between communities and treatment methods
Reis et al. [44]	Phenazone, ibuprofen, ketoprofen, phenylbutazone, betamethasone, ranitidine, loratadine, cimetidine, clarithromycin, erythromycin, paroxetine, scopolamine, omeprazole, trimetroprim, atenolol, fenofibrate, gemfibrozil, atorvastatin, fluconazole, prednisone, metformin, amoxicillin, ampicillin, caffeine, and enoxacin	Trace levels of pharmaceuticals were detected in superficial and drinking water Conventional drinking water treatment plants were not able to remove the pharmaceuticals completely Drier periods were related to the highest concentration of the pharmaceuticals Drinking water treatment plants' removal efficiency shows a great variation over the year
Greenham et al. [45]	Acetaminophen, caffeine, atorvastatin, lorazepam, cotinine, metformin,	Twelve of top-used pharmaceuticals and 2 metabolites were assessed

(continued)

Table 1 (continued)

Reference	Pharmaceuticals	Remarks/highlights
	metoprolol, paraxanthine, naproxen, quetiapine, ramipril, salbutamol, venlafaxine, and warfarin	Primary treatment was significantly less efficient than other technologies Removal efficiencies of pharmaceuticals with 9 different treatment technologies were tested
Kleywegt et al. [46]	Paroxetine, sertraline, carbamazepine, penicillin, acetaminophen, codeine, ibuprofen, naproxen, oxycodone, atorvastatin, metoprolol, amlodipine, diltiazem, furosemide, and verapamil	Direct discharges from pharmaceutical facilities are a crucial source of pollution to receiving sewer sheds Elevated concentrations of pharmaceuticals are detected in effluents from manufacturers The manufacturer facilities may be discharging several kilograms of lost products directly to the sewers daily
Kołecka et al. [47]	Ibuprofen, paracetamol, flurbiprofen, naproxen, diclofenac and its metabolites	Pharmaceuticals' distribution in wastewater treatment plant plus sludge treatment reed beds differs between season and chemical type Ibuprofen, naproxen, and paracetamol were eliminated by the conventional wastewater treatment plant
Hanamoto et al. [34]	Caffeine, theophylline, acetaminophen, lincomycin, sulfamonomethoxine, metoprolol, ofloxacin, ketoprofen, bezafibrate, and roxithromycin	In-stream attenuation of pharmaceuticals was observed by a mass balance approach Source was estimated based on populations for pharmaceuticals conservative in the river Three pharmaceuticals were substantially affected by household septic tanks
Afonso-Olivares et al. [48]	Trimethoprim, ofloxacin, metronidazole, ciprofloxacin, sulfamethoxazole, atenolol erythromycin, propanolol, ranitidine, omeprazole, fluoxetine, carbamazepine, metamizole, ketoprofen, naproxen, ibuprofen, diclofenac, bezafibrate, gemfibrozil, clofibric acid, nicotine, paraxanthine, caffeine, atenolol d7, sulfamethoxazole d4, ibuprofen d3	Twenty-three pharmaceuticals were monitored in sewage from wastewater treatment plants in Gran Canaria (Spain) Removal efficiencies of pharmaceuticals from two different wastewater treatment plants were evaluated Environmental risk assessment of pharmaceuticals was determined
Liu et al. [49]	Amoxicillin, erythromycin, clarithromycin, ofloxacin, roxithromycin, norfloxacin, levofloxacin, lincomycin, sulfamethoxazole, ibuprofen, trimethoprim, flumequine, metronidazole, metoprolol, caffeine, chlortetracycline, clofibric acid, diclofenac, salicylic acid, and carbamazepine	Caffeine showed the highest influent concentration than other pharmaceuticals Wastewater treatment plants in north China had a higher influent level of total pharmaceuticals Several high-risk pharmaceuticals to the environment were identified

4 Case Studies of Diffuse-Based Source Pollution

The unidentified movement of several organic contaminants that include active pharmaceutical compounds enters the soil zone and aquatic environments by numerous direct or indirect routes, with bioactive sludge being one of the furthermost essential diffuse sources [7, 50]. The excessive utilization of bioactive sludge, as a biofertilizer, in the agricultural settings is a dominant diffuse-based source of the pharmaceuticals which can then run off to the ground and enters the groundwater and freshwater resources [15]. Generally, bioactive sludge, also termed as biosolid which is commonly used as a biofertilizer, is a type of active residue obtained from the leftovers of the wastewater treatment plants. Such sewage sludge, as a biofertilizer, is typically used for soil amendment. For instance, it has been estimated that around 8 × 106 dry tons of sludge are produced, and 50% of the obtained sewage sludge is applied to the agricultural land in America. However, the maximum concentration of the pharmaceuticals found in the biosolid, i.e., thiabendazole, is 5,000 μg/kg, and other varieties of pharmaceuticals, e.g., caffeine and carbamazepine, can also be found in the sewage sludge. Owing to this high concentration of active pharmaceutical compounds and high solubility of halogenated hydrocarbon in sewage sludge, it leads to groundwater pollution from the application of biosolid to soil and surface runoff of the biosolid containing soil [15]. The controlled or uncontrolled excessive consumption of biologically active compounds in the agricultural settings is the foremost contributor, through activities such as agricultural runoff, the application of fertilizers and pesticides, tillage practices, habitat alteration, animal waste, and soil erosion. Thus, far more complex challenges are being posed by the diffuse-based source of the pharmaceuticals, which is also known as nonpoint source pollution. Considering the complexity, the diffuse-based source or nonpoint source pollution is a leading water quality problem, in recent times, as compared to the point-based source pollution. Moreover, the diffuse-based source or nonpoint source pollution from agriculture and the urban periphery is a most intractable dimension [51, 52], which is progressively being recognized by policymakers and regulatory authorities. According to the European Union Article 11(3)(h), the Water Framework Directive sets out lowest compliance requirements, i.e., "for diffuse sources liable to cause pollution, measures to prevent or control the input of pollutants. Controls may take the form of a prerequisite for prior regulation, e.g., a proscription on the entry of pollutants into water matrices, prior authorization based on general binding rules where such a prerequisite is not otherwise provided for under Community legislation" [53].

5 Pharmaceuticals' Fate in the Environment

Owing to the complications of diffuse-based source or nonpoint source pollution, the real information on the fate of various pharmaceuticals in the environment is limited [54–56]. Another possible reason behind this vague fate of pharmaceuticals in the

environment could be the low-level volatility of pharmaceuticals. Therefore, the main distribution/transportation of pharmaceuticals in the environment majorly occurs by aqueous transport via a point-based source route. The occurrence of pharmaceutical-related ECs in the environment is low but consistent/persistent. However, pharmaceutical-related ECs are ubiquitous in aqueous matrices. Such consistency/persistence of pharmaceuticals in the aquatic environment is mainly because the release rate is higher than the transformation rate [56, 57]. From the persistence viewpoint, sulfonamides and fluoroquinolones are the most persistent and then macrolides, tetracyclines, aminoglycosides, and β-lactam antibiotics. Among them, sulfonamides and fluoroquinolones are easier to adsorb than macrolides, sulfonamides, aminoglycosides, and β-lactams by the soils and sediments, which make them the most persistent [58].

The major transformation fate of biologically active pharmaceuticals occurs in WWTPs to soils via sludge usage as biofertilizer. However, such WWTP-based transformation significantly depends on the overall sewage composition, treatment conditions, and the design and operational factors of the wastewater treatment process [59]. The ultimate fate of pharmaceutical-related ECs and/or their active residues/metabolites in WWTPs could be mineralization to carbon dioxide and water. In the case of lipophilic-type pharmaceutical compounds, the end fate is adsorption on suspended solids or release in the effluent as broken-down residues or as degraded products [60]. From WWTP effluent sources, the persistent pharmaceuticals can afterward be transported to groundwater and/or surface water matrices, whereas the pharmaceutical products used in the aquaculture are directly released into the surface water bodies [61–63].

6 Concluding Remarks and Outlook

In conclusion, based on the above-discussed literature with suitable examples, environmental contamination with a range of emerging anthropogenic pollutants has become a global problem. This chapter is of particular interest, which spotlights a diverse source of pharmaceuticals such as analgesics/anti-inflammatories (narcotic analgesics, nonnarcotic analgesics, and nonsteroidal anti-inflammatory drugs (NSAID)) and synthetic antibiotic (β-lactams, macrolides, fluoroquinolones, aminoglycosides, sulfonamide, and tetracycline). Some of them are β-lactams (amoxicillin and penicillin)), cardiovascular pharmaceuticals (β-blockers/diuretics), estrogens and hormonal compounds (estriol, estradiol, estrone, and 17-α-ethinylestradiol), and antiepileptic drugs (carbamazepine) in the water matrices. A brief transmission fate of pharmaceutical-related ECs and their metabolized compounds to soils, groundwater, and surface water bodies is also given from point-based source and nonpoint-based source pollution. The growing water contamination with a controlled or uncontrolled discharge of incompletely or inadequately treated industrial wastes and WWTP effluents harshly distressing the whole living ecosystem. Considering the above-discussed scenarios, there is a dire need to

develop highly efficient bioremediation strategies that are clean, green, sustainable, and environmental-friendly and can replace the in-practice inefficient remediation approaches.

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Environmental, Economic, and Ethical Assessment of the Treated Wastewater and Sewage Sludge Valorization in Agriculture

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Contents

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Abstract Sewage sludge is a by-product of the sewage treatment plants. Because of its richness in nutrients and for several environmental and economic reasons, this waste by-product is widely used as a fertilizer for agricultural purposes under specific conditions. This practice might be hazardous since this waste includes many known and unknown non-biodegradable and harmful pollutants, especially emerging organic contaminants (EOCs), as well as cultivable and non-cultivable pathogens. The present work is aiming at (1) providing information on the nature of sludge in terms of persistent pollutants and cultivable and non-cultivable microbiota generated by the currently implemented treatment processes, (2) analyzing the consequences of the agricultural valorization on ecosystem biodiversity and soil fertility, and (3) addressing and discussing the economic, ethical, and environmental benefits or damage of this type of valorization. These issues need full consideration by policy makers regarding the agricultural use of this waste by-product in terms of irrigation with treated domestic wastewater or sludge land spreading as soil fertilizer and plant growth promoter.

Keywords Economy, Ecotoxicity, Emerging organic contaminants, Ethics, Irrigation, Land spreading, Metagenomics uncultured pathogens and parasites, Microbiome, Micropollutants, Sewage sludge

1 Introduction

The human access to drinking water and sanitation is a universal right since water is a vital resource for life and indispensable for the economy and the development of nations. The protection of water resources and their sustainable use is becoming a global issue to thwart consequences of population growth, increased urbanization, and tourism and climate change with the risk of drought. The treatment and "reuse" of sewage is among the possible solutions to solve the problem of water availability for future generations as the world's demand for safe and healthy food and water is rapidly increasing [1]. According to the United Nations, the world's population is estimated to be 9.7 billion in 2050. Hence, preventing the degradation of soil ecosystems and water resources must be a priority to consider for the next decades [2].

Current estimates show the daily water consumption of an individual varies according to the country and the environment (urban or rural); it can be excessive (600 L day^{-1}, in the USA), average (150 to 165 L day^{-1}, in France), low (80 to 120 L day^{-1}, in developing countries), or very low (<40 L day^{-1}, in rural areas) [3]. After use, the wastewater (WW) is discharged into the sewage system to join WW treatment plants (WWTPs) where it will be purified. WW undergoes a series of mechanical, physicochemical, and biological processes to produce clean water that meets environmental quality standards, preventing stream and river eutrophication

and groundwater contamination. The first stage of the treatment consists in physical separation of the pollutants of large size using mechanical tools to retain and harvest solid and bulky waste such as floating grease, plastic bags, leaves, sheets, condoms, etc. carried by raw WW. These materials are insensitive to biological treatment and harmful for downstream installations (pumps and pipes). Decanted materials are referred to as primary sludge, which are then collected [2, 4]. After the decantation step, the water loaded with dissolved organic matter (OM) and suspended colloidal particles (diameter < 200 μm) flow through to the biological treatment processes where it will be assimilated and metabolized by a complex and diversified microbiota including aerobic, anoxic, and anaerobic processes [2, 4, 5] (Fig. 1). WW's microbiota assembles into aggregated structures called flocs, where exopolymeric substances (EPS) are establishing the bonds between microorganisms. Aggregation and accumulation of mineral and organic compounds to and within the flocs make their weight increase, allowing them to settle into the bottom of the WW basins [2, 4, 6]. Free-living microorganisms, which do not aggregate to the flocs, grow at the expense of dissolved OM and constitute suspended matter (SM) [7]. At the end of the biological treatment, after assimilation or elimination of carbon, nitrogen, and phosphorus, as well as other biodegradable elements, the WW undergoes a second decantation in the clarifying basin. Following sludge decantation, purified water is discharged into the natural environment or reused mainly for irrigation [8]. After this treatment, the secondary or activated sludge is collected from the clarifier, which mainly consists of microbial cells and other SM, with the eventual chelation of sewage compounds leading to the formation of so-called "biological" or secondary sludge (Fig. 1).

It was reported that the United States is generating 40 Mt of sewage annually, while in the European Union, the figure is 50 Mt, representing about 7.5 Mt of dry matter [3, 9, 10]. In the countries located near the Baltic Sea watershed, the generated sewage sludge is about 3.5 Mt of dry solids annually. Germany is the highest sludge producer, followed by the United Kingdom and France. It is estimated that these countries with Spain and Italy generate altogether nearly 75% of the European sewage sludge [11]. In France, this production is of the order of 1.8 Mt SM $year^{-1}$, which represents 15–19 kg $inhabitant^{-1}$ $year^{-1}$ of SM [3]. Considering the organic waste recovery, French policy predicts an increase of 55 to 65% over the period going from 2020 to 2025 [10, 12].

Treated WW and sludge quality are specified by national and international standards and guidelines according to the receiving environment or their use for different purposes [13, 14]. Physicochemical parameters including organic pollutants or micropollutants and minerals, among which are metal trace elements (MTE), and pathogenic microorganisms indicating fecal contamination and nematode eggs are analyzed (Fig. 2). The analyses are achieved to ensure that there will be no negative effect on the quality of the receiving environments, such as streams, rivers, or soil and groundwater, when spreading the sludge or following agricultural fields' irrigation with treated WW [15–17].

Based on the economic point of view, recent cost analysis data estimate that half of the total operating cost of a WWTP accounts for sewage sludge management

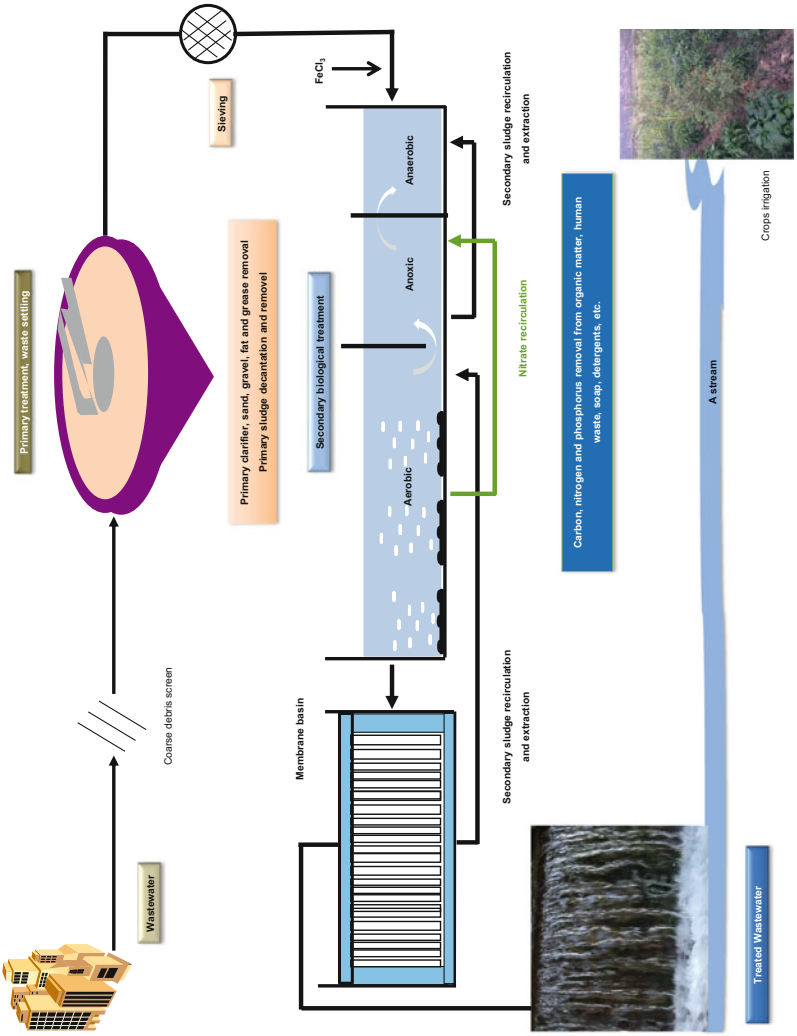

Fig. 1 Urban wastewater treatment using activated sludge technology

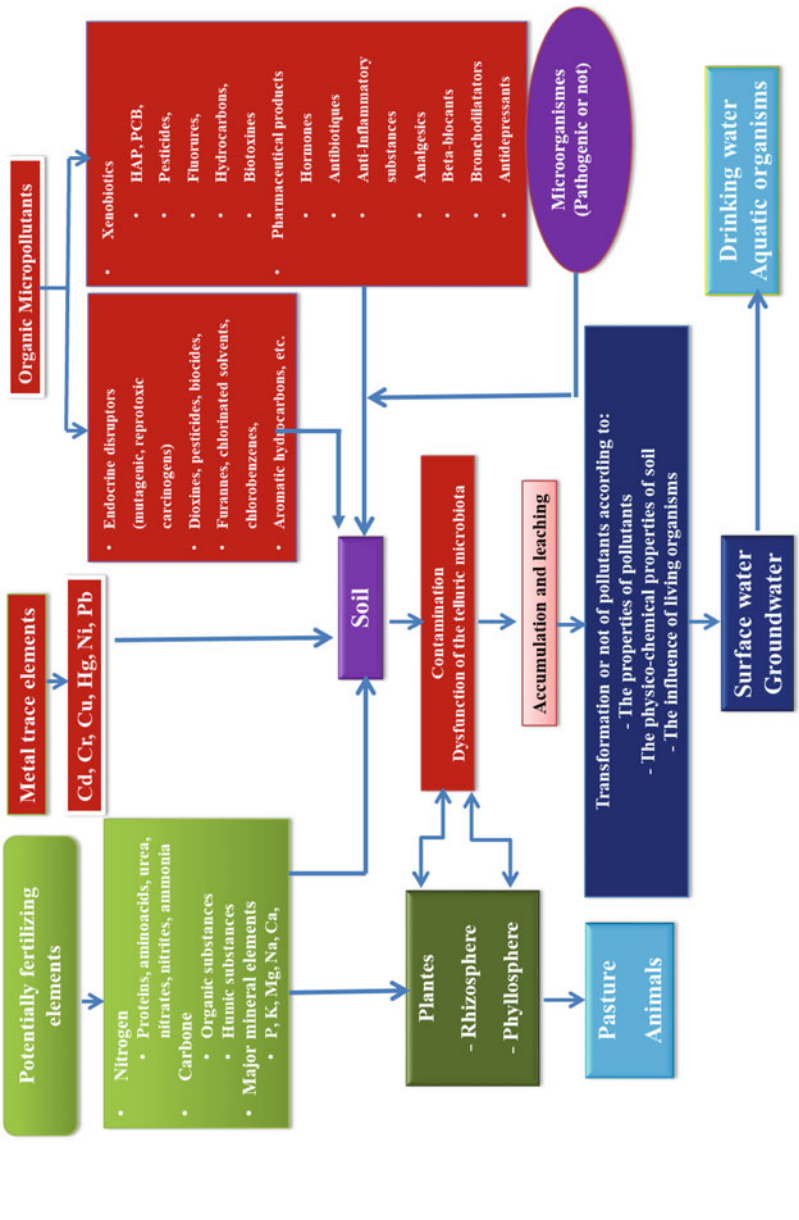

Fig. 2 Overall pollutants diversity in the sludge and possible routes through the soil ecosystem after land spreading

[18]. For this, many techniques were deployed to manage the sewage sludge including ocean disposal, incineration, landfilling, and agricultural valorization. In fact, there are many strategies to use sewage sludge but also many restrictions on the use of the given management method [11]. However, these routes of waste disposal may have numerous drawbacks and environmental hazards. To preserve marine ecology, ocean sewage sludge disposal was prohibited by the London Convention 97 protocol [19]. The presence of heavy metals, organic pollutants, pathogens, and other trace elements confines the sewage sludge use as agricultural fertilizer. For the best suitable option, some basic criteria have been described in the circular economy "from waste to resources" sense. The importance of sewage sludge as a valuable source of matter and energy and a potential risk related to the application of those strategies have been appreciated [11]. Moreover, to reduce freshwater consumption, as well as the discharge of effluents into freshwater ecosystems, treated WW (re)use may be a valid option [20]. Consequently, WW and its sludge become a valued resource rather than a waste product in line with circular economy rules. However to the best of our knowledge, the applied guidelines do not cover the impact and consequences of the introduction of known or unknown biological contaminants such as microbial pathogens or microorganisms resistant to antibiotics, heavy metals, or persistent and emerging micropollutants such as endocrine disruptors on the soil microbiome and its metabolic activity [9, 21, 22]. Consequently, the complex network of telluric microbial communities and mesofauna established based on tight interrelations between soil abiotic and biotic parameters may be affected [7, 23]. The introduction of nonindigenous potential invasive species may cause adverse effects at several levels of biological organization, inducing the elimination of indigenous microorganisms by competition, parasitism, or following changes in soil physicochemical properties [9, 16, 23, 24]. At the current state, to fill these gaps of knowledge, such studies remain to be achieved.

2 Biological Sewage Sludge Formation Within the WWTP

Biological treatment of WW converts dissolved OM into biosolids that consist of highly hydrated flocs. Oxygenation allows the dissolved OM conversion into microbial cells that eventually settle in the WWTP basins, following a physical collision and flocculation, based on the aggregation of colloidal particles. Consequently, the flocs characteristics (shape, size, density, and porosity) affect its sedimentation rate [6, 22]. WW sludge may be in liquid, solid, or pasty form and contain OM (carbon, nitrogen, and phosphorus), mineral elements, and heavy metals [10] (Fig. 2).

The sludge structure is closely related to that of the flocs. Microorganisms in the purifying biomass excrete complex mixtures of high molecular weight polymers [6, 12, 23]. Microbial EPS are an abundant and important group of compounds that can be secreted by *Archaea*, *Bacteria*, *Fungi*, and algae [25–27]. Cultivable bacteria of the genera *Bacillus*, *Pseudomonas*, and *Klebsiella* count among the hyper-producers of EPS [28]. In addition to EPS, *Zoogloea* is producing poly-beta-

hydroxy-butyrate, nitrate reductase, urease, and gelatinase and hydrolyzing benzoate forming a gelatinous structure. Other gelatinous matrices produced by phytoplankton and associated bacteria can flocculate under stressful conditions [10].

In sludge, the EPS concentration ranges from 73 to 139 g L^{-1}. These EPSs consist of sugars, proteins, lipids, nucleic acids, glycoproteins, phospholipids, as well as minerals and metals (Ca^{2+}, Mg^{2+}, Fe^{2+}, Al^{3+}, Cr^{6+}, and Cd^{2+}), contributing to the flocs formation [1, 10, 29]. EPSs allow bacterial adhesion to external surfaces, forming a protective layer, and fill the space between prokaryotes and eukaryotes inhabiting aggregates, where a variety of microorganisms develops and produces more specific EPSs [7, 23, 24, 28, 30]. EPSs also play the role of scavenger, transport and transform, store, and facilitate the degradation of nutrients in the environment, help detoxify xenobiotics following their sorption, accumulate toxic metal ions, and retain water [6, 10, 23].

For an efficient use of nutrients by microorganisms, the optimum C/N/P ratio should be 100/5/1. The optimal production of sludge is obtained for a C/N ratio of 9 to 21; its deterioration occurs when the C/N ratio ranges between 21 and 43. Moreover, under C and N limiting conditions, the biosolids' hydrophobicity decreases and dehydration becomes difficult [6]. The size and shape of the floc vary according to the interactions between the types of particles (microorganisms, organic and inorganic particles, and ions) and their affinities. These parameters influence the growth and organization of flocs, resulting in hydrated clusters of aggregates of living and nonliving organisms, interacting with the EPS. Nitrogen limitation affects EPS production. In fact, for a high C/N rates, the microbial activity decreases with the release of ammonia [4, 6]. However, floc size increases at high C/N ratios. Sludge color may vary from white to brown, depending on the constituents and types of bacteria present, aggregated particle density, oxygen availability, and age of aggregates [24, 31]. To facilitate sludge handling, its volume is reduced by various processes including thickening, dehydration, and drying, which may reduce its management costs [10, 24].

3 Chemical Composition of the WW Sludge

3.1 Macro- and Micropollutants as Plant Nutrients

The WW sludge microbiome partly assimilates carbon, nitrogen, phosphorus, and residual nutrients; the excess quantities are found in sludge which, together with all the non-metabolizable pollutants, make out of it a matrix rich in fertilizing material (C, 32–38%; N, 2.9–5.2%; P, 1.5–2.7%) [29, 32]. This matrix contains also elements at low concentrations more or less biodegradable [28, 33], which are at the origin of the problem raised when it comes to the urban WW sludge valorization [23, 31, 34]. In fact, the fertilizing elements of the WW sludge are organic carbon in its different forms, resulting from human activities, some of which is found in the form of humic substances adsorbed on the EPS, which represent 20% of the sludge carbon

[34, 35]. Nitrogen is the second compound present at relatively high concentrations (2–8% of DM). It is found in organic form (63%: proteins, amino acids, and urea) and ionic (37%: nitrates and nitrites) [23, 24, 26, 28, 29, 36, 37]. Phosphorus is also an important fertilizer in sludge (2–8% of dry matter). It results from exogenous contributions of detergents, pesticides, agricultural fertilizers, or microorganisms' storage (polyphosphates) [35, 38]. Sludge also contains other macro-elements such as K, Ca, Mg, and Na that result from urban activities [38, 39] (Table 1).

3.2 Metal Trace Elements (MTE) and Nanomaterials

Heavy metals found in WW or its sludge are associated with various products of domestic, industrial, or agricultural use or come from road leaching. The most frequently found MTEs are Zn, Cu, Ni, Pb, Cd, Hg, and Cr [42]. The concentrations of the MTEs, which are often important, depend on the countries and the type of effluents discharged into the urban network (Table. 2). They are characterized by their persistence, their bioaccumulation, and their toxicity vis-à-vis the soil and the environment (Fig. 2). Ferreiro-Domínguez et al. showed that land spreading of sewage sludge increases copper content of plants grown in agronomic and forest soils; this effect is more pronounced in unseeded areas of forest soils [42]. Their accumulation in the soil known as "terraccumulation" is defined as the concentration of pollutants in soils from land application of contaminated biosolids generated by agricultural practices, water, and WW facilities. It occurs when the soil ecosystem becomes unable to metabolize contaminants supplied by biosolids or water [45]. This may cause toxicity effect on plant and may disturb the autochthonous microbial communities (e.g., reduction of microbial biomass or alteration of the community structure).

Due to their widespread use in commercial products, nanomaterial (NM) research continues to expand, and their impacts on the environment are documented by numerous reviews and papers, describing new methods of detection, environmental occurrence and fate, as well as toxicity. Using wetland mesocosms with aquatic plants, Colman et al. published an interesting study on the impact of silver nanoparticles on ecosystems. They investigated two diameters of nanosilver (12 and 49 nm) compared to ionic silver (Ag^+) in 19 wetland mesocosms. Over 30 days of exposure, they concluded that all three silver treatments were toxic to the aquatic plants, leading to a significant release of dissolved organic carbon and chloride following exposure. Despite widely different toxicities observed in controlled laboratory tests, toxicities in the outdoor mesocosms were very similar [36].

Table 1 Urban wastewater sludge chemical characterization

Reference	pH	Organic carbon	Total nitrogen	[a]NH_4^+	[a]NO_3^-	PO_4^{3-}	Ca	Mg	Na	K	Fe
Tabatabai and Frankeraber [40]	6.9	24.0	2.25	899	163	0.9	9.3	1.1	0.25	0.31	1.95
EPA [41]	>5.5	ND	3.00	ND	ND	1.5	4	0.4	ND	0.3	1.70
Mantovi et al. [33]	7.9	29.6	4.25	0.62	ND	1.8	ND	ND	0.40	0.71	ND

ND not determined

[a]In ppm, the rest is expressed as a percentage of dry matter

Table 2 Heavy metal concentrations in sludge of various origins (mg kg^{-1})

Country	Zn	Cu	Cr	Pb	Ni	Cd	Hg
USA (1977) [32]	1,740	850	890	300	82	19.0	3.0
USA (2002) [41]	705	511	35	65	23	2.3	1.5
France (1999) [38]	761	286	4.5	107	35	4.5	2.1
China (2018) [43]	674	204	236	26	334	0.9	0.9
Concentration limit EPA [41]	7,500	4,300	ND	840	420	85	57
Sludge limit values, France[a] [44]	60–200	20–100	30-100	70–100	15–70	0.5–1.5	0.1–1.0

ND not determined
[a]In ppm; the rest is expressed as a percentage of dry matter. Values likely to amend a soil

3.3 Organic Micropollutants

In addition to the easily quantifiable known elements found in treated WW or sludge, the most problematic source of pollution is represented by persistent and emerging micropollutants, among which are synthetic molecules such as plastics, hydrocarbons, certain drug molecules (hormones, antibiotics, analgesics, etc.) and endocrine disruptors (e.g., pesticides, metabolites, toxins, plasticizers, surfactants) [9, 21, 34, 38, 44] (Fig. 2). The European Union has compiled a list of 143,000 industrial chemicals, known as emerging, persistent, and ecotoxic chemicals, and criteria for the agricultural use of biosolids have been established [32]. Among these molecules, some are stable and recalcitrant to degradation and are characterized by a dose effect, which would impose their control to avoid the related environmental problems [7]. These contaminants were found in sludge and surface water at a concentration between 1 μg and 1 mg L^{-1} [9]. Motoyama et al. found that residues of 12 drugs were found in recycled sludge in agricultural soil [39]. Recently, a fungicide (carbendizine) was found in treated WW and soil [21], and there are interactions between different pollutants and sludge formation conditions affecting their adsorption [23]. In addition, bisphenol A and irgasan were detected in untreated sludge with removal of 60% for bisphenol A [46]. Recent studies have demonstrated that conventional WW treatment using the activated sludge process is insufficient to remove persistent micropollutants, and 32 pharmaceutical compounds were found in sludge [47]. The fate of some of these molecules in soils is still unknown [38]. In the long term, irrigation with treated WW could lead to the accumulation of organic pollutants and micropollutants in soils such as phenolic compounds, surfactants, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pharmaceutical products, and MTEs with a disastrous impact on the soil microbiota and soil physicochemical properties. The risk may then arise with their gradual accumulation in the soil over the years of sludge application or through the irrigation process. For example, phenanthrene, a model compound for polyaromatic hydrocarbons, has negative effects on several bacterial groups, reducing soil richness and

evenness [48]. Endocrine disruptors and a myriad of pharmaceutical products, antimicrobials, personal care products, lipid regulator agents, anti-inflammatory drugs, beta-blockers, cancer therapeutics, contraceptives, and other hormones are contaminants of emerging concern [22, 49]. Some of them are not degraded at all, or degraded at low level by the WW microbiota.

A very interesting recent discovery made by Susan D. Richardson et al., from the University of South Carolina shows that compounds used for medical imaging (X-ray contrast media) can react with chlorine or chloramines in drinking water treatment to form the most toxic iodinated disinfection by-products (DBPs) identified to date [50]. Even though X-ray contrast media are nontoxic to humans in their parent form and are excreted within about 24 h, these iodinated disinfection by-products are very resistant to degradation in the WWTP, such that high levels are released to rivers and streams (up to 100 ppb) and can enter drinking water sources to form these highly toxic DBPs.

In the United States, concerns grow over tainted sewage sludge spread on croplands because of the perfluoroalkyl and polyfluoroalkyl substances (PFAS) in biosolids. PFASs are extensively used in the manufacture of surfactants, lubricants, polishes, textile coatings, and fire-retarding foams. On September 12, 2019, John Flesher and Michael Casey of the Associated Press reported that in Maine, a dairy farm was forced to shut down because of high levels of PFAS in the milk, after sludge land spreading. The concern is that certain PFAS chemicals, which studies have associated with an increased risk of cancer and damage to organs such as the liver and thyroid, could be absorbed by crops grown in soils treated with polluted sludge and wind up in foods. The Food and Drug Administration agency reported finding substantial levels of the chemicals in random samples of grocery store meats, dairy products, seafood, and even off-the-shelf chocolate cake, although the study did not mention any connection to sewage waste (https://apnews.com/32c65a5b3c27468ea2cdd2ce97848825). The significant loading of PFAS to US soils further increases concern about groundwater and surface water contamination [51].

Hence, collaborative projects are needed between environmental chemists, microbiologists, and engineers to harness the potential of new screening techniques for assessing the environmental and ecological impact of micropollutants, their metabolic end products, and their derivatives on overall ecological health.

4 The Sludge Microbiome Composition: An Untapped Diversity – Potential Consequences of WW Irrigation and Sludge Spreading on Agricultural Soil

Sludge from WWTP concentrates 85 to 90% of solid and contains a large amount of prokaryotic microorganisms, which account for 95% of the microbiota. These microorganisms include functional groups that ensure carbon removal by oxidation

or fermentation; nitrogen by nitrification, denitrification, or assimilation; and phosphorus in the form of polyphosphate polymers, stored as a source of energy for the WW biome. Thus, functional properties of sludge microbiota help in avoiding eutrophication and deterioration of recipient surface waters such as rivers and streams. Within this anthropized ecosystem, prokaryotes live in tight association with eukaryotes comprising protists, fungi, metazoans, metaphytes, and viruses. They form a complex microbiome with a large part (>95–98%) still awaiting culture and characterization [52–54].

Culture-independent molecular analysis of the sludge microbiome circumvents conventional culture methods. It gives valuable information about microbiome composition, community structure, and function. Thus, high-throughput sequencing of 16S and 18S rRNA genes has depicted the high complexity of microbial populations. Our most recent studies using 16S and 18S rDNA barcoding show that at the phylum level, we found at least 40 and 15 prokaryotic (*Archaea* and *Bacteria* domains) and eukaryotic phyla, respectively (Morin et al., 2020).

4.1 Eukaryotic Components

The eukaryotic compartment of the sludge microbiome is predominantly composed of at least 15 phyla. The most predominant are *Nucletmycea*, *Holozoa*, *Amoebozoa*, *Rhizaria*, *Alveolata (Ciliophora*, *Apicomplexa)*, *Stramenopiles*, *Discoba*, *Chloroplastida*, and *Protalveolata*, representing up to 90% of the total species, or "operational taxonomic units (OTUs)." Unknown and multi-affiliation phyla are making up to 10% of the total OTUs. The remaining six minor phyla affiliate with *Apusozoa, Rhodophyta*, *Cryptia*, *Haptisia*, *Metamonada*, and *Dinoflagellata.* They altogether totalize 1% of the total OTUs (Fig. 3a). In terms of abundance, seven predominant phyla, *Holozoa*, *Nucletmycea*, *Amoebozoa*, *Rhizaria*, *Ciliophora*, *Discoba*, and *Stramenopiles*, represent >98% of the total V9 18S rDNA sequence reads (Fig. 3a, b). The remaining ten eukaryotic phyla made up only 1.20% of the total eukaryotic V9 18S rDNA sequence reads (Morin et al., 2020). These phyla comprise an abundance of non-cultivable species and lineages (60–90%).

Among novel lineages the phylum *Cryptomycota*, formerly known as LKM11 and LKM118, was found in abundance, making up to 76% of the total fungal population within a domestic WWTP. The *Cryptomycota* are currently not represented by only one cultivated species [54] (Fig. 3a, b; Morin et al., 2020). Pathogenic fungi such as *Olpidium*, *Paecilomyces*, *Aspergillus*, *Rhodotorula*, *Penicillium*, *Candida*, *Synchytrium*, *Phyllosticta*, and *Mucor* have been isolated from WWTPs and would be very dangerous to human health since treated WW in some cases is not only used for irrigation but also to produce drinking water [55]. In our study of domestic WWTP sludge, we detected 45 potential human fungal genera-containing pathogen species. *Candida* and *Pichia* were the two most important genera reported for *Ascomycota* phylum, while *Lichtheimia* and *Rhizopus* were observed for *Mucoromycota* phylum. These genera represent the most persistent

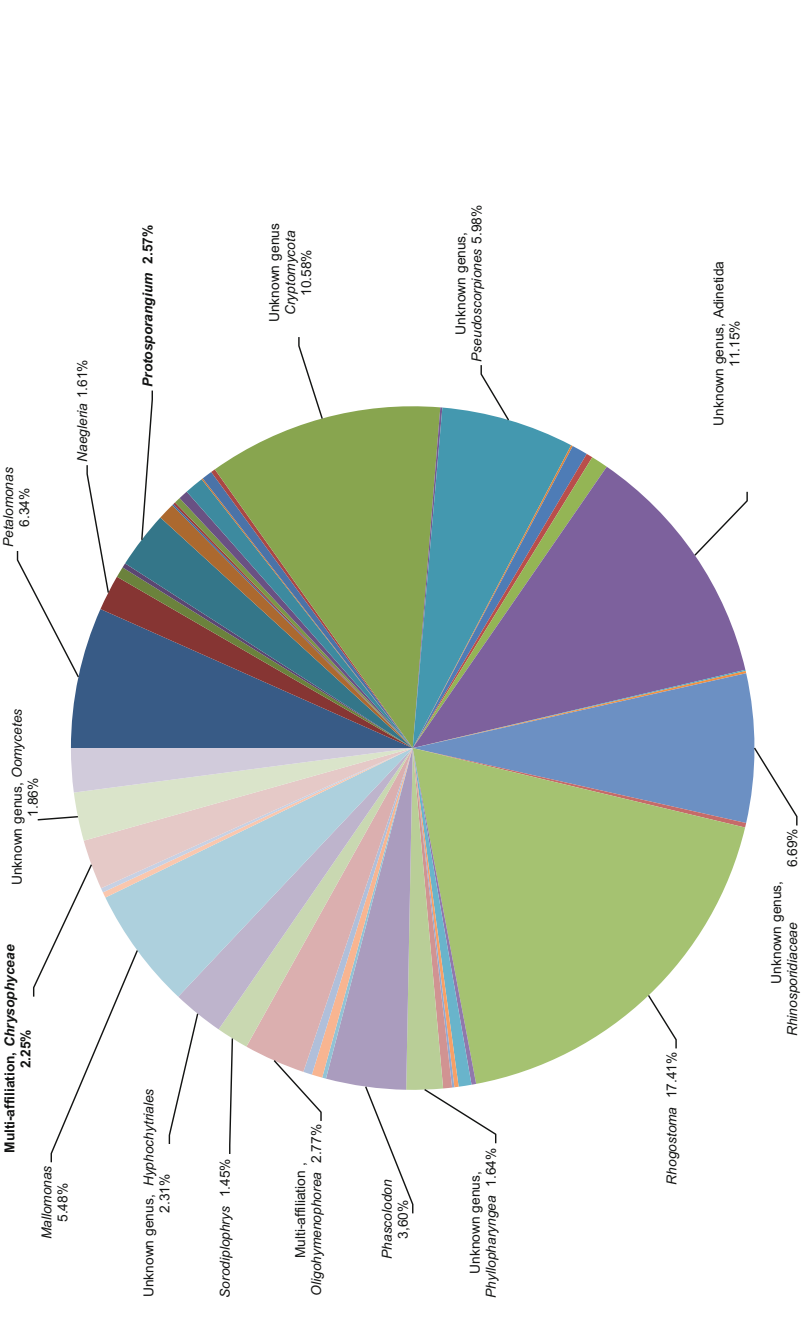

Fig. 3 (**a**) Eukaryotic diversity of domestic wastewater sludge at the genera level based on the phylogenetic analysis of the V9 region of the 18S rDNA sequence reads. Metagenomic DNA was extracted from 23 time series samples over 236 days. Only reads $\geq$5% of abundance average were included in the analyses (Morin et al., article in preparation). (**b**) Bacterial diversity of domestic wastewater sludge at the genera level based on the phylogenetic analysis of the V4 region of the 16S rDNA sequence reads. Metagenomic DNA was extracted from 23 time series samples over 236 days. Only reads $\geq$1% of average abundance average were included in the analyses (Morin et al., article in preparation)

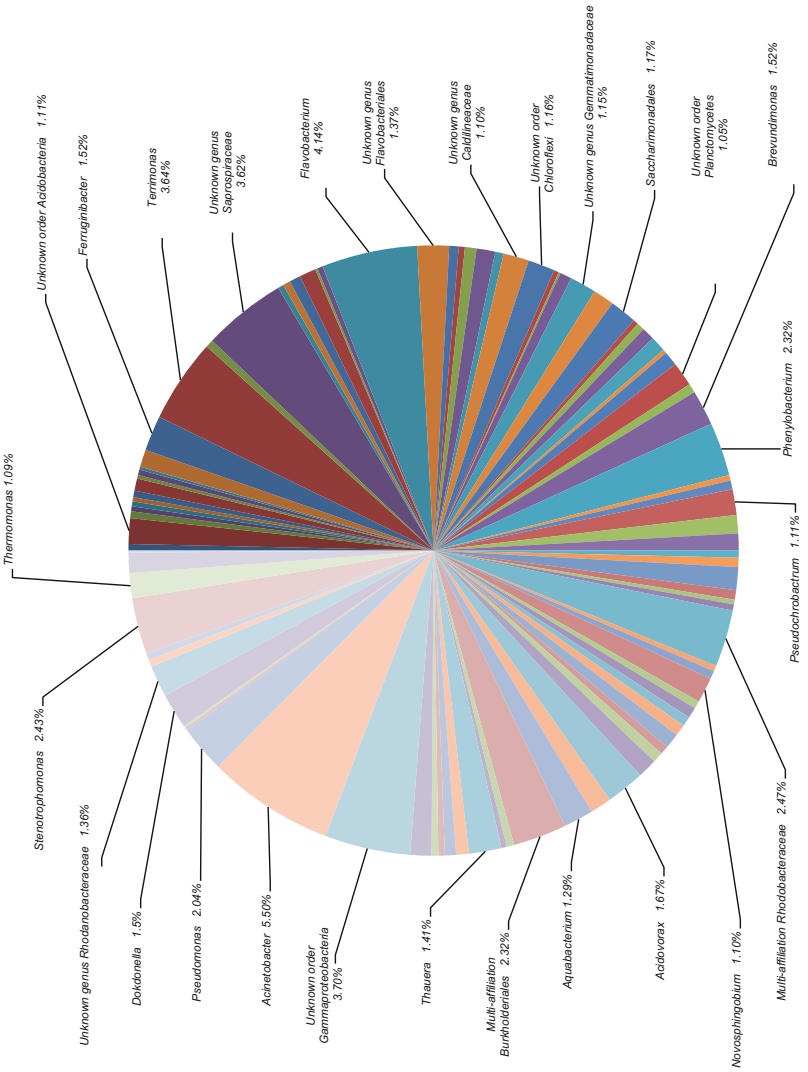

Fig. 3 (continued)

fungal pathogens within the WWTP microbiome, over the 236 sampling days. In the WWTPs, some pathogenic fungi are of a great interest, they can be a serious threat to human health and an issue of concern because fungi are not completely removed by conventional WWTP and they are not included in regulatory frameworks. Furthermore, some species may produce mycotoxins, which are toxic to humans. These pathogenic fungi may have also a significant impact on crop and plant life, affecting food security and the whole ecosystem [43].

Cysticercosis is a parasitic infection caused by larvae of *Taenia* solitary worms. In Europe and in cold or temperate regions, the species *Taenia saginata* is predominant. In Africa, we find *Taenia solium*. These worms are most commonly found in cattle and pigs respectively, and may infect humans. They may pose a serious risk of ocular or neurological damage. The problem posed by *Taenia* is that its eggs are particularly resistant and can survive the different treatments of sewage sludge [56]. This is why the persistence of helminth eggs is a criterion taken into account in the law of 1998 on the approval of sewage sludge as fertilizer. The transmission of worms is the consequence of sludge spreading on plots intended for pig, sheep, or cattle breeding. Ascariasis is a parasitic infection caused by the nematode worm *Ascaris* parasitizing the small intestine of mammals. *Ascaris lumbricoides* is found in sewage sludge, and its eggs contaminate the soil and plants after sludge spreading, resulting in the infection of any organism that consumes them [1]. *A. lumbricoides* is the largest intestinal roundworm and is the most common helminth infection of humans worldwide. Infestation can cause morbidity by compromising human nutritional status [57] or affecting cognitive processes [58] inducing tissue reactions such as granuloma to larval stages and causing intestinal obstruction, which can be fatal. This worm weakens its host by eating the ingested nutrients. Knowing that this worm is particularly virulent in the tropics, it causes problems of undernutrition in countries having trouble feeding. Thus, it decreases the hosts' immune defenses making them vulnerable to other diseases. In endemic areas, massive infections (200 to 1,000 worms) cause serious damage including intestinal obstruction that can lead to death. The worms in abundance eventually cross the intestinal wall to spread throughout the body and cause multiple problems (irritation, lesions, and edema). Hookworms *Ancylostoma duodenale* and *Necator americanus* are two nematodes parasitizing mammalian duodenum and jejunum. As with most parasitic nematode worms, they cause skin lesions, respiratory irritation and lack of nutrition with inflammation of the intestine. However, hookworms differ from other intestinal helminthiases in attacking male adults rather than children. Giardiasis is also an intestinal disease, including diarrhea, caused by *Giardia intestinalis*, a flagellated protozoan [59]. This parasite can form cysts that are particularly resistant to WW treatment. It can be transmitted to humans by non-potable water or by ingestion of contaminated food. In the case of sewage sludge, it can be transferred to livestock after soil sludge application. *Giardia* is asymptomatic most of the time. However, it can cause diarrhea from a certain concentration of parasites. These diarrheas can become serious when the protozoan is present at high quantity. A recent study by Amorós et al. [60] in raw and treated sewage sludge showed that *Cryptosporidium* oocysts and *Giardia* cysts were present in 26 of the 30 samples (86.6%) of raw sludge

samples in Spain. In treated sludge samples, oocysts have been observed in all WWTPs analyzed (25 samples) with different stabilization treatment (83.3%). This study provides evidence that oocysts are present in sewage sludge end products from WW treatment processes with the negative consequences for public health [60].

4.2 Prokaryotic Components

The predominant prokaryotic phyla are affiliated mainly with ***Hydrobacteria*** represented by *Proteobacteria*, *Bacteroidetes*, *Planctomycetes*, and *Verrucomicrobia* and ***Terrabacteria*** represented by *Chloroflexi*, *Firmicutes*, and *Actinobacteria* (Fig. 3b). *Archaea* domain is accounting for 0.02% of the total 18S rDNA reads, represented by 70% of human intestinal methanogens (Morin et al., 2020).

Prokaryotes and eukaryotes are involved in important biogeochemical processes such as carbon, nitrogen, sulfur, or phosphorus biogeochemical cycles of which a large number of microorganisms are always represented by so-called candidate species (Fig. 3b) [52]. These microbial phyla would be of great importance for the transformation of chemical elements, making them available to plants, thus contributing to soil fertility. Some of these non-cultivable microbial lineages are affiliated with obligate intracellular bacteria such as the *Alphaproteobacteria* and *Rickettsiae* [61], while others are affiliated with *Bacteroidetes* [62] or close to *Chlamydiae* [63]. Within this latter family, two new genera, *Parachlamydia* and *Neochlamydia*, have been described [62, 64].

Although only 12–15% of the 16S rRNA gene sequences extracted from the sludge microbiota are affiliated with the human gastrointestinal tract microbiota, 97% of the fecal taxa preserved in the sludge reflect the population structure of the human or animal fecal microbiota [65, 66]. Among *Proteobacteria* and *Bacteroidetes* phyla, members of the orders *Aeromonadales*, *Alteromonadales*, *Enterobacteriales*, *Legionellales*, *Pseudomonadales*, *Vibrionales*, *Xanthomonadales*, *Actinomycetales*, *Bacillales*, *Clostridiales*, *Lactobacillales*, and *Bacteroidales* are common members of the human microbiome (NIH Human Microbiome Project catalogue, http://www.hmpdacc.org/catalog/) and described as carriers of multiple determinants of antibiotic resistance [67]. Some human or animal pathogens detected in the final effluent or constituting a part of the sludge generated during WW purification process can survive the treatment process. This is also the case for several commensal or saprophytic microorganisms that can adopt a pathogenic lifestyle in inappropriate ecological niche, or whenever the host defenses are weakened or compromised. The possibility of a transfer of these microorganisms to humans, animals, and plants through inappropriately treated WW or sludge use cannot be ruled out [68].

It is well recognized that several plant pathogens, either bacteria, fungi, viruses, parasitic nematodes, or oomycetes (e.g., *Phytophthora* and *Pythium*) are waterborne [69, 70]. Phytopathogenic bacteria are present in treated WW used in irrigation

systems and include, among others, *Pseudomonas syringae*, *Ralstonia solanacearum*, *Corynebacterium flaccumfaciens*, *Erwinia* spp., and *Xanthomonas* spp. [70]. Some species of *Xanthomonas*, *Pseudomonas*, and other phytopathogenic agents such as *Herbaspirillum* and *Acidovorax* or *Staphylococcus* and *Pseudomonas* are particularly monitored because they can originate from hospitals' WW [71]. Evidence of possible risks are supported by reports involving the use of recycled freshwater in plant nurseries and greenhouses, concluding that this can be identified as an important source for the spread of plant pathogens [72]. More studies on the presence of phytopathogens in treated WW need to be done for assessing the risks posed by WW irrigation.

4.3 Viral Components of the Sludge Microbiome

In a typical WWTP, the number of viral particles varies from 10^8 ml^{-1} to 10^{10} ml^{-1}, 10–1,000 times higher than in other aquatic environments [73–75] suggesting that WWTPs can be considered as an important reservoir of viruses. Despite viruses' importance in WWTPs and their potential impact on surrounding environments after discharge, little is known about their diversity, function, and fate throughout the treatment of a typical WWTP. The few metagenomic studies revealed novel diversity and function of DNA viral communities in the influent, activated sludge, anaerobic sludge digester, and effluent of a domestic WWTP. The few molecular studies show that many viruses found in the WWTP are novel, resulting in only <5–20% of the sequence reads being phylogenetically or functionally assigned [76]. Viruses of the families *Tombusviridae*, *Geminiviridae*, and *Nanoviridae* were identified [77, 78]. The most recent studies of the sludge microbiome show an important underestimation of diversity of sewage sludge viruses infecting humans. These studies demonstrate the prevalence of respiratory viruses in sewage sludge. Herpesviruses and coronaviruses were found to be responsible for infections, one of which is latent and the other acute and can be fatal [8]. Viruses are also predicted to account for the most significant fraction of human illnesses in sewage-contaminated water under specific exposure scenarios [79].

4.4 Antibiotic-Resistant Bacteria, Genes, and Mobile Genetic Elements

WW effluents generally retain a variety of antimicrobial components, antibiotic-resistant bacteria (ARBs), antibiotic resistance genes (ARGs), as well as mobile genetic elements even after treatment [23, 30, 80]. In soils, through WW irrigation or sludge fertilization, antimicrobial compounds can disrupt the structure and activity of telluric microbial communities [22, 48, 81]. In fact, disturbances of the nitrogen

cycle, methanogenesis, and sulfate reduction have been reported [81]. It was also observed that degradation products of amoxicillin contaminate groundwater in an agricultural field irrigated with WW [82].

With regard to the ARGs, studies by Wang et al. demonstrated that the WWTP could not effectively remove super antibiotic resistance genes (SARGs) with high amount being discharged into the Yangtze River. They were transported into the drinking water treatment plant (DWTP), and the persistent SARGs in the effluent would probably be transferred into human, thus imposing great threats on public health [83]. About 40% of the erythromycin resistance genes and 80% of the tetracycline resistance genes could not be eliminated from the WW even after chlorination [84, 85]. In a recent study, using metagenomics approaches Chu et al. have also shown a large abundance of ARGs belonging to the aminoglycoside and phenicols groups, including chloramphenicol and its florfenicol derivatives and thiamphenicol, in river water that has received effluents from the WWTP [86]. Indeed, they tracked genes specific to antibiotic resistance and mobile genetic elements and their associated organisms, from WWTPs to lake sediments, based on two different WWTPs microbiomes with different treatment processes. Thus, a thorough risk assessment of antibiotics and ARGs within the sewage sludge and treated water is required [87] (Fig. 4). WW and sludge antibiotic-resistant populations can proliferate in soil or plants, behaving as invasive species; some antibiotic resistance genes may then be horizontally transferred by conjugation, transduction, or transformation, from WW bacteria to soil or plant rhizo- or phyllosphere.

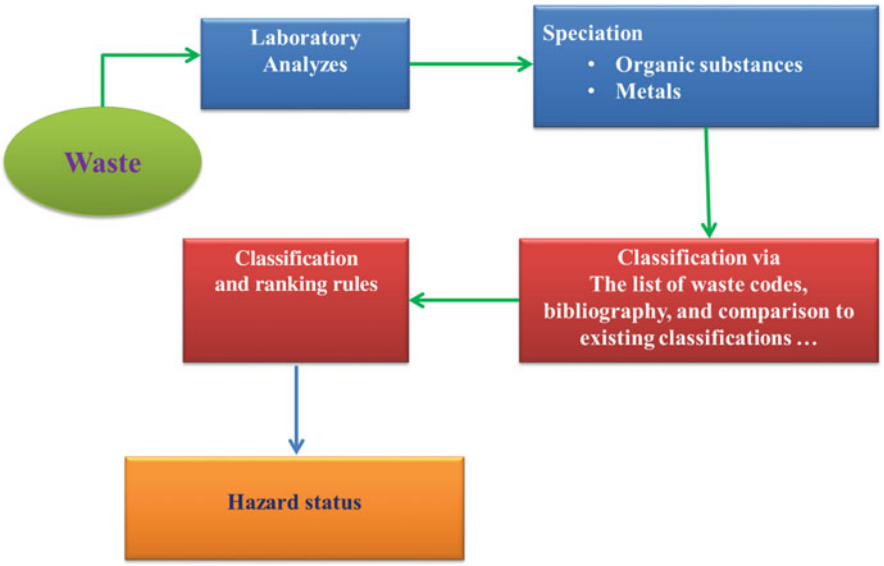

Fig. 4 Diagram of the principle of characterization of waste hazard according to the National Institute for the Industrial Environment and Risks [88]

5 Sewage Sludge Management

Searches of the International Scientific Indexing database (ISI) for articles dealing with sludge and biosolids yielded 4,030 articles published between 1976 and 2008 and 6,410 between 2009 and 2018. The articles dealing with application on agricultural land were concerned with the emission of gases (CH_4, NO_2, NH_3), with 15% of ammonia (NH_3) [31, 89]. The evaluation of sludge life cycle highlights their richness in nitrogen and phosphorus explaining their valorization as fertilizer in several countries. Within EU countries, there are three main sludge disposal possibilities. Sludge valorisation as fertilizer remains a priority, and in France, 60% of WW sludge is used as fertilizer in agricultural fields [89], even though different existing methods for sludge disposal are based on landfilling, composting and incineration are practiced [90, 91] (Fig. 4). Sludge is used as fertilizer for agriculture in Portugal, Ireland, the United Kingdom, Spain, Norway, and Albania. However, 2/3 of sludge was composted in Estonia (2013 data) and Hungary (2015 data). Incineration is the main mode of sludge disposal in the Netherlands, Germany, Slovenia, and Austria, as well as Switzerland. Controlled landfill is the main and only mode of treatment used in Malta and Serbia, as well as in Bosnia and Herzegovina. Other sludge treatment routes are applicable such as composting, and especially lombricomposting (Fig. 5) [34, 43]. Methane production and biomineralization represent very interesting alternative routes over land spreading.

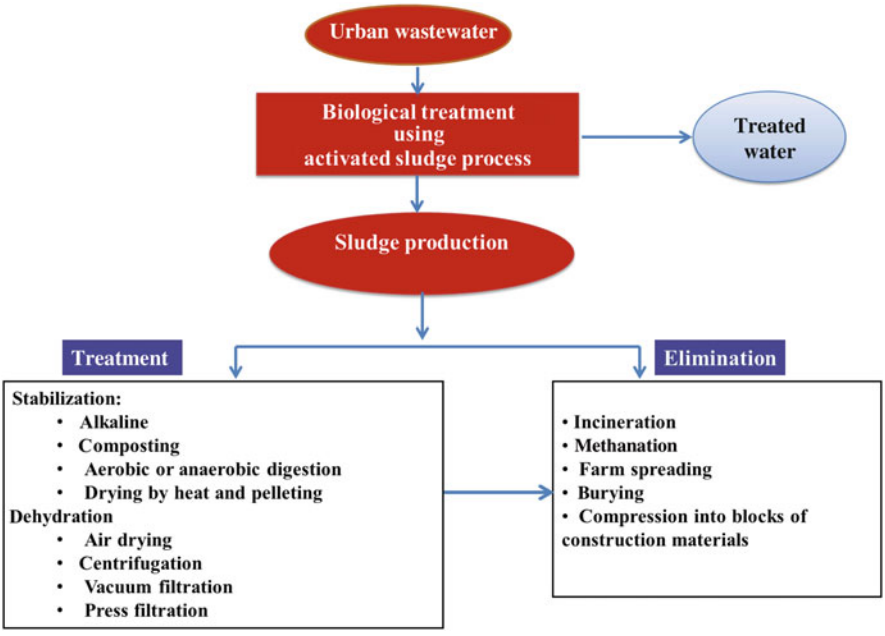

Fig. 5 Sewage treatment and sludge disposal pathways

Methane production from anaerobic digestion (AD) of sludge is commonly practiced, providing a residual carbon content of 35%. AD process is naturally present in many ecosystems such as the digestive tract of insects (e.g., termite) and mammalians (e.g., cows, pigs, sheep, gazelle) and humans as well as in natural and cultivated ecosystems like wetlands, marine sediments, and rice fields where it is actively involved in biogeochemical cycles of matter. Indeed, It has been applied since the end of the nineteenth century for the treatment of household WWs in septic tanks of slurries in digesters. AD not only enables to reduce and stabilize the volume of sludge to be disposed, but it is also a way to recover energy from WW process. This leads at least to reduce the energy demand for the aeration on the water line to reach energy self-sufficiency for WW treatment plants. Because of these different aspects, there is no doubt that AD is a mature and exciting process that gathers many advantages that is worth to optimize and to promote. As for incineration, it produces CO_2 and energy (12–20 MJ/kg of sludge's calorific value) [23]. Nurrokhmah et al. showed that direct application to agricultural soil presents the lowest cost compared to the other options, whereas co-incineration had the highest cost [92].

Biomineralization represents a novel route of sludge valorization based on microbial-induced calcite precipitation. Biomineralization is a physiological process that allows living organisms to develop a mineral structure, called the biomineral, which is distinguished from its purely mineral equivalent by the presence of organic molecules that give it specific properties such as better resistance to fracture. This phenomenon occurs naturally and chemically but over several thousand years to give rise to rocks such as sandstone or stromatolites. Bio-calcification occurs when microorganism metabolic activities lead to the precipitation of calcium carbonate ($CaCO_3$). This process has been utilized in broad spectrum of applications using different bacterial communities, such as calcium removal in WW, carbon sequestration, soil stabilization, and concrete durability improvement as well as cement manufacturing alternative fuel [93, 94]. Bacterial precipitation of calcium carbonate can be accomplished quickly (day-hours) by the hydrolysis of urea via a biological enzyme catalyst, the urease. This leads to the alkalization of the microenvironment, allowing the precipitation of carbonate ions in the presence of calcium ions. The alkalineophilic bacterium *Sporosarcina pasteurii*, with high intracellular concentrations of urease, is often used to catalyze the biomineralization process. This process is carried out without energy supply and releases ammonium, a source of valuable nitrogen fertilizer. Biomineralization may be an efficient alternative for recycling WW sludge. Calcium carbonate precipitation by sludge microorganisms would be used in applications for environmental protection, material technology, and other applications. Recently a strain called *Microbacterium* sp. GM-1, isolated from active sludge, was investigated for its ability to produce urease and induce calcium carbonate precipitation in a metabolic process. Xu et al. evidenced that *Microbacterium* sp. GM-1 can biologically induce calcification, and they suggested that this strain may play a potential role in the synthesis of new biominerals and in bioremediation or biorecovery [95].

Due to the sludge biochemical characteristics, it has been suggested that biomineralization could potentially provide an eco-friendly cost-effective alternative for

sludge treatment through its conversion into construction materials. Recently, in Korea, a study was conducted for evaluating CO_2 fixation through biomineralization of sludge underneath landfill cover soil [96]. It was proven that sludge could potentially serve as an effective medium for biomineralization and can naturally and efficiently mitigate the CO_2 emitted from landfills.

The challenges for the use of sludge by-products in France and Europe are to build the transition strategies of their territories through the establishment of a circular economy. The recycling of by-products from two sectors: water treatment (sand, biomass, and leachate) and building site waste brick aggregates, concrete and mortar to use as a substitute of the other materials, may limit pressure on raw materials.

6 Sewage Sludge: An Ambiguous Status

Based on legal texts, sewage sludge has a waste status according to the Article R211–28, within the meaning of the legislative provisions of the Environment Code 23 [89]. Sludge land application is specified in the scope of Articles L. 214-1 to L. 214-6 of the Public Health Code with regard to hygiene rules. These regulations define the technical provisions for land application (R211-38). If the treatment plant polluting flow is greater than 120 kg day^{-1} in terms of chemical oxygen demand, special adjustments are made (R211-39); the spreading period and the applied quantities are adapted according to the soil type (R211-40) and the climatic conditions (R211-41). The spreading technical rules are also legislated (R211-43). The sludge must be subject to a physical, biological, chemical, or thermal treatment, in order to reduce their fermentable capacity and the health risks associated with their use. The authority designated in the code for public security (CSP) article L. 1313-5 shall issue the marketing authorization and the permit for the introduction of a fertilizing material, or a fertilizer adjuvant. The authorization is issued following an evaluation which must reveal the absence of any harmful effect on human and animal health and the environment and its effectiveness with regard to plants and plant products or soils (Fig. 2) [17, 90, 97].

The sludge is also considered as a product according to the rural code (CR law no 79-595 of the 13/07/79 relating to the organization of the control of the fertilizing materials and the supports of culture, articles L. 255-1 to 255 -11 CR). According to the French Decree of 21/12/98, Article L 1323-1, the CSP entrusts ANSES (The French Agency for Food, Environmental and Occupational Health & Safety) with their evaluation and their approval as fertilizing materials and culture media; these products' safety through regular testing is required and specified by legislation [43].

In addition, Article L255-7 modified by the ordinance no 2015-615 of June 4, 2015 – art.1 defines fertilizers as "products intended to ensure or improve plant nutrition or the soil physical, chemical and biological properties." These products include amendments intended to modify or improve the properties of soils or materials which, when applied to the soil or around plants, stimulate natural nutrition

processes. Their purpose is to facilitate or regulate the nutrients absorption by plants or improve their resistance to abiotic stress.

The apprehension of the legal definitions of the terms waste and products applied to WWTPs sludge as fertilizing materials brings out a harmony and the coherence between these terms for the use of sludge as fertilizer for agricultural applications after their stabilization, as required and legislated. Nevertheless, before being considered as a recoverable waste that can be integrated into a circular economy process, sludge should be safe for human health and without any harm to the environment, creating hence no risk for surface water or groundwater, neither air nor soil or flora and fauna (L125-1) [12]. Therefore, an assessment of the hazardous properties should be made taking into account the complex composition of the sludge [1].

Considering the sludge formation process, its composition closely related to that of the initially treated effluent, and the nature of its pollutant load, various types of compounds with negative impacts on health and soil could be present (Fig. 2). The persistent substances concentration effects and their mixing may reinforce the danger of the product resulting from the WW treatment. The key point of ethical issues about sewage sludge valorization is related to the nature of the product emanating from a depollution process to become a fertilizer with added agricultural value. **In fact, the sludge use will depend first on its legal status:** Is it a by-product of effluent treatment used as an input into a second activity that is agriculture according to the circular economy principles? Should we first ensure the safety of this by-product before considering its recycling in a circular economy?

A comparison of recent data available on the dangerousness of persistent compounds that can be found in sludge is required with the implementation of direct tests and methods of specific analysis and calculation considering the different hazardous properties to be evaluated [1].

Taking into account the limitations of the biological treatment of certain polluting substances such as endocrine disruptors and metalloids, as well as the risk of cultivable and non-cultivable pathogenic microorganisms' presence [91, 98] for humans, animals, and the environment, could we continue to exploit agricultural sludge without concern? In addition, without being exempted of toxic compounds or pathogens whose quantification should be possible, mustn't we take a strong decision regarding their safe recycling?.

As it is known, a standard is a viable tool that responds to a specific problem. Should it be required to revise the standard specific to sludge reuse in agriculture (NF U 44-095) [99] and update its specifications to meet a crucial need for safety and of the environment protection?

In France, most of the produced WW sludge is used in agricultural farming (47%), particularly in cereal crops (3% of the agricultural area). WW sludge is also mixed with green waste and composted (26%); of the ten million tons of sewage sludge produced each year in France, three million are now valued this way. The compost is then sold to farmers with the objective to reduce the use of chemical fertilizers, although this practice is still limited to 1% of the useful agricultural area. However, **the "Waste Directive" adopted in 2018** by the European Union, Directive (EU) 2018/850, requires Member States to reduce significantly waste disposal

by landfilling to ensure that economically valuable waste materials are recovered through proper waste management. Use of landfills should remain exceptional rather than the common way. Furthermore, the Member States will take the necessary measures to ensure that by year 2035, the amount of municipal waste disposed of in landfills is reduced to 10% or less of the total amount of municipal waste generated. Although it undergoes a process of stabilization of the organic matter, the compost obtained is not free from any toxic risk, since the persistent molecules as well as pathogens including emerging ones and helminth eggs are not completely eliminated. In addition, composting sludge also concentrates heavy metals. The obtained compost must be then homologated according to NF U 44-095 standard, which sets the concentration of organic matter and the threshold values of a few MTEs and conventional pathogens as well, without worrying about endocrine disruptors or pathogenic microorganisms recently identified in such composts. **Nevertheless, the use of these types of compost obtained from WW sewage sludge co-composting remains unauthorized in organic farming**.

It is becoming necessary to set up a balance between the utilitarian position that favors beneficence such as the agronomic value of sludge, a short-term economic solution that is not viable in the long term and might be of terrible consequences, and the principle of prevention that promotes non-maleficence by cumulative pollution for a sustainable solution. The invention of novel water and sludge treatment processes as well as very advanced and more sophisticated analytic tools is necessary for favoring one or the other solution and helps political decision-making.

7 Sewage Sludge: Questions at the Crossroads of Ethics and the Economy

Economic growth often relies on depleting natural resources that are either consumed or destroyed. If current trends persist, the tomorrow world will be even more crowded, more polluted, more ecologically unstable, and more exposed to disturbances than it is today. It is in this context that the notion of "sustainable development" or "non-destructive development" has been coined. In its 1987 Report entitled Our Common Future, the World Commission on Environment and Development defined sustainable development as "development that meets the needs of the present without compromising the ability of future generations to meet their own needs." The central idea of sustainable development is that the needs of present and future generations must be taken into account, and many international instruments affirm our responsibility towards future generations. An example is the Rio Declaration on Environment and Development adopted in 1992. The ethical foundations of this principle are set out in the Declaration on the Responsibilities of the present generations towards future generations, adopted by UNESCO in 1997. Article 4 of this Declaration proclaims, "The present generations have the responsibility to bequeath to the future generations Earth that is not irreparably damaged by human

activity." Finally, we need to understand that we are part of nature; our prosperity depends on that of nature and the wise exploitation of the ecosystems services they may offer. We have a duty to preserve and protect the integrity of the ecosystem components and their overall biodiversity. Each generation should give the next generation equal opportunity to lead a happy life and therefore leave them a healthy planet.

Ethics in general has little influence as a formal decision support tool relative to law and economics. This is because ethicists have no standard classification system; the acceptability of an act depends on its effect; the basis of utilitarian ethics and utilitarian interest may extend well beyond economic welfare. People may be willing to give up on economic resources out of sympathy to avoid repugnant experiences in accordance with the deontological stance, which advocate human obligation to protect the land and ecosystems.

Considerations of consequences are almost inevitable; the decision to sacrifice the soil for sewage sludge disposal must be based on the determination that consequences of disposing it in other ecosystems will be more severe or other alternative solutions are totally depleted. Increasing stakeholder influence in the business point of view of water and sludge management operators, farmers, loggers, and ranchers, in general, tends to accentuate ecological health problems and to diminish ecological issues. Scientists should participate in stakeholders-informed decision processes by clearly presenting the results of their research and assessments. They must learn to work with economists, lawyers, risk managers, and policy analysts independently of human emotion.

8 Concluding Remarks and Future Recommendations

The WW irrigation or sludge application impact on soil microbial communities and soil properties depends on the direct effects of the exogenous microbiota, WW physicochemical characteristics, and biosolids pollutants contents. These factors may be responsible for the elimination of indigenous soil microorganisms through competition or parasitism, or through modification of the soil physicochemical properties, inducing disturbances of the indigenous soil microbial and mesofauna community. These impacts are rarely characterized if not characterized at all, constituting profound gaps in our knowledge. One of the challenges for future research programs is to understand if and how the two parameters will affect the soil micro- and macrobiome, which represent the backbone of soil fertility and productivity, but also the capacity of the exogenous WW organisms to survive in the soil and constitute a health risk to livestock and humans.

Currently culture-independent methods have a big advantage over traditional cultivation of microorganisms, resulting in an improved representative picture of a community, including the "viable but non-cultivable" fraction. Multi-omics approaches (e.g., metagenomics, metatranscriptomics, and metaproteomics) combined to a myriad of molecular ecology techniques (e.g., isotope probing,

fluorescence in situ hybridization, flow cytometry, NanoSIMS, etc.) should provide valuable information on the sludge and soil microbial communities' structure, dynamics, and metabolic activities. These tools combined to statistical methods may help in establishing correlations of these parameters to the effectiveness of any soil amendment with sludge or WW irrigation. This way we will be able to effectively track not only the fate of pathogenic bacteria in irrigated soil or to which sludge is applied, in terms of survival, leakage, and spread to both groundwater and surrounding crops, but also the modification of overall soil microbial activity. Novel chemical analytical methods to detect and quantify organic and mineral micropollutants such as gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry, combined to different ionization modes, total organic halogen (TOX) analysis, and ion chromatography as well as high-resolution MS, represent effective tools for measuring trace levels of compounds in complex environmental matrices. These tools, combined, may eventually lead to the development of reliable qualitative and quantitative microbiological and chemical risk assessments that are invaluable in the prediction of the potential risks of WW use and application of sewage sludge [100].

Scientists must move from the reductionism analyses to a more holistic view of nature to give a realistic view to the public and policy makers in order to help them to take the right decisions. Governments and environmental agencies should update their technical standards making use of the above methods for assessing overall ecological health risks and should further study whether treated sewage sludge causes health problems for farmers who apply it to their land and for residents who live nearby. These studies nowadays are affordable through the application of the above novel molecular analytical approaches and those to come.

The destruction of biodiversity can be very rapid, on a human scale; however, the resilience goes through processes that are often very long on an ecological scale and depends on the intrinsic properties of an ecosystem and damage amplitude. The recovery is usually long and incomplete. Avoiding ecosystems destruction is safer and less expensive than their rebuilding.

Finally, because of improved understanding of the functions of the complex systems that are human cells and organs, the practice of medicine had already begun to move from reactive treatment of patients' symptoms to proactive and personalized care, and so we advocate for our environment!

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Wastewater Reuse in Agriculture: Effects on Soil-Plant System Properties

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Contents

Abstract The use of non-conventional water resources can help to mitigate water stress and can support the agricultural sector. Treated municipal wastewater is one of the most readily available alternative water resources, and its use in agriculture has been adopted to reduce fresh water usage in several countries, under their respective water quality regulations. This chapter reviews the results of past and current research on the reuse of treated wastewater (municipal and agro-industrial) for irrigation and the corresponding effects on soil and plant systems. Particular attention has been given to research efforts highlighting the effects of chemical-physical

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wastewater characteristics (e.g. nitrogen, phosphorus, potassium, sodium, and heavy metals) and the corresponding microbiological indicators (e.g. *Escherichia coli* and *Salmonella*) on irrigated crops and soils. The selection of irrigation methods is another topic discussed in this chapter. Drip and subsurface irrigation methods are considered the more suitable irrigation techniques to be used with treated wastewater; they minimise toxicity hazards for plants, reduce the contamination of edible crop products, and mitigate human health risks by minimising direct contact between wastewater and plant.

Keywords Agro-industrial wastewater, Crop irrigation, Microbiological risks, Municipal wastewater, Wastewater reuse

1 Introduction

Wastewater reuse has potential benefits for agriculture and water resources management but can also determine substantial risks to public health. Moreover, chemical risks for plant and environment could occur due to soil and groundwater pollution. Indeed, the main problems related to wastewater reuse can be linked to the possible environmental dispersion of macro- and micronutrients, soil and plant accumulation of heavy metals and the contamination due to microbial pathogens.

Once crops are exposed to chemicals, the potential uptake and accumulation in the edible parts (fruits and vegetables) need to be controlled in order to assess their introduction into the food chain.

Water quality criteria and guidelines for effluent reuse in irrigation have been implemented and can act as protecting measures to farmers health as well as to public health; moreover, the quality criteria can prevent problems such as soil salinity and toxicity and other phenomena that can generate issues for soil and crop production.

This chapter does not address the effects of the emerging pollutants (i.e. contaminants of emerging concern, CECs) detected in wastewater sources on the soil-plant system. This research topic is reported in detail in other chapters.

1.1 Legislative Framework

The use of reclaimed wastewater for crop irrigation could play a strategic role in mitigating the problems of decreasing water availability for the agricultural sector and competition with civil and industrial water uses. The use of reclaimed wastewater in agriculture may also increase the environmental sustainability of crop production. However, reuse of wastewater in this sector is constrained by relevant

legislative frameworks, which can in turn represent an important driving force in its adoption.

In 2006, the World Health Organization (WHO) released the guidelines for the safe use of wastewater in order to improve its use in agriculture and provide clear guidance for local decision-makers. The purpose of the WHO's guidelines was to support the definition of specific government regulations related to wastewater use and management, in consideration of each country governance [1].

In 2012, based on global data, the United States Environmental Protection Agency (US-EPA) completed the "Guidelines for Wastewater Reuse" that includes an updated overview of water reuse regulations adopted in the USA, current advancements in wastewater treatment technology, international water reuse practices, and other factors supporting the safe and sustainable expansion of wastewater reuse.

The treated wastewater reuse guidelines mentioned above (WHO, FAO, and US-EPA) constitute the basis for the formulation of local regulations in other countries [2].

In the European Union (EU), there currently are no guidelines for wastewater reuse, but according to UE Directives (91/271/EEC, 2000/60/EC) and other international guidelines, several Member States have produced legislative regulations for water reuse applications. Standards differ among and within the Member States, in response to different socio-economic conditions at the regional and local levels.

The Spanish legislation (RD 1620/2007 – *The legal framework for the reuse of treated wastewater*) includes the following different uses of reclaimed water: urban, agricultural, industrial, recreational and environmental. As for the Portuguese guidelines (NP 4434 2005 – *Reuse of reclaimed urban water for irrigation*), they only refer to urban areas irrigation, and the main applications are for agricultural and landscape purpose (e.g. golf courses irrigation).

The French standards on wastewater reuse (JORF no. 0153, 4 July 2014) define water reuse for the irrigation of agricultural lands and green areas and exclude industrial and urban uses and aquifer recharging, while Cypriot regulation (Law 106 (I) 2002 – *Water and Soil pollution control and associated regulations*) does not allow any industrial or urban use of reclaimed water (Water Reuse in Europe, UE 2014).

The Cyprus, Greece and Spain wastewater guidelines include also aquifer recharging with reclaimed water by percolation or direct injection, with the aim to reduce the depletion of groundwater and to mitigate the impacts of saline intrusion in coastal zones.

In Italy, the agricultural reuse of municipal and agro-industrial reclaimed wastewater is regulated by Legislative Decree no. 152/2006 of the Ministry for the Environment, which presents restrictive limits for microbiological parameters (e.g. *Escherichia coli*) [6, 7], but does not define different limits according to the risk associated with different destinations for reuse (e.g. irrigation of food or no-food crops).

To maximise the benefits and minimise the risks related to the use of treated wastewater, uniform legislative frameworks should be adopted [3]. To this regard, a

new proposal for European regulation of minimum requirements for water reuse [4, 5] is currently pending approval by the European Parliament and the European Council. This new proposal defines, for the first time, the minimum water quality acceptable for reclaimed water destined for crop irrigation in European Countries.

1.2 Characteristics of the Municipal and Agro-Industrial Wastewaters

Wastewater effluents can originate as by-products from the civil, industrial, and agricultural sectors. Following purification, treated wastewater represents an important and readily available water source to meet the increasing demands of crop irrigation, particularly in water-scarce countries. Indeed, wastewater recycling in agriculture has gained importance as a component of the agricultural water supply in some regions (e.g. Mediterranean area) [8].

Wastewater reuse provides significant amounts of irrigation water and helps reduce the environmental impacts related to the discharge of municipal and/or agro-industrial effluents into water bodies [8, 9]. Municipal and agro-industrial wastewater contains approximately 0.1% solid substances, represented by organic and inorganic solids and microorganisms [10]. The chemical-physical characteristics of the wastewater effluent depend on its origin and vary with climate, social and economic situation and with the habits of the population of origin.

Organic substances can include carbohydrates, lignin, fats, proteins and their decomposition products, as well as various organic chemicals of natural and synthetic origin derived from industrial processes [11].

Inorganic substances include potentially hazardous compounds and heavy metals, which may be present in wastewater at phytotoxic levels and cause health risks [11, 12]. In particular, municipal wastewater can contain wastes from domestic, small-scale craft and livestock activities.

The quality of agro-industrial wastewater is closely related to the type of vegetable products and to the processing systems adopted; such wastewater typically contains organic substances that are suspended and partly dissolved (sugars, proteins, fats, and residues of plant and animal products).

Agro-industrial wastewaters can contain heavy metals, although the concentrations are unlikely to reach levels dangerous for crops and consumers [13]. Health risks associated with the use of wastewater for crop irrigation are primarily due to microbial pathogens (bacteria, viruses, and protozoa) [14, 15]. To mitigate hazards and damage to human and environment health, wastewater should be adequately treated before use in irrigation.

The positive or negative effects of treated wastewater application on the soil and on plants are primarily dependent upon the quality and quantity of the organic and mineral chemical substances (in particular plant nutrients, such as N, P, K, heavy metals, and salts) in the solution.

2 Main Advantages and Risks of Treated Wastewater Reuse in Agriculture

2.1 Supply of Mineral Nutrients for Crop Growth

The quantity of crop nutrients supplied to the soil by wastewater (municipal or agro-industrial) in irrigation must be carefully considered. The concentrations of the primary mineral nutrients necessary for plant growth (such as N, P and K) in municipal and agro-industrial wastewater vary significantly according to the quality of the wastewater. Generally, nitrogen concentrations vary from 20 to 35 mg L^{-1}, phosphorus concentrations from 3 to 10 mg L^{-1} [10, 16–19], and potassium concentrations from 10 to 25 mg L^{-1} [18, 20].

Wastewater effluents can also contain high levels of micro-nutrients (e.g., boron, iron, copper, zinc, manganese, and molybdenum), which are essential for the growth and development of crops. The effects of macro- and micronutrients supplied with wastewaters can change in relation to the crop cycle (i.e., vegetable annual crop vs tree crops) and to different intake rate of nutrients.

As for vegetable crops, in a study carried out on a succession of processing tomato (*Lycopersicon esculentum* Mill.) and broccoli (*Brassica oleracea* L. var. *italica*) crops, secondary and tertiary treated agro-industrial wastewaters were compared with groundwater irrigation. The findings indicated that treated agro-industrial effluents supplied greater amounts of mineral nutrients ($N\text{-}NH_4$, $N\text{-}NO_3$, and K^+) to the crops [20]. The authors suggested that the use of treated agro-industrial effluents as irrigation water could reduce the need for supplementary mineral compounds through chemical fertilisation.

Other research has shown that treated wastewater irrigation of vegetable crops (lettuce) could successfully increase the availability of irrigation water and increase the concentration of some soil nutrients (K, Ca, H, Al, and S) [21].

In a study of the effects of secondary and tertiary treated wastewater irrigation on globe artichoke crop performance, Gatta et al. [19] reported that the total inorganic nitrogen (i.e. $N\text{-}NH_4$, $N\text{-}NO_3$) supplied to the crops through treated irrigation water was on average 95 kg ha^{-1} and 66 kg ha^{-1} for the secondary and tertiary wastewaters, respectively, representing approximately 25% to 17% of the nitrogen requirements of the artichoke crop.

As for tree crops, Vivaldi et al. [22] reported the effects of irrigation by treated wastewater and the deficit irrigation strategy on almond trees (*Prunus dulcis* L.). The results of this study showed that the nutritional contribution of treated wastewater to irrigated soil was 35.8 kg ha^{-1} of NO_3^-, 4.41 kg ha^{-1} of PO_4^{3-}, and 149.9 kg ha^{-1} of K^+. Other similar studies on tree crops irrigated by reclaimed water have reported that high concentrations of macro- and meso-nutrients supplied to soil through wastewater could facilitate a significant reduction in fertiliser application [23–25].

The supply of mineral nutrients by wastewater application to cultivated species represents a significant agronomic value of this water resource. However, this benefit must be assessed with particular attention to the possibility of plant nutritional

imbalances and phytotoxicity [26]. Each cultivated species is characterised by specific nutrient requirements depending on the phenological phase. Excesses or deficiencies in some mineral elements can significantly affect crop production, both in quantitative and qualitative terms [27].

2.2 *Heavy Metal Accumulation in Soil and Crops*

Another possible outcome of wastewater reuse for cultivated species is the accumulation of heavy metals in the irrigated soil. The presence of heavy metals in the soil at very low concentrations is necessary for the growth plants, but at high concentrations, they can be toxic and harmful [12, 28]. Although the heavy metal content in wastewater used for irrigation is subject to legal limits, continuous use of wastewater can lead to their buildup in the soil [29]; this can cause stress to plants due to interference with the metabolic activities and physiological functions in the plants [30].

Excessive levels of metals can also degrade soil quality, reduce marketable crop yields, and reduce the quality of marketable agricultural products; these effects can also pose significant hazards to the health of humans and the surrounding ecosystem [12, 31].

The leaves and vegetative tissues of cultivated plants tend to accumulate higher amounts of heavy metals than the fruits [32]. Studies have shown that heavy metals taken up through plant roots can be transported to the shoots through the xylem vessels [33] but have poor mobility in the phloem [34]. Crop storage organs (primarily fruits and seeds) are characterised by low transpiration rates and do not accumulate heavy metals because they are largely phloem-loaded. Harmful effects on human health from soil and vegetables contaminated with heavy metals have, however, been widely reported [12, 35].

Heavy metal activity in the soil is highly influenced by chemical and physical soil characteristics (pH, texture, and organic matter content) and the effect of heavy metals on plants varies by crop. It is therefore challenging to set precise limits and thresholds of tolerance and/or hazard deriving from the presence of heavy metals.

The risk to human health related to the uptake of heavy metals by food crops depends more on the increase in their available fraction than on their total concentration in the soil [36]; moreover, it is dependent on the heavy metal speciation and solubility and on the crops cultivated in contaminated soil [37].

High contents of heavy metals are normally found in industrial wastewater, whereas the concentrations of these metals in domestic wastewater are generally low due to the settling of solids during treatment [29, 38].

Generally speaking, the use of treated municipal wastewater in agriculture may result in heavy metal accumulation in the surface layers of the soil [1, 39, 40] following very long periods (decades) of application, after which concentrations that can cause adverse effects on crop growth are possible [41–43]. However, regardless of the heavy metal content of the wastewater, the metals are only taken

up by the crop at certain concentration thresholds, and uptake can only occur if the metals are in the mobile phase. Additionally, the concentration of heavy metals in the soil solution is influenced by soil characteristics.

Heavy metals are less available at soil pH over 6.5, due to precipitation phenomena and the presence of higher amounts of organic substances. On the contrary, at lower pH values, heavy metals become mobile in the soil and can be absorbed by crops [44]. Not all heavy metals are easily absorbed by plants; lead, chromium, and mercury, for example, are bound by soil particles and very slowly absorbed by crops, even when accumulated in the soil. Copper, boron, and zinc are more easily absorbed by plants, sometime reaching levels of accumulation ten times higher in a plant than in its originating soil [45]. In this regard, cadmium and nickel represent the highest risk to human health. The impact of heavy metals on crops is complex, because antagonistic reactions can occur the influence their absorption [46].

Table 1 shows the heavy metal concentrations in wastewater-irrigated soils reported by several authors, considering the irrigation length period (short and long term), type of wastewater, and the effects on soil and plant. The concentration of some heavy metals exceeds the international threshold values, especially in long-term experimental trials, with consequently accumulation in plants.

Table 2 shows a classification of heavy metals added to the soil with wastewater when used for crop irrigation, according to risk characteristics and classes relative to their effect on plant nutrition and human health.

2.3 Microbiological Risks

One of the primary obstacles to the widespread use of reclaimed wastewater for irrigation of agricultural crops is the possible persistence of pathogenic microorganisms through treatments and their potential contamination of vegetable crops, causing outbreaks of foodborne illness. The risk of biological contamination is primarily related to the spread of bacteria, viruses, helminths, and protozoa that are harmful to humans through the soil environment and onto crops which are then ingested.

Bacterial pathogens like *Salmonella* spp., *Shigella* spp. enterohaemorrhagic *E. coli* serotypes, and *Vibrio cholerae* are of major concern for public health systems worldwide. The helminths *Ascaris* and *Tenia* spp. and the protozoans intestinal *Giardia* and *Crysptospridium* are also of public health concern. The waterborne viruses HAV, HEV, rotavirus, and adenovirus are reported to have the greatest risk of transmission through reused wastewater [1]. The direct detection of such a wide array of pathogen microorganisms (whose levels are generally low and fluctuating) by laboratory methods is not an efficient monitoring strategy in terms of monetary cost and time required for the microbial methods of isolation and confirmation. The use of microbial indicators of faecal contamination has therefore been considered for decades by health and environmental authorities worldwide to be the most reliable method of monitoring water quality and the performance of water treatment systems [47, 48].

Table 1 Heavy metal concentrations of wastewater irrigated-soils detected in several field studies, considering the irrigation length period (short and long term), type of wastewater (source), and the effects on soil and plant

	Soil guidelines (mg kg^{-1})					Effect on	
Metal	EUs[a]	IR[b]	Case study (mg kg^{-1})	Source	Period	Soil	Plant
Cd	3.0	3.0[c]	[d]0.2 [41]	PTE effluent	L-T	+	
			3.8 [42]	UE	L-T		+↓
			5–13 [39]	TE	S-T	+	
			[d]0.037 [40]	STE	S-T	–	
Cr	150	100[c]	112.8 [42]	UE	L-T		+↓
			936.5 [43]	UE	L-T	+	–
Cu	140	100[c]	[d]35.0 [41]	PTE	L-T	+	
			75.0 [42]	UE	L-T		+↓
			8–16 [39]	TE	S-T	+	
			[d]1.25 [40]	STE	S-T	–	
			[d]94.4 [43]	UE	L-T	+	+↑
Ni	75	50[c]	[d]5.76 [41]	PTE	L-T	+	
			703.2 [42]	UE	L-T		+↓
			[d]0.80 [40]	STE	S-T	+	
			55.6 [43]	UE	L-T	+	+↑
Pb	300	100[c]	[d]12.8 [41]	PTE	L-T	+	
			135.6 [42]	UE	L-T		+↓
			126 [39]	TE	S-T	–	
			[d]2.0 [40]	STE	S-T	+	
			94 [43]	UE	L-T	+	+↑
Zn	300	300[c]	78.2 [42]	UE	L-T		+↓
			70–80 [39]	TE	S-T	–	
			[d]1.25 [40]	STE	S-T	–	
			285.37 [43]	UE	L-T	+	+↑
Mn		2,000[e]	[d]5.0 [40]	STE	S-T	+	
			77.5 [43]	UE	L-T	+	+↑

PTE primary treated effluent, *UE* untreated effluent, *TE* treated wastewater, *STE* secondary treated effluent, *L-T* long-time trial, *S-T* short-time trial

+, the concentration increases compared to control; **+↓**, the concentration increase than control, below guideline thresholds [96]; **+↑**, the concentration increase than control, above guideline thresholds [96]; **–**, the concentration does not increase compared to control

[a]*EUs* European union standards (EU 2002) [93]

[b]*IR* international references [94, 95]

[c][95]

[d]Extractable content (DTPA method)

[e][94]

Rapid and sensitive molecular methods (mostly PCR based) introduced in the 1990s to detect pathogens in water [49] are still facing issues related to sampling DNA preparation, standardising protocols, and assessing only viable. Consequently, the use of indicators is still necessary for the assessment of both water quality and

Table 2 Risk characteristics and classes of heavy metals present in urban wastewater used for crop irrigation [97, 98]

Risk characteristics and classes	Metals
Low risk	Mn, Fe, Zn, Cu, Se, Sb
High risk	Cr, As, Pb, Hg, Ni, Al, Cd
Essential micronutrients for plants	Cu, Fe, Mn, Mo, Zn, Ni
Important elements for some crops	Co, Na, Si
Can accumulate in crops at toxic levels for consumers	Cd, Cu, Mo
No toxicological threshold established for irrigation reuse	Hg
Thresholds high enough for irrigation reuse	Cu, Fe, Mn, Zn
Low absorption by plants	Co, Cu, Mn, Zn

water management [50]. The most important and widely used microbial indicator for faecal contamination of water and wastewater is the species *Escherichia coli*, supported by the previously more frequently considered indicator groups of faecal coliforms and enterococci.

In many countries, the legislation of microbial parameters for wastewater reuse is affected by different interpretations of the concept of microbiological risk; such legislation frequently constitutes a simplification that is inadequate to exploit the full potential of wastewater reuse in agriculture [51].

Table 3 details the dissimilarities of limits for wastewater reuse for various regions, including consideration of different microbial indicators and threshold levels, different methods of detection, inclusion of nematode eggs, and consideration for different types of crops as restricted or unrestricted (herbaceous vs arboreous, food vs feed, etc.).

Ideally, wastewater treatment could effectively and reliably reduce the microbial risk through relatively simple and inexpensive means, justifying the use of reclaimed water as an alternative to higher quality water and securing that supply for conservation or other uses. Wastewater from different sources generally differs to a great extent in its physicochemical and biological characteristics, and any evaluation of possible sustainable reuse must therefore rely on the determination of the specific chemical and biological qualities of the water and their interactions with the field environment and with irrigated crops.

Urban wastewater tends to harbour a higher number of microorganisms of faecal origin and, therefore, to represent a major potential source of human pathogens. Industrial wastewater possesses an intrinsically higher variability due to the different processes for which it is used. In the last 20 years, many studies have focused on the evaluation of microbiological safety in the reuse of treated wastewater for crop irrigation. Assessments of the microbiological quality of agricultural crops irrigated through different methods and by different types of wastewater have frequently reported that possible health risks due to *E. coli* and helminth eggs were not directly correlated with the use of wastewater for irrigation [15].

In some reported cases, crops irrigated with wastewater from various depuration technologies were microbiologically equivalent to the crops irrigated with well

Table 3 Microbial limits for wastewater reuse in different countries

Country or organism		Total coliform (CFU 100 mL)	Faecal coliform (CFU/100 mL)	*E. coli* (CFU/100 mL)	Nematode eggs (no./L)
US – EPA	UR R		Absent 2×10^2		
WHO (2006)	UR R			10^3 –	≤ 1 ≤ 1
Italy	ND			10^2	
France	UR R		4[a] 2–3[a]	2.5×10^2 10^4–10^5	
Spain	UR R			10^2 10^3–10^4	0.1 0.1
Portugal	UR R		10^2 2×10^2–10^4		
Australia	UR R	10 10^2–10^4		10^2–10^4	
Israel	ND		10		
Saudi Arabia	UR R	2.2[b] 10^{3b}			1 1
China	UR R		2×10^4 4×10^4		
Mexico	UR R		240[b] $10^{3\ b}$		

Adapted from Becerra-Castro et al. [51]
UR unrestricted, *U* restricted, *ND* no distinction
[a]Log reduction
[b]MPN/100 mL

water, as assessed by faecal indicators (*E. coli*, faecal coliforms, and enterococci) and pathogens like *Salmonella* spp. *E. coli* O157:H7 and *Listeria* spp. [52, 53].

Orlofsky et al. [54] carried out field experiments to monitor the human pathogenic bacteria, protozoa, and viruses in parallel trials of tomatoes watered with treated wastewater or with fresh water using a combination of microscopic, cultivation-based, and molecular techniques. The results revealed that microbial contamination on the surface of the tomatoes was not associated with the source of the irrigation water. In the specific case of greywater (domestic wastewater that excludes wastewater from toilets), contradicting results regarding increasing levels of faecal coliforms in soils following long-term greywater irrigation have been reported [55].

Efficacy of removal of pathogens and indicators is strictly dependent on the type of treatment conducted prior to irrigation reuse of wastewater: engineered systems that may include membrane filtration and UV disinfection units typically achieve the highest performance but also have higher installation and upkeep costs. Conversely, constructed wetlands and phytodepuration systems are considerably less technologically and energetically demanding, but their performance is consequently more variable [56].

In addition to the efficacy of the water treatment prior to reuse, it must be considered that the survival rate of potential pathogenic microorganisms in the treated water is highly variable, depending on the type of microorganism, water stocking and distribution system, environmental conditions, and other variables. Moreover, irrigation water is only one of the potential sources of contamination for cultivated crops, and the safe reuse of wastewater alone is not sufficient to eliminate risks related to other sources of potential contamination [57]. Therefore, an integrative approach aiming to assess and evaluate risks through microbiological laboratory tests, epidemiological studies, and quantitative microbiological risk assessment (QMRA) is necessary to elaborate novel safety rules for wastewater reuse in agriculture [2]. A balance must be struck between excessive restrictions that hamper the reuse of wastewater reuse and a lack of safety procedures that could lead to foodborne outbreaks; balanced legislation should therefore be a priority on the agenda of national and international regulation agencies. For example, a scientific study aiming at modelling the impact of recent FDA rules on the microbial safety of lettuce production illustrated how less stringent microbial parameters could maintain safety levels suitable for consumption [58].

3 Agronomic Practices Related to Treated Wastewater Reuse: The Role of Irrigation Methods

The use of treated wastewater for crop irrigation requires proper management of these resources at the farm and at the district level. The irrigation strategies for crops should consider the characteristics of the available water sources. Irrigation variables such as irrigation interval, irrigation volume, and seasonal irrigation volume should be established according to the adopted irrigation method and regime (full irrigation or deficit irrigation), temporal availability of the water source, climatic trends, and crop phenological stage.

In the use of treated municipal and agro-industrial wastewater, the definition of irrigation variables is based on the same principles and criteria used for conventional water [59]. For this reason, the irrigation scheduling strategies adopted for conventional water use are also considered valid for wastewater irrigation reuse. Regardless, the selection of adequate irrigation techniques for wastewater use plays an important role in the qualitative and quantitative increase of yields, water resource preservation, and environment protection.

The choice of irrigation system is related to factors including the socio-economic condition and the orographic characteristics of the agricultural area, the adopted crop system, the level of agricultural mechanisation of the farm, and the availability and qualitative properties of the irrigation water resource.

For irrigation with treated wastewater, the most suitable methods are drip and subsurface irrigation systems. These methods offer several advantages. First, the irrigation water efficiency of drip irrigation systems is very high, achieving

efficiencies of 90–95% and allowing up to 30–40% water saving as compared to sprinkler and surface irrigation systems. Localised irrigation systems save water through mitigation of water loss through evaporation from the soil.

Drip irrigation systems are characterised by more frequent applications of smaller water volumes, thus ensuring a low and nearly constant soil water tension in the root zone (about −0.1/−0.2 MPa). These aspects mitigate the problems of salt concentration in the root zone due to possible salt addition from wastewater application.

Finally, drip irrigation is considered the more suitable irrigation method to use with treated wastewater because it minimises toxicity hazards for plants, reduces the contamination risk for edible crop products, and mitigates hazards to human health by minimising direct contact with the wastewater. These latter advantages can also be achieved by adopting the sub-irrigation method, with the concurrent benefits of lower water loss to evaporation (15–30%), maximum irrigation efficiency, and lower visual/environmental impact of wastewater distribution.

Experimental trials comparing wastewater application by drip and subsurface irrigation methods did not show significant differences in the irrigated crop yields or for the microbiological contamination levels of the soil and the marketable crop [3, 19, 60].

Drip irrigation methods for crop irrigation with wastewater present a suitable and useful technical solution applicable to all types of soil and crops. However, wastewater containing high total dissolved solids (TDS) can cause nozzle clogging in drip irrigation systems and require the adoption of appropriate filtering systems and treatment of the irrigation water with acid and/or chlorine [61].

Table 4 details the factors influencing the choice of irrigation method, the safety measures that farmers must adopt for treated wastewater, and recommendations for use relative to crop type.

4 Effects of Wastewater on Soil-Plant System

4.1 Effects on Physical and Chemical Characteristics of the Soil

Many studies have been carried out to determine the effects of wastewater use on various soil characteristics. Particular emphasis has been placed by some authors on long-term variations in soil physical and chemical properties [62, 63].

The physical and mechanical properties of the soil (such as porosity, stability of aggregates, water retention, infiltration, and permeability) are very sensitive to organic matter and exchangeable ion types present in the irrigation water [64, 65].

The organic materials added by wastewater irrigation can accumulate in the soil fraction where intense microbial degradation and transformation of these substances occur, leading to the formation of humic compounds and the release of mineral elements. The mineral elements released in the soil following organic substance

Table 4 Suitability of irrigation methods for applying wastewater

Irrigation method	Factors influencing the choice of the irrigation method	Safety measures to adopt for irrigation wastewater reuse	Suitable crops to irrigate with wastewater
Submersion	• Low irrigation water efficiency • Low cost • No soil preparation	• Maximum protection for field workers and consumers	• Use on non-food crops is recommended
Furrow infiltration	• Low cost • Required soil surface levelling	• Maximum protection for field workers and consumers	• Use on non-food crops is recommended
Sprinkler irrigation	• Good irrigation water efficiency • No soil preparation	• Requires protection for workers • Distance of filed from the houses is needed	• Non-food crops • Irrigation of golf courses • Irrigation of parks and gardens
Subsurface irrigation	• High irrigation water efficiency • High plant cost • Difficult control of irrigation lines	• No precaution Wastewater can be used in all cases	• Irrigation of all the food and non-food herbaceous and tree crops
Drip irrigation	• High irrigation water efficiency • High plant cost • No soil preparation	• No precaution Wastewater can used in all cases	• Irrigation of the food and non-food herbaceous and tree crops

degradation enrich the soil with elements useful for plant nutrition, while the humic compounds improve the soil structure and increase its stability, facilitating the movement of water and air along the soil profile.

The integration of the organic substances into soil aggregates guarantees the physical protection of organic substances, limiting their mineralisation by microorganisms and enzymes [66]. This increase in structural stability is due to the cementing action of substances originating from the decomposition of the organic fraction of wastewater and primarily consists of organic polymers (polysaccharides), which form bonds between the soil particles.

In Mediterranean agricultural areas, the soils are frequently characterised by low organic matter contents due to intensive cropping systems and high temperatures that facilitate organic matter mineralisation. In these agricultural contexts, the use of wastewater for crop irrigation could reduce the depletion of soil organic matter.

Once applied to the soil, soluble and insoluble compounds contained in wastewater are involved in numerous physicochemical and microbiological processes that influence their mobility and biodegradability [67].

Field research carried out in the Apulia region of Italy aimed to verify the effect of secondary and tertiary treated municipal wastewater on artichoke crops in comparison with conventional groundwater. No significant differences were found for several chemical parameters (pH, EC, NO_3-N, NH_4-N, and organic matter content) in the upper 30 cm layer of the soil irrigated with the three different water sources [68]. On the contrary, Vergine et al. [20] and Libutti et al. [60] compared the use of

groundwater with secondary and tertiary treated municipal wastewater to irrigate a succession of tomato and broccoli crops and found that the soil irrigated with secondary treated wastewater resulted in a significant increase of pH and NH_4-N, as well as of Na^+, SAR, and EC, although these were below the threshold value used to define the soil as saline. According to the authors, the EC increase due to salt accumulation was particularly evident during the cultivation of tomatoes, while this condition changed completely during the cultivation of the broccoli due to the leaching effect of the autumn and winter rains.

However, other researchers have noted that use of low-quality irrigation water requires the addition of a leaching fraction to avoid salt accumulation in the root zone. Basic recommendations to appropriately manage low-quality water and avoid salinity hazards and soil degradation have been provided by Ayers and Westcot [69] and Rhoades and Loveday [70]. Moreover, numerous studies reported the impacts of salinity control measures in irrigated agriculture [60, 71–73].

In terms of the accumulation of heavy metals in soil, Campi et al. [74] carried out a 3-year field study within the In.Te.R.R.A. project in which annual energy crops were irrigated with secondary and tertiary treated municipal wastewater and compared with crops irrigated with conventional water. The results showed that the concentrations of heavy metals in the soil were characterised by negligible mean variations and no significant differences were found among the experimental treatments.

These results confirm those reported by other authors in studies conducted over middle-term time scales. Surdyk et al. [75] in the European FP6 SAFIR project examined heavy metal comportment in soils irrigated by treated wastewater over 3 years in Serbia, Crete, Italy, and China. The authors reported that, when properly treated, wastewater influent with higher heavy metal contents can be used over the middle-term (about 3 years) without visible degradation of the soil, even if long-term cumulative effects cannot be excluded. To this note, some long-term studies have reported significant increases in heavy metal concentrations in soil surface layers, although these were below the critical thresholds established by the international guidelines [62, 76, 77].

Other authors define the effect of wastewater in the long term. Dere et al. [78] and Lucho-Constantino et al. [79] evaluated the effects of heavy metal concentrations in soils irrigated over several decades (up to 100 years) with the low-quality wastewaters (raw sewage) of two cities (Paris and Mexico City). They found significant heavy metal accumulation, especially in wastewater-irrigated soil zone.

Similar research conducted in three different areas of Zimbabwe [34] has demonstrated that the concentrations of the analysed heavy metals (i.e. Cu, Zn, Cd, Cr, Pb, and Ni) in the wastewater-irrigated soils were significantly higher respect to the concentrations found in the non-irrigated soils. These results highlighted that the application of wastewater had enriched the soils with heavy metals, and the authors concluded that soil contamination by wastewater use presents long-term environmental and health risks.

The effect of wastewater on the accumulation of heavy metals in soil profiles and groundwater was examined by monitoring zones irrigated with wastewater for

different years (20, 30, and 40 years) in China [80]. The authors reported that long-term wastewaters irrigation does not constitute heavy metals pollution in soil and groundwater; however, they suggested that the monitoring of Hg, Pb, and Cu concentrations should be evaluated in areas that use treated wastewater irrigation to avoid health risks.

4.2 Effects on Soil Microbiological Characteristics

The soil microbiota is an essential component of the soil system, interacting with inorganic components, the atmosphere, soil organic matter, and with other organisms of the soil ecosystem, including plant crops. Microorganisms provide many functions in the soil ecosystems that are essential for the quality and productivity of agricultural products, including plant growth promotion, organic matter turnover, availability of nutrients, and plant pathogen suppression. The application of treated wastewater to agricultural soil can affect the structure and functions of the soil microbiome in two primary ways: first, through the introduction of exogenous microorganisms (with repeated events following each irrigation treatment) leading to changes in microbial diversity and dynamics; and second, through changes in the physicochemical properties of soil due to the composition of the wastewater and the consequent intake of salts, inorganic nitrogen and phosphorous, metals, and micropollutants, with unavoidable impacts on the properties of the soil and on cultivated crops.

There is still a lack of information regarding the stability of exogenous microbial communities from wastewater. One study [3] found that short-term applications of treated industrial wastewater could cause a shift in soil microbial communities during a single tomato crop season. More recently, Dang et al. [81] found that irrigation with treated industrial wastewater had a greater impact on microbial community structures than domestic wastewater; irrigation significantly affected the composition of indigenous soil microbial communities at different soil depths and might therefore introduce exogenous microbes into the soil environment.

The composition of wastewater can effect of the degree to which its application modifies the physicochemical properties of the soil; generally, any deviation from the native soil structure and composition (pH, salinity, etc.) tends to reduce microbial diversity, and bacteria are particularly sensitive to soil perturbations. However, an increase in microbial diversity does not necessarily imply an increase in functional diversity, in terms of microbial metabolic activity within the soil. As an example, Cheng et al. [82] found that aquaculture wastewater irrigation of agricultural soil reduced the functional activity of microbial communities (primarily due to increased salinity) but induced a higher richness of microbial taxa.

4.3 Quantitative and Qualitative Response of Crops to Irrigation with Wastewater

The quality of water used for crop irrigation can impose a major constraint to agricultural productivity because of its influence on soil fertility and crop yields. Therefore, the knowledge of the effects of the water quality used for irrigation on crop yield response is critical to the understanding of necessary management criteria for long-term productivity as well as to develop the most suitable irrigation schedule to get the optimum plant yield and the desired profit from irrigation. These aspects are of particular importance if considering that the continuous decrease in water resources in the world in general, and in arid and semi-arid regions in particular, is continuously forcing farmers to use wastewaters and modify the conventional irrigation practices.

The plant responses in terms of yield and quality of agricultural products to wastewater are often contrasting; generally, either positive or negative and sometimes even neutral effects have been reported for crops. Positive effects of treated wastewaters reuse on the growth and production of cultivated crop, as well as on the chemical and microbiological characteristics of crop products, were observed within the experimental trials of the already mentioned In.Te.R.R.A project.

Field experiments were carried out in different pedoclimatic conditions of the Apulia region (Southern Italy) with the aim to evaluate the quali-quantitative response of several horticultural and vegetable crops to the irrigation with municipal and agro-industrial-treated wastewaters [3, 17, 18, 60]. At the same time, the hygienic traits of crop products, such as the presence of Coliforms, *Escherichia coli* and *Salmonella*, and the risks for human and environmental health related to the reuse of this irrigation source, were verified. Horticultural crops, such as processing tomato (*Lycopersicon esculentum* Mill.), artichoke (*Cynara cardunculus* L. subsp. *scólymus* Hayek), broccoli (*Brassica oleracea* L. var. *italica*), and vegetable crops for fresh consumption, such as lettuce (*Lactuca sativa* L.), fennel (*Foeniculum vulgare* Mill.), melon (*Cucumis melo* L.) and cucumber (*Cucumis sativus* L.), were examined. The results obtained indicated that the qualitative traits of crop products (e.g. dry matter content, diameter, soluble solid content, titratable acidity, pH) were similar to those obtained when the crops were irrigated with conventional water.

In some cases, when the amounts of nutrient elements (N, P, K) supplied to the crops by wastewater were higher than those supplied by conventional water, also the productive response of the crops, in terms of both total and marketable yield, was positive. During the considered crop cycles, a sporadic presence of pathogenic microorganisms was observed in the soil and, only in rare cases, on the aerial part of the plants. However, in no case the presence of pathogenic microorganism was possible related to the wastewaters microbiological quality, because other potential contamination factors, such as vector insects, aerosols, wild animals, birds, processing and harvesting personnel, endogenous environmental factors, can likely have played a role in microbial contamination of soil and plants [83]. As

consequence of irrigation with wastewater, a reduction of the crop cycles, ranging from 7 to 10 days, was also observed within these experimental trials, but without compromising crop qualitative and quantitative performances. This was due to the high amount of nutrients, especially nitrogen, not removed by depuration processes in the considered treated wastewaters that were frequently applied to the plants (2–3 days interval), up to a few days before harvesting.

In this regard, it is well known that treated municipal wastes, if not undergone to denitrification treatment, contain high quantities of nitrates, ammonia salts, mineral perphosphates, and macro-elements than conventional water. High nitrogen supply to the plants, especially close to product harvest, affects crop cycle duration, delaying the reproductive phase and reducing the vegetative phase. Therefore, the plants whose crop cycle ends with the reproductive phase (seed production), under the irrigation with wastewater, tend to lengthen the crop cycle; on the contrary, species that are harvested before the production of seeds (horticultural crops whose product is intended for cooked and/or raw consumption), such as fennel, lettuce, chicory, cucumber, and barter, can reduce their crop cycle [84]. All the quali-quantitative crop response observed within the In.Te.R.R.A research activities confirm the results of previous studies aimed at evaluating the agricultural reuse of municipal wastewaters as alternative irrigation water source, carried out in Apulia region (Southern Italy) [85, 86].

A field experiment carried out by Qaryouti et al. [87] showed the increase of some cucumber and tomato crop parameters when wastewater from industry rich in organic matter and nutrients, particularly K, was reused for irrigation. Especially in cucumber plant the height, fruit yield and average fruit weight, as well as the tomato leaf area and plant dry weight were even significantly increased due to the replacement of K-chemical fertilizer by the wastewater. Also Al-Lahham et al. [88] reported an increase in tomato fruit size and weight when irrigated with reclaimed domestic wastewater. Any adverse effect on chemical quality of several vegetable crop fruits, such as okra (*Abelmoschus esculentus L.*), bean (*Phaseolus vulgaris* L.), corn (*Zea Mays* L.) and sunflower (*Helianthus annuus* L.) was observed when grey treated wastewater, characterized by high BOD and COD values, was used for irrigation [89].

In addition to plant nutrients, wastewaters may contain various potentially toxic mineral elements (see Sect. 2.2) with harmful effects on human and animal health [90]. The transfer of heavy metals to plants irrigated with wastewater may cause accumulation in plant tissues, and in some cases, the content of these metals may reach phytotoxicity thresholds. Particularly, high concentration of heavy metals can result in the reduction in marketable crop yield and/or poor quality of marketable agricultural products [12]. To this regard, Gatta et al. [29] observed heavy metal contents (Al, Cd, Co, Cr, Cu, Fe, Ni, Pb, Zn, and Mn) of artichoke heads, harvested after irrigation with secondary and tertiary treated wastewaters, lower than the international threshold values. They also found low bioaccumulation factors for the edible part of the artichoke crop and not significant health risks (hazard index <1.0) to adults and children after the consumption of the artichoke heads.

On the contrary, other authors [13] found concentrations of Ni, Pb, Cd, and Cr in the edible portions of okra vegetable crop grown on a soil irrigated with treated wastewater well above the safe limit and a Health Risk Index (HRI) higher than 1, indicating a potential health risk. Similarly, heavy metal concentrations (Zn, Pb and Cd) several times higher than the WHO prescribed permissible limits were observed by Uddin et al. [91] in edible portions of red amaranth (*Amaranthus cruentus* L.) and tomato irrigated with industrial wastewater.

The results of several research activities on wastewaters (untreated and treated) clearly highlight that these irrigation sources are a rich source of nutrients and most crops give higher than hypothesized yields with the adoption of a wastewater irrigation system, diminishing the requirement for synthetic fertilizers and bringing about reduction of investment cost to farmers. Nevertheless, if the estimated nitrogen provided for harvesting by the wastewater system exceeds the required dosage for the ideal yields, it may reinforce vegetative development, but it may delay growth and cause adverse effect on crop yield [92]. The presence of toxic elements in wastewater must be also considered since they are hazardous and might be poisonous to plants in high amounts and to human health. Therefore, evaluating the impacts of wastewater irrigation sources on characteristics of crop yield in the different agronomic situations is fundamental in a perspective of wastewater irrigation system development, under the best agronomic and water management practices.

5 Final Considerations

In this chapter, the possibility of recovery of wastewater for irrigation purposes was illustrated and discussed, highlighting the advantages, disadvantages, and possible risks of the practice. Interest in wastewater reuse is continuously developing and derives from the growing worldwide demand for water resources, particularly for food crop irrigation, and not restricted to countries characterised by water scarcity.

The primary results of past and recent research on irrigation reuse of treated wastewater, both of municipal and agro-industrial origin, can be summarised as follows:

- The reuse of treated municipal and agro-industrial wastewater in agriculture can mitigate the increasing scarcity of conventional water resources for a particularly water-demanding sector such as agriculture; agricultural wastewater use could improve the chemical fertility of the irrigated soils; treated wastewater could represent a resource of strategic importance in terms of nutrient availability (e.g. N, P, and K) for the irrigated plants. However, this benefit must be assessed with particular attention because can cause plant nutritional imbalance and phytotoxicity.
- The use of treated wastewater in agriculture (with particular reference to industrial or agro-industrial wastewaters) can lead to heavy metal accumulation in the soil following long periods of application. Therefore, the monitoring of heavy

metals in wastewater-irrigated soils and crops is very important for the prevention of potential environmental and human health risks.
- The application of treated wastewater to agricultural soil can affect the structure and functions of the soil microbiome, mainly owing to the introduction of exogenous microorganisms, with effects on some physical-chemical properties of the soil.
- Soil salinization can become a problem in the long term, particularly if wastewater irrigation takes place in soils already affected by salinity problems.

Finally, the assessment of the suitability of treated wastewater in the agricultural sector cannot be separated from specific scientific studies on the effects of emerging micro-pollutants (e.g. contaminants of emerging concern, CECs) both on the soil-plant system and on human health.

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Uptake and Translocation of Pharmaceuticals in Plants: Principles and Data Analysis

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Contents

Abstract Pharmaceuticals originating from reclaimed wastewater or biosolid-, livestock manure- or sewage sludge-amended soils can enter crops by irrigation and fertilization. Generally, the putative uptake occurs through the plants' roots and

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can lead to the bioaccumulation in different plant parts. The uptake and translocation therefore is dependent on multiple parameters, i.e. physicochemical properties of compounds, plant physiology and environmental factors. This book chapter combines a theoretical background on the main principles of uptake and translocation of pharmaceuticals by plants and a critical evaluation of current available literature, by analysing studies for the bioconcentration and translocation factors of different pharmaceutical groups in several plant species. Thereby, interesting results were obtained by looking at the translocation of various pharmaceuticals in radish and at cationic compounds in soil studies. Comparing the different studies, the relevance of testing not only high but also real environmental concentrations became obvious, since for some pharmaceuticals, higher uptake and translocation ratios were achieved with lower applied concentrations. Basic guidelines could provide a possibility to make scientific data more comparable and reliable and to avoid the exclusion of potential reasons for the missing uptake or translocation of pharmaceuticals. This book chapter provides recommendations for future research studies to generate more valid conclusions within the scientific community.

Keywords Bioconcentration factor, Hydroponic studies, Ionic compounds, Sequestration, Soil studies, Translocation factor

1 Background

Ecosystems are often exposed to natural or synthetic substances that have no direct nutritional value or significance for metabolism but can have a negative impact on the function and performance of biota. Commonly, these substances enter the aquatic environments through wastewater treatment plant effluents as a consequence of partial and/or inefficient removal during wastewater treatment processes. Recent studies, supported by powerful analytical screening analyses, described a high number of emerging pollutants in those effluents; they can range from pesticides, pharmaceuticals and personal care products (PPCPs), illicit drugs, endocrine disruptive compounds, flame retardants, food additives, disinfection by-products through all possible metabolites and transformation products (TPs) [1, 2]. Although only low concentrations (ng/L–μg/L) of these organic molecules were frequently found in surface and groundwater, they can be considered as 'pseudo-persistent' because of their continuous discharge and deposition into the environment [3]. These substances can also enter the terrestrial environment by agricultural practices, i.e. the irrigation of plants with treated wastewater or fertilization with manure; after their exposure to agricultural soils, compounds can be taken up by crops and therefore enter the food chain. In case of pharmaceuticals, long-term exposure to low concentration levels can induce toxic or metabolic dysregulation in terrestrial and aquatic organisms [4, 5].

Due to their chemical properties, a topic that will be also discussed further in this chapter, pharmaceutical residues, metabolites and TPs might be adsorbed to soil particles and taken up by plants [6]. In order to be able to estimate the effects not only on biota but also on human health, an understanding of the absorption and transport processes in plants is of ample relevance.

This chapter will provide readers with an overview of the most important uptake mechanisms in plants, in addition to the transport of pharmaceutical compounds through the plant vascular system. Concepts will be resumed from soil and chemical properties ending up in plant biotransformation and sequestration mechanisms and environmental factors that can influence the pharmaceuticals' uptake.

This article will cover the main pharmaceutical groups, i.e. antibiotics, hormones, analgesics, anti-inflammatory, lipid regulator agents, antidiabetic, anticonvulsants, stimulants, psychotropic drugs and antihypertensives (e.g. beta-blockers, calcium channel- or angiotensin receptor blockers) since these compound classes are in continuous debit into the environment and due to their chemical characteristics that make them prone to plant uptake.

Data on pharmaceutical uptake and translocation published from year 2013 on were analysed to take conclusions based on different experiments and conditions.

2 Which Factors Can Influence the Uptake of Pharmaceuticals by Plant Roots?

Soil properties, like ionic strength, pH and organic matter (OM) content, are determining factors in the fate of emergent compounds (as pharmaceuticals) in soil-plant systems. OM is an important sorbent for pharmaceuticals, which changes their bioavailability/bioaccessibility for root uptake [7, 8] (see Fig. 1). According to Miller and co-authors [9], polar and ionizable pharmaceuticals can engage in interactions beyond hydrophobic partitioning, including electron donor-acceptor interactions, cation and anion exchange, protonation, water bridging, cation bridging and surface complexation. Moreover, for ionizable compounds, several physico-chemical properties strongly influence the degree of association with soil particles.

Abiotic transformations like redox reactions may occur in the clay fraction through reactive mineral phases and influence the molecule's integrity. Photolysis can likewise be involved in processes close to soil surfaces, but it has a lower relevance due to strong light attenuation deeper in soils [9].

Synergistic effects between different pharmaceuticals can also play an important role. Especially, when crops are irrigated with treated wastewater, plants are not only exposed to one but to a cocktail of pharmaceuticals and other compounds. The co-occurrence of carbamazepine and lamotrigine in crops showed that synergistic effects enhanced the uptake of lamotrigine when carbamazepine was present, but the uptake of carbamazepine was not affected in presence of lamotrigine in cucumber plants (*Cucumis sativus*) grown under hydroponic conditions [10]. Moreover, the

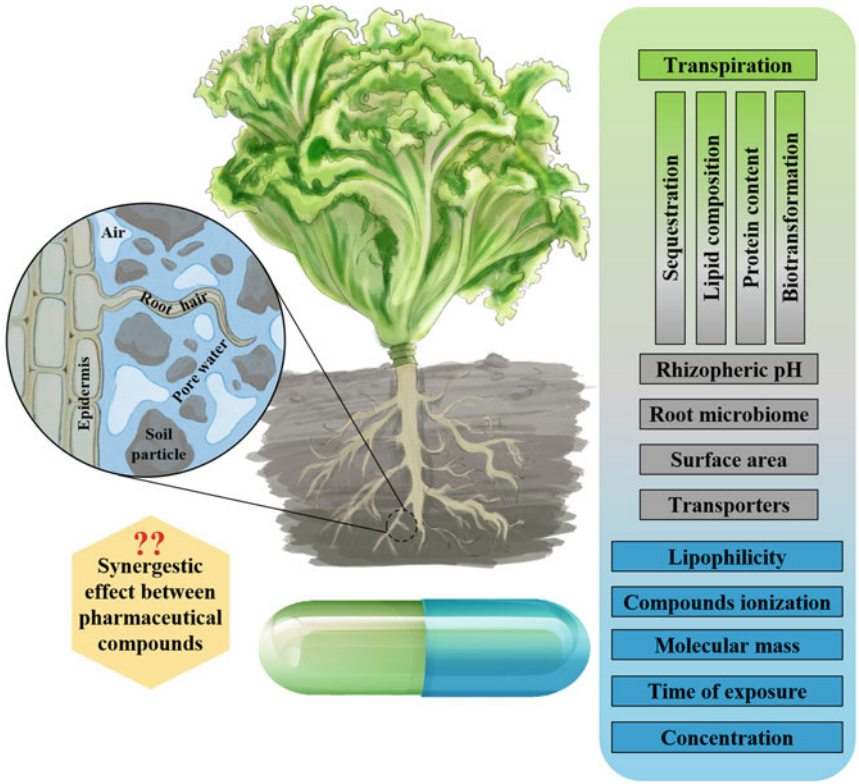

Fig. 1 Multiple parameters, which play a critical role on plants' uptake of pharmaceuticals along with their distribution among different plant organs

uptake of pharmaceuticals, when applied in a mixture compared to single compound exposure, also differed between plant species. The concentration of atenolol was higher during the single compound exposure in the roots of lamb's lettuce (*Valerianella locusta* L.) whereas on arugula (*Eruca sativa* L.) and radish (*Raphanus sativus* L.) did not show higher values compared to the mixture application. The uptake and translocation of other substances were in contrast similar between plant species in the single and mixture application of pharmaceuticals [11]. However, as this study was performed in soil, the additional soil effects might influence the uptake of these pharmaceuticals, which was also shown in the same study. Furthermore, interactions between pharmaceuticals, heavy metals and metalloids were detected in beet root (*Beta vulgaris* L.). The concentration of sulfamethoxazole in beet root increased with increasing concentration of a mixture of heavy metals (Mn, Zn, Cu, Cd, CO, Cr, Ni and Pb). In contrast, the accumulation of metoprolol decreased with increasing heavy metal concentration. For other compounds, the changes were negligible or no clear trend was observed [12]. To conclude,

interactions between different pharmaceuticals but also pharmaceuticals and heavy metals could be observed, which are not always favouring an increased or decreased accumulation in plants. This uptake is rather influenced by additional parameters like physicochemical properties of the compound, plant physiology or soil composition.

Biodegradation is considered the most important process for eliminating the majority of xenobiotics (e.g. pharmaceuticals), where microorganisms – as important degraders – provide products to other organisms in the food web. However, these processes are only significant when the molecules' toxicity does not inhibit microbial activity. Although, known for a long time, the biodegradation of drugs and their effects on ecological processes driven by microorganisms is quite scarce but may be also too complex to be fully addressed in this book chapter [13]. Besides the potential transformation of pharmaceuticals by soil organisms, their bioavailability might also be reduced by the microbial communities at root surfaces – so-called rhizobacteria – which can act as enhancers of phytoremediation efficiency; the same concept has been proposed for endophytic bacteria inhabiting root tissue. Moreover, the latter can interact closely with their host plant boosting the degradation pathways and metabolic activities and then decreasing both phytotoxicity and evapotranspiration of volatile organic compounds [14–16].

Various microbial species and strains may perform differently under different environmental and growth conditions, determining their efficiency and hence their usefulness [17, 18]. Although many microbial species are still unidentified, Agrawal and co-authors [17] listed a wide range of pollutant-degrading microorganisms that have been spotted by culture-independent techniques and could be harboured in the root environment of various plant species. The full metabolic capacity of the plant associated bacteria (plant endophytes and rhizosphere bacteria) has not been completely resolved yet, although first experiments indicate that microbial activities can have a strong influence on biotransformation processes of pharmaceuticals [15, 19] (more details are provided in chapter "Impact of PhACs on Soil Microorganisms").

Another factor that has been mostly neglected is the direct availability of active metabolites that may be excreted from animals or humans. Generally, it is assumed that 90% of an active compound are metabolized from a mammalian body within 48 h, after treatment. In any case, the availability of parent compounds and major metabolites will be decisive for their further fate in plants.

2.1 Compounds Properties

One of the primary criteria that influences uptake into roots and translocation in plant tissue is the *molar mass* of the pharmaceuticals [20]. Low-molar mass organic compounds can easily enter the soft rhizodermis and move through the porous mesh of the cell wall. Hence, organic substances with molar mass $<$1,000 g/mol are easily absorbed by the apical sections of plant roots [21]. However according to Chuang and co-workers [20], only molecules below 300 g/mol can, in general, enter

the roots easily, when compared to large-sized pharmaceuticals (molar mass >400 g/mol).

In the living parenchymal tissue deeper inside the root, and towards the delicate younger apical roots, the cell wall and the biomembrane (plasmalemma) may function as filters (*membrane permeation*) limiting the uptake or movement of organic molecules based on their size.

Besides that, physicochemical properties of the molecules, like *lipophilicity* and *ionic strength* (polarity H bonding), will dictate their fate, even before uptake and transport into the plant vascular translocation system (xylem and phloem) occur. A significant proportion of pharmaceuticals are ionizable meaning that they can assume neutral, cationic, anionic or zwitterionic form under different pH conditions [22]. This means that the difference in lipophilicity between the neutral and ionic forms varies within compounds and is difficult to predict. Usually, a single log K_{OW} value (also called *P*) is determined, reflecting only the lipophilicity of neutral species [23]. So, it has been discussed that for ionic forms, log D_{OW} seems to be more appropriate to express the lipophilicity of these molecules because it accounts for pH dependence (i.e. acid dissociation constant (p*K*a)) of a molecule in aqueous solution [24].

In early research on the topic, Briggs and co-workers [25] established a linear relationship between K_{OW} of non-ionized chemicals and the observed root concentration. Albeit only shown for industrial pollutants and herbicides [26], this relationship seems to hold true also for other synthetic molecules like pharmaceuticals. It is crucial to consider that pharmaceuticals have been specifically designed to penetrate through biological borders and membranes, to ensure their rapid delivery at the site of action. Wild and co-workers [27] pointed out that non-ionic organic chemicals with log $K_{OW} > 4$ seem to exhibit high retention in plant roots, while Cousins and Mackay [28] suggested that for organic chemicals with log $K_{OW} < 2$ and a Henry's Law constant of less than 100 $cm^3\ cm^{-3}$, the water filled intercellular space seemed to be the main storage compartment [29]; the topic has been extensively covered by Schröder and Collins [30].

2.2 Uptake of Pharmaceuticals by Plant Roots

In the first step, compounds from the surrounding medium or pore water (usable water in soil for plants) become available for root uptake by diffusion, where compounds properties like *solubility*, lipophilicity, molar mass, *compound concentration* and characteristics from the surrounding environment as temperature and soil humidity (if the case) will influence the uptake performance [21] (Fig. 1). Here, soils with high proportions of clay minerals might be a significant temporary sink for charged molecules and build up local hotspots of organic pollutants. In a second phase, compounds are available to root uptake: due to a negative water potential in soils at field capacity, a net movement of pharmaceutics towards plant rhizospheres might prevail. The root surface and its extensions are key compartments for uptake

of organic compounds: roots of perennial plants (except monocots) typically develop a rigid protective structure called periderm (replaces the normal rhizoderm), which comprises a large component of bark and the most outer layer called phellem, consisting of suberized-dead cells [31]. These bark-like materials contain accumulations of lipophilic substances and may hence act as a sink for lipophilic compounds. In this context, the role of the protective root cap and its mucilage has not been investigated as sink in depth.

Although chemical features of a molecule may be important predictors for the uptake, the physiology of the plant root itself and its composition can also have significant influence. Trapp and Pussemeir [32] critically reviewed the relationship derived by Briggs and co-workers [25] as an overestimate of the uptake of some herbicides by common bean (*Phaseolus vulgaris*) [33]. We are still lacking knowledge about the factors determining such differences.

Among all biological factors, root extractable lipid content seems to have the strongest influence on the emerging compounds' uptake [34]. Either way, lipophilic compounds are expected to partition to root lipids (membrane and storage lipids) and thus concentrate in roots, until an equilibrium between the chemical concentration in the aqueous phase within the plant root and the external solution is reached. The strong affinity of charged compounds or their metabolites in roots retards pharmaceutical transport to shoots and results in a significant accumulation in roots, making tuberous vegetables critical sources of food and fodder [35]. However, protein content was found to have a greater influence on the prediction of uptake than the lipid content as described by González García and co-authors [36]. For weak acids like ibuprofen, ketoprofen and naproxen, higher concentrations in roots than in leaves were quantified, suggesting the adsorption to proteins and consequently retention in roots, which supported their model.

Once a solute enters the root – through the growing tip of the root hair epidermis passing by cortex, endodermis and pericycle, ending up with the entrance into the vascular tissue – it can take two pathways to reach the xylem, along which it is transported to the aerial plant parts:

In the *apoplastic pathway*, the solute travels along cell walls through the intercellular space of the epidermis and cortex region of the root and across cell membranes at the endodermis. Non-ionic pharmaceutics are able to cross cell membranes easily and thus have higher potential to be taken up by the roots due to their higher lipophilicity [37]. However, compounds taken up exclusively by the apoplastic route cannot cross the Casparian strip; that is, they must cross at least one lipid bilayer to enter the xylem or phloem; if not, they tend to accumulate in roots [9]. Little research has been directed towards elucidating xenobiotic uptake mechanisms and pathways, knowledge that is needed to develop models to predict uptake and accumulation. Chemical sorption to lipophilic root structures may be a significant factor influencing the available concentration.

In the *symplastic pathway*, the solute crosses cell membranes of root hairs, epidermis and cortex and moves to the vascular cylinders by the plasmodesmata and/or by membrane permeation [38], which means that only a small fraction of the compounds is transported via the symplastic movement into cellular vacuoles

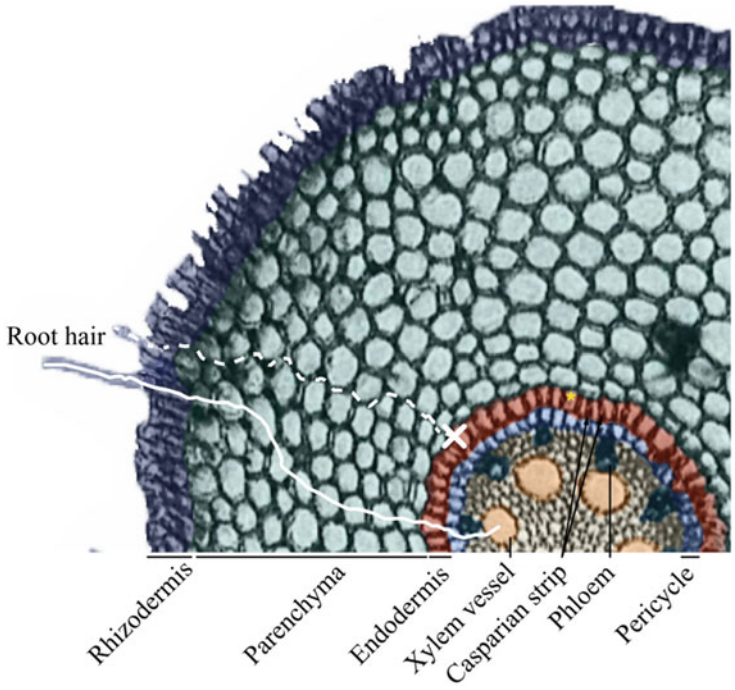

Fig. 2 Cross section of an iris (*Iris pseudacorus*) root. Diffusive uptake of chemicals can occur via the apoplast, i.e. through the cell wall continuum (dotted line). However, at the endodermis with its thickened suberized cell walls (Casparian strip; red), diffusive apoplastic transfer is stopped. This mechanism is responsible for the accumulation of various pollutants in the root. Chemicals can only penetrate into the central tissues after active passage to the symplast, i.e. the continuum of living cells (solid line). Their passage into the central cylinder with its access to vessels is facilitated by passage cells (asterisk in yellow) lacking the suberized wall deposits

[39]. Once in the symplast, these compounds can move through the xylem in the direction of the transpiration stream and accumulated mostly in transpiring organs (i.e. leaves) [8, 37]. Ionizable compounds may be subject to additional processes such as ion trapping and electrostatic interactions with cell walls [9] (see Fig. 2).

As large numbers of pharmaceuticals, as well as endogenous metabolites, are organic ions, it seems that uptake, distribution and sequestration of these compounds highly correlates with the expression of the transport system [40]. It is well known that the major facilitator superfamily (MFS) and/or ATP-binding cassette (ABC) transporters are responsible of conveying organic compounds (like sugars or amino acids) throughout the plant [41]. Members of solute carrier 22 family (SLC22), which have been initially found in animals [42], are plasma membrane transporters that belong to the MFS and strongly contribute to organic ions homeostasis. The SLC22 family encompasses organic cation transporters (OCTs), organic cation/zwitterions transporters (OCTNs) and organic anion transporters (OATs) [43]. Transporters of multidrug and toxic compound extrusion (MATE) are cation antiporters,

which are considered as one of the major transporter families in plants [44]. It has been reported that the first isolated MATE transporters from in plants (specifically in *Arabidopsis*) were involved in the detoxification of xenobiotics [45, 46]. Li and co-authors [46] succeeded to characterize the first multi-specific MATE transporter and named it AtDTX1 (for *Arabidopsis thaliana* detoxification 1). Moreover, they demonstrated that AtDTX1 serves as an efflux carrier for the antibiotic norfloxacin during functional screening with *Escherichia coli* KAM3 mutant. Furthermore, they suggested that AtDTX1 is localized in the plasma membrane and consequently will mediate the efflux of exogenous or plant-derived toxic compounds from the cytoplasm. PvOCT1 is the first protein linked to the SLC22 family and has been identified in *Phaseolus vulgaris* [47]. The expression of PvOCT1 is upregulated after exposure to the drought stress, and this presumes that it plays a role in stress adaption. In 2007, Lelandais-Briere and co-workers [48] discovered AtOCT1 (a PvOCT1 homologous) that is localized in the plasma membrane of *Arabidopsis* and can be characterized as carnitine transporter. The other five members of *A. thaliana* OCT family (AtOCT2-AtOCT6) are localized in the tonoplast, and their functions are still unknown; nevertheless, the expression of these genes was upregulated during the exposure of *Arabidopsis* plants to drought, cold and salt stress [49]. In a recent study, it was suggested that OCTs might provide an important route for delivery of the antidiabetic drug metformin (MET) [50], showing that MET transport was significantly affected in common cattail (*Typha latifolia*) roots after addition of quinidine (OCTs inhibitor in mammals).

2.3 *Translocation of Pharmaceuticals Within Different Plant Parts*

After organic contaminants (e.g. pharmaceuticals) entered the root, translocation might occur to the aerial part of the plant via the vascular tissue. These compounds can be transported upwards with water and other solutes by *transpiration* through vessels and tracheids in the xylem (Fig. 2). Transpiration flow, driven by root pressure and transpirational pulling, was shown to be the main driving force of the translocation of pharmaceuticals [51].

During photosynthesis and to protect plants from overheating, stomatal apparatus – specific ventilation pores – mostly present on the abaxial side of the leaf are open for gas exchange or evaporative cooling. Mesophyllic cells located above the stomata are transpiring water, leading to water deficiency and increased negative water potential. To compensate this effect, the cell takes away water from neighbouring cells, which results in a spreading suction force towards leaf vessels, to xylem tracheids and finally to roots to take up water from the surrounding environment. A high *light intensity* (higher photosynthesis rates), *warm temperature* (which increase saturation level of water vapour within leaves) and *dry air or wind* enhance transpiration rates. Transpiration rates determine the flux of water and solutes and

depend on plant species and shoot height. Environmental factors are also influencing the daily transpiration rates. As it has been mentioned before, the molecular size of pharmaceuticals can determine their diffusion rate through root cell membranes. A good example of a pharmaceutical being translocated by xylem flow is carbamazepine. The uncharged compound with intermediate hydrophobicity (log K_{OW} 3.64) is known to be frequently detected in higher concentrations in aerial parts rather than in roots [20, 52, 53]. Moreover, carbamazepine was detected through the whole plant in xylem sap and even found in transpiration waters in the ambient air [10, 54].

Pharmaceuticals could be also transported via sieve tubes of the phloem, as shown already for several herbicides [55, 56]. Compared to the unidirectional flow from roots to leaves in the xylem, compounds in phloem can be translocated in two directions: together with photosynthates (photosynthetically derived carbohydrates) from leaves to the plant below (branch, shoot, root) and above (young developing leaves, apical meristem, fruits). As generally alleged, phloem mass flow is driven by an osmotically generated pressure gradient by the accumulation (active loading) of sugars in the photosynthetically active leaves (source) and their deliverance (unloading) to the place of consumption (sink). Therefore, it is hypothesized that neutral compounds, which are mainly translocated by water flow (xylem), can be generally found in higher concentrations in mature leaves [53], in contrast to xenobiotics being transported via phloem to younger leaves, as suggested by Hsu and Kleier [57]. In this respect the abovementioned carbamazepine, which is known to be transported by xylem, was detected in higher concentrations in old leaves compared to young leaves of cucumber plants. In contrast, the anionic antibiotic tetracycline was quantified in similar concentrations in both kinds of leaves [58].

However, for non-ionic compounds, like the insecticide fipronil or some neonicotinoids, the ion trap theory does not apply, and the active ingredient can move freely between phloem and xylem according to its membrane permeability [59]. Herbicides with high ability to cross membranes may equilibrate between phloem and xylem but are preferentially transported by xylem because of the higher water flow [60]. Although only described for agrochemicals, this concept may as well influence the pharmaceutical compounds transport in plants.

The *transpiration stream concentration factor* (TSCF) is a descriptor for the quantitative uptake of contaminants. It is defined as a ratio of contaminant concentration in the xylem to the concentration in nutrient media, and this ratio varies between 0 and 1 [61]. The hydrophilic compound caffeine had a higher TSCF value than the more hydrophobic compounds triclocarban or endosulfan in zucchini (*Cucurbita pepo* ssp. *pepo*), soybean (*Glycine max* L.) and squash (*Cucurbita pepo* ssp. *ovifera*). Hence, hydrophilic pharmaceuticals, after passing the Casparian strip, seem to be translocated faster than hydrophobic ones [62]. The TSCF can give useful information about the translocation of compounds although not many studies exist measuring the pharmaceutical concentrations in xylem sap. Thus, the *translocation factor* (TF) describing the ratio between the pharmaceutical concentrations in the leaf compared to the root is often used to characterize the translocation of compounds. However, it is not taken into account if compounds are translocated by xylem or phloem.

Another difference between xylem and phloem, which influence the translocation of environmental contaminants (e.g. pharmaceuticals), is the *pH*. Phloem juice is about 8.0, which is similar to cytoplasmic pH (6.9–7.6), but inside xylem vessels, and also in the apoplast and intracellular spaces, the pH is about 5.0 [63]. Translocation of emerging contaminants is also interlinked to physical and chemical properties of the organic compounds. p*K*a values, influencing the charge of some pharmaceuticals at a specific pH is highly relevant (see previous section about root uptake). Accumulation of lamotrigine in leaves correlated with uncharged lamotrigine in pore water; thus, the pH-dependent charge of the molecule in the soil had an impact on its translocation to aerial parts of durum weed (*Triticum durum*) [64]. Such as the p*K*a, also the *lipophilicity* of compounds plays a crucial role, as moderately lipophilic neutral substances, with log K_{OW} (1–3.5) or log D_{OW} (0.5–3), are preferably translocated [65, 66]. Collins and co-workers [33] pointed out that for some uptake models, the lipid content (in their case, of the leaves) represents the most sensitive input parameter for lipophilic chemicals. It has not yet been investigated whether this is also valid for the root compartment, although several experimental studies showed missing or very low translocation of lipophilic compounds to aboveground parts [67, 68], but an exception exists. Astonishingly, zucchini is able to take up and translocate different highly hydrophobic polychlorinated dibenzodioxins and furans (PCDD/F) congeners to leaves and to the entire fruit, whereas for pumpkin and cucumber, contaminants were shown to be restricted to the outer part of the fruit [69]. It was hypothesized that zucchini might release a binding substance for PCDD/Fs with root exudates, which forms a hydrophilic complex with the pollutant to enable the uptake by the plants' roots. Furthermore, molecules in leaf extracts and in the xylem sap of zucchini and melon (*Cucumis melo* L.) were detected with the ability to increase the apparent aqueous solubility of tetrachlorodibenzodioxin (TCDD) by forming a reversible binding [70]. More recently, 17-kD proteins (probably major latex-like proteins (MLPs)) in xylem sap of zucchini were suggested to influence the translocation of hydrophobic organic contaminants, as the expression of the *MLP-GR3* gene in *C. pepo* cultivars correlated positively with the presence of the 17-kD proteins and BCFs of dioxins and dioxin-like compounds [71]. The translocation of hydrophobic pharmaceuticals to shoots was as well enhanced in zucchini plants compared to soybean and closely related squash. Additionally, higher xylem sap solubilities of these chemicals were detected in zucchini, leading to the hypothesis of an involvement of xylem sap proteins in the enhanced translocation of pharmaceuticals to aerial tissues like for other ECs [62].

Dilution by growth is another factor influencing the concentration in plant parts, which is especially important for the prediction of the foliar uptake of organic compounds [29]. The resulting increased plant biomass leads to a potential dilution of the pharmaceutical concentration relative to the flux of their uptake. In contrast, expanded plant leaf area provides a larger surface for the *foliar uptake* of emerging contaminants from ambient air [30, 33]. The uptake of organic contaminants by aerial tissues was shown for many pesticides, polycyclic aromatic hydrocarbons (PAHs) or polychlorinated contaminants [72–75]. To enter the leaf, chemicals have

to either cross the cuticle or to enter through the stomata. Therefore, cutin, cuticular waxes and other cellular lipids act as a lipophilic barrier that might absorb different substances. A correlation could furthermore be detected between the surface wax concentration and the resistance to foliar penetration [76]. Although spray irrigation with treated wastewater contaminated with pharmaceuticals serves the possibility that these molecules are deposited on plants' leaves and could therefore be taken up, we are not aware of studies about the leaf penetration of these chemical contaminants. Some hints for the possibility of pharmaceuticals uptake by leaves are given [77]. Comparing the bioaccumulation in roots and leaves of a submerged and a free-floating plant species, differences in allocation of several pharmaceuticals could be detected. Highest concentrations of these chemicals were found in the plant tissue, which was exposed to the contaminated environment. Free-floating common water hyacinth (*Eichhornia crassipes*) having their roots exposed to different pharmaceuticals in water exhibited a higher concentration in the roots rather than leaves, except for carbamazepine which is known to be translocated to the leaves very fast [20]. For the submerged plant, burhead (*Echinodorus horemanii*), where leaves are surrounded by contaminated water, the tested compounds accumulated in the leaves in a higher proportion compared to roots. Even though submerged plants show differences compared to higher terrestrial plants (e.g. no transpiration, reduced xylem, thin cuticle), this study gives useful initial information about the possible uptake of pharmaceuticals by plant leaves.

Many pharmaceuticals are susceptible to *photodegradation*, which is an advantage in the wastewater treatment process to degrade them by UV treatment [78, 79]. As leaves are exposed to intensive light intensities, photodegradation within plants is theoretically possible, although no evidence about photodegradation of pharmaceuticals in plants is available till now.

2.4 Role of Biotransformation in the Translocation of Pharmaceuticals

The *biotransformation* of pharmaceuticals plays an important role in their translocation and risk assessment. From the intensive research about herbicide resistance in weeds, herbicide detoxification in crops and the removal of organic xenobiotics by phytoremediation, it has been known that plants possess an elaborate detoxification system for organic xenobiotics and agrochemicals, comprising of a metabolic cascade proceeding in three phases [80–82] (see Fig. 3). During phase I, xenobiotics can be activated by oxidation, reduction or hydrolysis depending on their molecule structure. The activated molecules can be conjugated to reactive groups, such as amino acids, glutathione or sugars by specific enzymes like glutathione *S*-transferases or glycosyltransferases to reduce the compounds reactivity and increase their water solubility during the consecutive phase II. Conjugated metabolites can afterwards be sequestered in vacuoles during phase III (*vacuolar sequestration*) or form

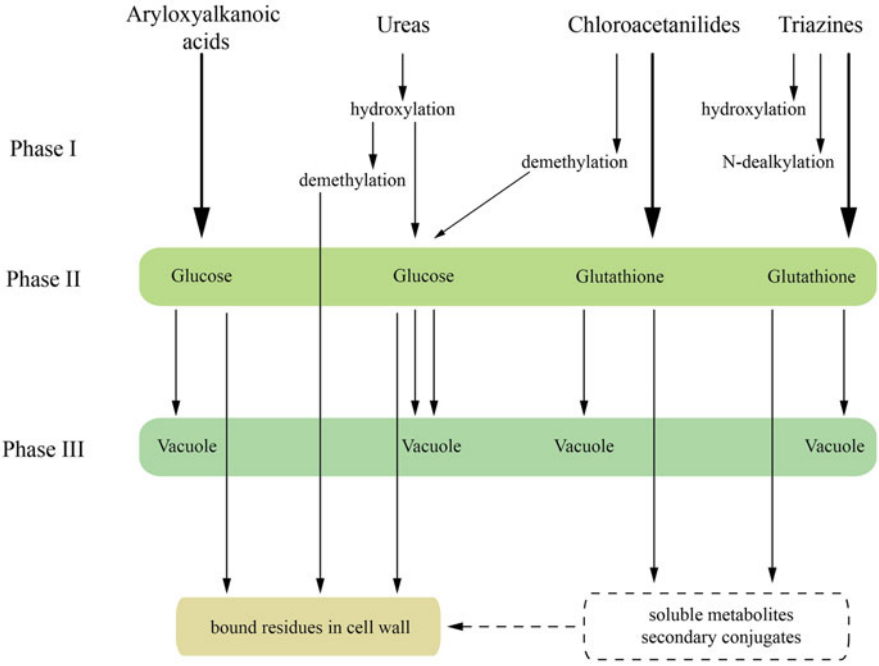

Fig. 3 The metabolic cascade of the green liver concept implies three phases for the fate of herbicides and foreign compounds in plants. It can be assumed that pharmaceuticals follow the same routes. While many compounds are finally bound to cell wall material to form insoluble residues, other xenobiotics may be stored in the vacuole as "soluble residues" and undergo further metabolism (adapted from [83])

insoluble residues in the cell wall (*bound residues*) [80] chapter "Metabolism of Pharmaceuticals in Plants and their Associated Microbiota". Several studies showed that this detoxification mechanism is also applicable for the metabolization of pharmaceuticals in plants [84–86]. The metabolization of particular pharmaceuticals can be differentially pronounced in plant tissues. Therefore, the metabolization of the anticonvulsant carbamazepine was noticeably higher in shoots than in roots, which might suggest a higher metabolism occurring in the leaves. However, one should bear in mind the fast translocation and the subsequent higher concentration of carbamazepine in shoots compared to roots [10]. Supporting this hypothesis, the phase I and phase II metabolites 4′-OH diclofenac, 4-O-glucopyranosyloxydiclofenac and 4-OH-glutathionyl-diclofenac were present in much higher concentrations in roots than shoots of cattail. These conjugates all originated from diclofenac, a pharmaceutical known to accumulate in roots rather than to be translocated to shoots [84]. In light of current literature, it is also possible that partially metabolized compounds, at least after phase I reactions, or even as conjugates can be translocated in plants via the vascular tissue [87, 88]. Nonetheless,

many recently published studies about the uptake and translocation of environmental contaminants overlook the concentration of metabolites. Neglecting pharmaceutical metabolites in environmental studies might lead to a severe underestimation of the uptake and translocation of pharmaceuticals in plants and eventually to an underestimated human exposure to these contaminants in food [89]. Therefore, it is always necessary to perform mass balance analysis because only this can provide clear-cut information to evaluate the potential metabolic routes of pharmaceuticals in distinct plant tissues.

Figure 3 displays the current knowledge about the detoxification cascade for herbicides [90]. While the traditional scheme of herbicide detoxification concluded in a phase III leading to bound cell wall residues has been well accepted for agrochemicals as the concept of the "green liver" [80], information on the fate of non-herbicidal pollutants and pharmaceuticals in plants is only poor and scattered. However, it can be assumed that pharmaceuticals undergo exactly the same metabolic steps since they possess similar molecular properties and sometimes derive from identical chemical families (e.g. triazines, sulfonylureas). Since experimental evidence indicated that xenobiotic glutathione or glucosyl conjugates may inhibit cytosolic processes [91], it has generally been accepted that xenobiotic conjugates are sequestered from the cytosol in higher plants during phase III.

2.5 Vacuolar Transport and Sequestration

Considering now the central dogma of xenobiotic metabolism in plants as valid that conjugation of xenobiotics may not be the end point of metabolism, a deeper look should be taken into plant storage processes. In fact, it seems that storage may be only intermediary for many substances and that further breakdown of these polar derivatives can lead to a complex set of processing reactions (Fig. 3), both in the vacuole and in the cytoplasm [92, 93]. One of the best studied routes of xenobiotic conjugate catabolism relates to glutathionylated pesticides [94]. An early report followed a chloroacetamide herbicide in cereals that could be tracked into the vacuole, where the respective detoxification products, glutathione conjugates, were cleaved by a carboxypeptidase to produce γ-Glu-Cys-alachlor conjugates [95].

Hence, it is not unlikely that ABC and MATE transporters in plasmalemma and tonoplast may also be involved in the detoxification of organic compounds other than herbicides, since enzymes involved in the synthesis of secondary compounds may also recognize and modify potentially toxic molecules taken up by the plant. Subsequently, molecules can yield cell wall residues or be transported into the vacuole for final detoxification (Fig. 3). Evidence for this latter sequestration step has been presented for several species and seems to be ubiquitous [96]. In a recent paper, the uptake and metabolism of the sun shield, oxybenzone, has been followed in umbrella papyrus (*Cyperus alternifolius*). Uptake and phase I and II metabolism followed the green liver concept, and it seems likely that some member of the ABCC subfamily was responsible for vacuolar delivery of the glutathionated phase II

metabolite [85, 97]. This is an important finding, since so far only plasma membrane-localized MATEs had been found to be involved in detoxification (reviewed in [98]). It is likely that further studies will reveal a role for vacuolar MATEs in cellular detoxification. Sequestration of detoxified compounds seems beneficial for the living plant cell, and the vacuole might be regarded as final storage compartment. Break down to smaller metabolites [95] or adding a malonyl residue alters the molecule so that backflush through the ABC transporters is prevented and final storage in vacuoles occurs [99, 100]. Interestingly, in umbrella papyrus the oxybenzone conjugate also undergoes partial cleavage and subsequent malonylation [85].

The significance of such phase III sequestration mechanism for the uptake of xenobiotics may be understood from the membrane potential across the tonoplast, which is −30 to −40 mV, and maintained by the activity of ATPases [101]. Since most ABC transporters are antiporters, the extrusion of cations leads to the accumulation of organic anions by a factor of 3 or 4 [96]. Such an efficient flow of xenobiotic metabolites will lead to a diminished cytosolic concentration of the active parent compound and hence be a strong driver for further diffusive uptake into the cell.

3 Experimental Section

For a bibliographic online search (using the search engine Google Scholar) of the scientific literature on plant uptake of pharmaceutical compounds, crossing 7 years of publications, authors used a combination of keywords as "plant uptake + pharmaceutical group" or "plant uptake + compound name" to obtain the highest number of articles within the topic and pharmaceutical group. Parameters like concentration applied in the study, time of exposure, type of experiment (hydroponic, pot or plate experiment), final concentration in the plant or plant part with clear units and plant species were used to decide which articles would be part of the study.

Field and lysimeter studies were not included due to their complexity and the number of external factors that can influence the results and therefore may not be compatible with the other studies. Experiments with different time points where concentrations in nutrient media/soil were not mentioned for the middle time points were also excluded, since it was not possible to calculate bioconcentration factors for these cases. Moreover, when no numerical data was provided in the studies, approximate values were extracted from figures with support of ImageJ software (version 1.52a) using the tools "set scale" and "analyse".

Chemical properties like molar mass (g/mol), logarithmic octanol-water partition coefficient (log K_{OW}) and water solubility (mg/L) were gathered from PubChem and/or DrugBank website, while the acid dissociation constant (pKa) and the logarithmic distribution coefficient (log D_{OW}) were calculated using the software SPARC Performs Automated Reasoning in Chemistry and values used according to the pH measured in each article.

The bioconcentration factor (BCF), which is the ratio of the concentration of a chemical in an organism to the concentration of the chemical in the surrounding environment [37], was calculated as:

$$\mathrm{BCF} = \frac{\mathrm{concentration}_{\mathrm{root}}\ (\mathrm{ng/kg})}{\mathrm{concentration}_{\mathrm{soil}}\ (\mathrm{ng/kg})} \quad \text{or}$$

$$\mathrm{BCF} = \frac{\mathrm{concentration}_{\mathrm{root}}\ (\mathrm{ng/kg})}{\mathrm{concentration}_{\mathrm{nutrient\ media}}\ (\mathrm{ng/L})}$$

The concentration in soil or nutrient media was applied as the difference between the spiked concentration and the concentration found at that particular time point; like this, a more realistic BCF can be obtained since it is only considered the concentration that was available for the plant.

The translocation factor (TF) or mobilization ratio was calculated to determine relative translocation from root to shoots (stem and/or leaves) [102]:

$$\mathrm{TF} = \frac{\mathrm{concentration}_{shoot}\ (\mathrm{ng/kg})}{\mathrm{concentration}_{\mathrm{root}}\ (\mathrm{ng/kg})}$$

Therefore, TF > 1 means that the target compound was effectively translocated from roots to shoots. In contrast, TF < 1 highlights an accumulation in the roots rather than a translocation to shoots.

For BCFs and TFs, plant-to-soil/nutrient media or leaves-to-root concentrations were both expressed in fresh weight (FW/FW) or dry weight (DW/DW). If not, data would be converted using the percentage of dry weight for each plant species.

3.1 Data Collected

A total of 53 ISI scientific articles and one technical report were used in this study. From all covered years, 2016 and 2018 presented the highest number of articles ($n = 11$) published on the uptake and translocation of pharmaceuticals in plants. Antibiotics ($n = 19$) and the psychotropic drugs ($n = 14$) were the pharmaceutical classes with the highest number of different compounds studied. Furthermore, antibiotics was the most frequent pharmaceutical class addressed in several articles (27.6%), followed by anticonvulsants (15.6%) and anti-inflammatory drugs (14.6%), which is showing a special interest by the scientific community in these chemicals. These numbers illustrate also, to which extent scientists are concerned about the presence and the potential effects of antibiotics, anti-inflammatory drugs, anticonvulsants and psychotropic drugs in the environment and in a second baseline, the publics' concern.

Regarding antibiotics, the main concern is the propagation of multiresistant bacteria and as a consequence, the dispersal of genes related to resistance against

those agents. This issue creates two main lines of scientific work: phytoremediation and human health risk assessment. From the collected articles, only 12.5% of the studies focused on phytoremediation [103–105], which shows a trend towards a focus on edible plants for further human risk assessments. In that respect, the most studied plant of the analysed studies was lettuce (*Lactuca sativa*) (29.65%), followed by radish (12.96%) and cucumber (7.41%), which are all economically relevant crops.

The duration of exposure in the collected studies varied between 6 h and 98 days; some showed only single time point measurements ($n = 32$) and others a time course with multiple time points ($n = 22$). Considering only single time points studies, in 71.9% of the cases, they tested a duration of at least 21 days. As it was mention before, only studies with multiple collection time points with given concentrations in nutrient media or soil at tested time points were used, to avoid overestimations of BCFs. It is also necessary to be aware of studies where nutrient solutions or soils were replenished/irrigated with solutions containing pharmaceuticals during the time course of the experiment when no information about volume, concentration and frequency of the added solution were mentioned to calculate the correct BCF. The tested concentrations of pharmaceuticals varied between 100 ng/L and 200 mg/L. In some studies, a single concentration was used, while in others, like Adeel and co-workers [106], several concentrations were studied ranging from 100 ng/L to 10 mg/L.

Taking into account all conditions and limitations presented above, data from selected publications was grouped and expressed as BCF and TF, according to the chemical properties and the ionic status of the compounds and additionally separated into trials done as hydroponic (a) and soil (b) experiments (Tables 1, 2, 3, 4, 5, and 6). Information is presented like this, because most of the concepts in the first part of this chapter can only be directly related to experimental data with controlled and/or few external interferences, as the hydroponic experiments. With the soil experiments, factors like the percentage of OM and even the soil constituents will interfere in the analysis, especially when comparing different studies, but on the other hand, the results will be closer to a realistic scenario.

The boxplots (designed using GraphPad Prism software, v 6.01) in Figs. 4, 5 and 6, which are showing the BCFs and TFs of the distribution of observations from different studies as well as minimum, median and maximum values, were also separated according to the ionic status of the compounds and the type of study (hydroponic and soil experiments), as mentioned above. One study can include several observations (shown by dots) by testing various conditions like duration, concentration or pH. Therefore, boxplots (Figs. 4, 5 and 6) provide a detailed picture of summarized data in Tables 1, 2, 3, 4, 5 and 6, and exceptions can be detected easily and considered for discussion to secure the validity of BCF and TF average values.

For the uptake and translocation of organic compounds, the molar mass with high possibility only plays a role for big molecules with molar mass $\geq$1,000 g/mol [21] or as hypothesized for pharmaceuticals with molar mass $\geq$400 g/mol [20]. None of the studied pharmaceuticals was $\geq$1,000 g/mol, and only eight of them can be

Table 1 Chemical properties of neutral pharmaceuticals, as well as the average BCFs and TFs calculated per compound in hydroponic studies

Compounds	log K_{OW}	pH	p*Ka*	log D_{OW}	BCF	TF	Authors
	Average values						
Analgesic							
Acetaminophen	0.46	5.60	0.00	0.09	1.43	0.49	[20, 58, 107–111]
Antibacterial							
Triclocarban	4.34	na	0.00	5.23	31.39	0.01	[109, 110]
Antibiotic							
Sulfamethoxazole	0.89	5.59	0.00	−0.06	0.55	0.13	[11, 109, 110, 112–114]
Sulfapyridine	0.35	6.53	0.00	4.21	3.29	0.03	[105]
Anticonvulsant							
Carbamazepine	2.45	6.23	0.00	3.64	0.93	2.05	[10, 11, 20, 110, 114–119]
Primidone	0.91	na	0.00	−1.23	1.61	0.17	[109]
Hormone							
17β-estradiol	0.20	na	0.00	4.33	2.01	1.11	[106]
17α-ethinylestradiol	3.67	5.30	0.00	4.94	0.98	1.04	[106, 112, 114]
Beta-estradiol	3.67	5.55	0.00	4.33	0.01	nd in leaf	[20, 114]
Estrone	3.13	5.55	0.00	4.23	0.13	0.07	[20, 114]
Levonorgestrel	3.48	na	0.00	4.27	17.26	nd in leaf	[101]
Lipid regulator							
Atorvastatin	6.36	na	0.00	2.38	0.48	0.26	[109]
Psychotropic drug							
Meprobamate	0.70	na	0.00	1.16	0.37	6.11	[109, 110]
Stimulant							
Caffeine	−0.07	5.68	0.00	0.95	0.32	12.06	[20, 109, 114, 116, 119, 120]

Symbols: na, means not available; *nd*, means not detected

considered as large-sized pharmaceuticals, as mentioned by Chuang and co-workers [20]. Of these pharmaceuticals, five were antibiotics (clarithromycin, streptomycin, oxytetracycline, tetracycline and lincomycin), two drugs against high blood pressure (verapamil and valsartan) and one lipid regulator (atorvastatin). All selected compounds can enter the roots, and only a minor amount of the tested pharmaceuticals could have difficulties to enter because of their high molar mass.

Table 2 Chemical properties of neutral pharmaceuticals, as well as the average BCFs and TFs calculated per compound in soil studies

Compounds	log K_{OW}	pH	p*Ka*	log D_{OW}	BCF	TF	Authors
	Average values						
Analgesic							
Acetaminophen	0.46	8.10	0.00	0.09	0.01	0.34	[102]
Antibacterial							
Triclocarban	4.34	6.42	0.00	5.23	0.02	0.50	[121]
Antibiotic							
Sulfamethoxazole	0.89	5.67	0.00	−0.06	0.96	0.58	[11, 112, 122, 123]
Anticonvulsant							
Carbamazepine	2.45	7.03	0.00	3.64	0.62	3.57	[11, 102, 112, 123–128]
Hormone							
17α-ethinylestradiol	3.67	6.60	0.00	4.94	0.61	0.06	[129]
Estrone	3.13	8.10	0.00	4.23	0.00	2.45	[20]
Psychotropic drug							
Oxazepam	2.24	6.30	0.00	3.42	0.04	17.15	[130]
Temazepam	2.19	6.30	0.00	4.71	0.01	5.99	[130]
Stimulant							
Caffeine	−0.07	7.87	0.00	0.95	0.23	20.03	[102, 126, 127]

Symbols: na, means not available; *nd*, means not detected

3.2 Data Analysis

3.2.1 Neutral Compounds

Neutral organic compounds were identified as having higher membrane penetration than ionized substances [140]. Therefore, it is expected, that these molecules can be taken up and translocated easily by transpiration via the xylem [51], resulting in TFs > BCFs. For compounds like meprobamate, caffeine and carbamazepine and, additionally, estrone, oxazepam and temazepam (in soil assays), this pattern was observed (Tables 1 and 2). Figure 4 shows that many observations and studies were made on the uptake and translocation of caffeine and carbamazepine, reflecting a TF > BCF, which clearly underlines their validity. However, this pattern is not clearly detected for the whole group of compounds.

Looking at the data in detail, triclocarban (antibacterial) stands out with an average BCF of 31.4, as a result from data reported in Sun and co-authors [109] and Wu and co-authors [110] (Table 1). In total, these two studies had nine observations, and none of them had TF higher than 0.08 (Fig. 4). BCFs (18.9–32.4) obtained by Wu and co-workers [110] for spinach and lettuce, when exposed for 21 days at two different concentrations (5 and 0.5 μg/L), were similar, and also Sun and co-authors [109] observed a relatively high BCF (12.6) when cucumber was exposed for 7 days to a concentration of 5.0 μg/L. Therefore, the

Table 3 Chemical properties of anionic pharmaceuticals, as well as the average BCFs and TFs calculated per compound in hydroponic studies

Compounds	log K_{OW}	pH	p*K*a	log D_{OW}	BCF	TF	Authors
	Average values						
Antibacterial							
Triclosan	4.76	5.47	−0.01	5.42	1.50	0.20	[9, 19, 41, 55, 117]
Antibiotic							
Ofloxacin	−0.39	7.80	−0.16	0.74	0.01	nm in leaves	[131]
Oxytetracycline	−0.90	5.97	−0.15	−6.49	0.59	0.02	[25, 55]
Sulfadiazine	−0.09	6.81	−0.88	1.04	14.87	0.15	[46, 87]
Sulfamerazine	0.14	6.81	−0.58	3.54	25.98	0.03	[105]
Sulfamethazine	0.89	6.85	−0.23	4.39	9.13	0.02	[105, 116]
Sulfamethoxazole	0.89	6.85	−0.002	−0.06	16.61	0.02	[105]
Sulfapyridine	0.35	6.91	−0.01	4.21	11.78	0.03	[105]
Tetracycline	−1.30	na	−0.08	−5.44	0.15	3.18	[103]
Anticonvulsant							
Dilantin	2.47	na	−0.003	1.71	0.95	2.98	[41, 110]
Anti-inflammatory							
Diclofenac	4.51	6.61	−0.98	1.85	2.82	0.24	[43, 55, 56, 65, 101, 132]
Ibuprofen	3.97	5.48	−0.90	2.25	0.21	1.52	[109, 110, 116, 133–135]
Naproxen	3.18	5.65	−0.89	2.09	0.61	0.90	[109, 110, 114, 119]
Lipid regulator							
Clofibric acid	3.32	6.00	−0.99	1.20	1.37	1.59	[119]
Gemfibrozil	4.77	na	−1.00	2.92	4.03	0.04	[109]

Symbols: *na*, means not available; *nm,* means not measured

dynamic between BCF and TF reported in the two studies was the opposite of what was expected (i.e. BCF > TF) but might be explained due to the high lipophilicity of triclocarban (log K_{OW} = 4.34). Indeed, several models purposed different ranges during which translocation is favoured or not. All of these models predicted a low transfer for compounds with around log $K_{OW} > 4$ [25, 61, 140, 141].

Another neutral compound that stands out from the proposed observation was levonorgestrel (hormone). Li and co-workers [101] described a BCF average of 17.3, where no compound was detected in stems and leaves. Therefore, further investigation is needed to scrutinize these results.

Sulfamethoxazole was another pharmaceutical, which was studied intensively in hydroponic and soil experiments. This antibiotic and the analgesic acetaminophen, which is also studied on several hydroponic experiments, showed a slightly higher average BCF > TF. Moreover, as many observations were showing similar results for these two pharmaceuticals, the validity is high. The reason for this might be their

Table 4 Chemical properties of anionic pharmaceuticals, as well as the average BCFs and TFs calculated per compound in soil studies

Compounds	log K_{OW}	pH	p*K*a	log D_{OW}	BCF	TF	Authors
	Average values						
Antibacterial							
Triclosan	4.76	7.05	−0.11	5.33	1.09	1.06	[103, 116, 121, 124, 125, 129]
Antibiotic							
Amoxicillin	0.87	7.01	−0.40	−2.05	0.00	na	[132]
Oxytetracycline	−0.90	7.50	−0.91	−6.64	0.00	0.58	[102]
Sulfadiazine	−0.09	7.50	−0.98	1.02	0.65	1.72	[102, 136]
Sulfamethoxazole	0.89	7.68	−0.03	−0.06	0.27	0.77	[11, 102, 123]
Tetracycline	−1.30	7.28	−0.66	−5.59	0.00	na	[132]
Trimethoprim	0.91	8.10	−0.44	0.67	0.00	5.38	[102]
Anti-inflammatory							
Diclofenac	4.51	6.25	−0.98	2.13	2.02	2.43	[125, 126]
Ibuprofen	3.97	7.42	−0.97	2.08	2.51	1.38	[126, 127]
Naproxen	3.18	na	−0.95	1.86	0.24	0.51	[126]
Blood pressure							
Furosemide	2.03	7.42	−1.00	0.73	1.27	nd in leaves	[127]
Lipid regulator							
Clofibric acid	3.32	7.42	−1.00	1.20	1.11	0.04	[127]
Psychotropic drug							
Diazepam	2.82	6.30	−0.99	4.73	0.03	3.13	[130]

Symbols: *na*, means not available; *nd*, means not detected

metabolization by plants [105, 118]. As mentioned before, the fast biotransformation of some pharmaceuticals should not be neglected to not underestimate BCFs and TFs of the parent compound.

3.2.2 Anionic Compounds

Among the anionic compounds, antibiotics are represented by the largest group of studied substances in both hydroponic and soil experiments. Within antibiotics, the sulfonamides (SAs), i.e. sulfadiazine, sulfamerazine, sulfamethazine, sulfamethoxazole and sulfapyridine, display the largest group. They are widely used for the control of infectious diseases, in both human and livestock care, and due to their stability – with a half-life over 81 days [105] – they are ubiquitously present in wastewaters. Therefore, SAs receive a special attention by the researchers since they are prone to increase the resistance of pathogenic bacteria and boost the spread of antibiotic resistance, hostile to aquatic environments and human health. According to Wang and co-authors [142], the uptake process of these molecules might be

Table 5 Chemical properties of cationic pharmaceuticals, as well as the average BCFs and TFs calculated per compound in hydroponic studies

Compounds	log K_{OW}	pH	p*K*a	log D_{OW}	BCF	TF	Authors
		Average values					
Antibiotic							
Clarithromycin	3.16	na	1.00	−1.98	9.91	0.04	[137]
Lincomycin	0.20	5.80	1.00	−4.84	0.33	0.08	[20]
Trimethoprim	0.91	5.80	0.88	0.09	2.45	0.23	[20, 97, 110, 112]
Anticonvulsant							
Lamotrigine	2.57	6.05	0.65	2.49	7.86	0.12	[138]
Antidiabetic							
Metformin	−2.64	6.00	1.01	−2.56	32.14	0.02	[65]
Beta-blocker							
Atenolol	0.16	7.80	0.98	−2.23	0.21	2.79	[11, 109, 131]
Propranolol	3.48	na	1.00	0.15	0.92	0.22	[116]
Lipid regulator							
Gemfibrozil	4.77	5.30	0.81	3.56	–	0.06	[114]
Psychotropic drug							
Amitriptyline	4.92	7.00	1.00	3.60	29.85	1.11	[117]
Clomipramine	5.19	na	1.00	2.35	0.18	0.62	[139]
Diazepam	2.82	na	0.01	4.73	3.21	0.45	[109, 110]
Fluoxetine	4.05	7.00	1.00	1.05	13.96	1.03	[110, 117]
Sertraline	1.37	na	1.00	2.27	0.43	0.12	[139]
Trazodone	3.21	na	0.10	3.97	0.09	2.89	[139]

Symbols: na, means not available; − not possible to calculate

slower, when compared to cationic and neutral compounds due to electrostatic repulsion between root surface and anionic substances. However, looking at data from the hydroponic experiment of Tai and co-workers [105] (Table 3, Fig. 5a), high BCF ratios of SAs, ranging from 9.1 to 26.0, were quantified in two wetland plant species (Indian shot (*Canna indica*) and yellow iris (*Iris pseudacorus*)) in a 7-day trial. In this work, authors suggested that plants take up SAs via active processes. However, the high BCF values might be related to the plant lipid content, since it is considered as the main storage site for hydrophobic organic contaminants, as hypothesized by the same group. To support this hypothesis, a positive correlation between the obtained BCF and the respective log D_{OW}, for several nutrient media and soil articles (cited in Tables 3 and 4), was calculated (0.29 and 0.42, accordingly ($p > 0.05$)). Nonetheless, for a specific antibiotic (tetracycline), the results were the opposite (i.e. TF > BCF), meaning that this compound is rather translocated to the aerial parts than being stored in roots [104], which can be explained by its hydrophilic behaviour (log D_{OW} −5.44).

As observed for SAs, high average BCF > TF values for triclosan, diclofenac and gemfibrozil were registered in hydroponic experiments (see Table 3). Several studies focused on the antibacterial pharmaceutical triclosan, but only in some of them, high average BCFs were obtained. It can be highlighted that highest BCFs were

Table 6 Chemical properties of cationic pharmaceuticals, as well as the average BCFs and TFs calculated per compound in soil studies

Compounds	log K_{OW}	pH	p*K*a	log D_{OW}	BCF	*TF*	Authors
		Average values					
Antibiotic							
Lincomycin	0.20	7.58	0.75	−3.35	0.00	9.96	[102]
Anticonvulsant							
Lamotrigine	2.57	8.10	0.18	2.70	0.03	1.93	[102]
Antidiabetic							
Metformin	−2.64	na	1.01	−2.56	0.34	0.61	[126]
Beta-blocker							
Atenolol	0.16	6.96	0.99	−2.60	0.39	3.51	[124]
Propranolol	3.48	6.63	0.99	0.59	2.59	1.97	[125]
Psychotropic drug							
Chlordiazepoxide	2.44	6.30	0.43	−0.12	0.04	6.58	[130]
Clonazepam	2.41	6.30	0.01	3.56	0.01	16.82	[130]
Fluoxetine	4.05	6.25	1.00	1.07	0.04	0.24	[125]
Flurazepam	3.80	6.30	1.00	3.77	0.01	1.24	[130]

Symbols: *na*, means not available

calculated for several plant species (cucumber, lettuce, spinach (in hydroponic experiments) and for ryegrass and lettuce (in soil)), with a time exposure ranging from 7 to 40 days [109, 110, 125, 127]. For all the selected cases, the applied concentrations were relatively low (2.7–69.0 μg/L), when compared to the rest of the studies (5.0–758.0 μg/L), which might indicate a more efficient uptake for lower applied concentrations. For the well-studied anti-inflammatory drug diclofenac, ten times higher average BCF > TF values were detected in hydroponic experiments (Table 3); nonetheless, four of thirteen studies had higher BCFs (3.2–17.7) than the rest of the studies (BCF ≈ 0.5; Fig. 5a) [101, 111, 112, 126]. Several works therefore reported that this pattern is caused by the hydrophobicity of diclofenac [115, 119], but as for charged molecules, the log D_{OW} rather than the log K_{OW} should be considered. Since this compound has a log D_{OW} of 1.85 translocation should be favoured, however it is not the case. As mentioned in the first part of the chapter, the protein plant composition might play an important role on storage of anionic compounds in roots, as discussed by González García and co-authors [36].

The same pattern (BCF > TF) was also obtained for gemfibrozil (lipid regulator) in a 2-week study with old cucumber plants [109] (Table 3); this result might be related to the high metabolism of young plants, since for different type of compounds (neutral, anionic and cationic) BCF > TF were registered in this study. In any case, further investigation is needed to evaluate the uptake results according to rigorous pH measurements, since this molecule dramatically changes its ionization status (p*K*a 0.8 to −0.99) in a very short pH interval (5.3–6).

In contrast to the behaving of most of the anionic compounds, dilantin (anticonvulsant) presented a higher average TF (2.9) when compared to its BCF (0.9)

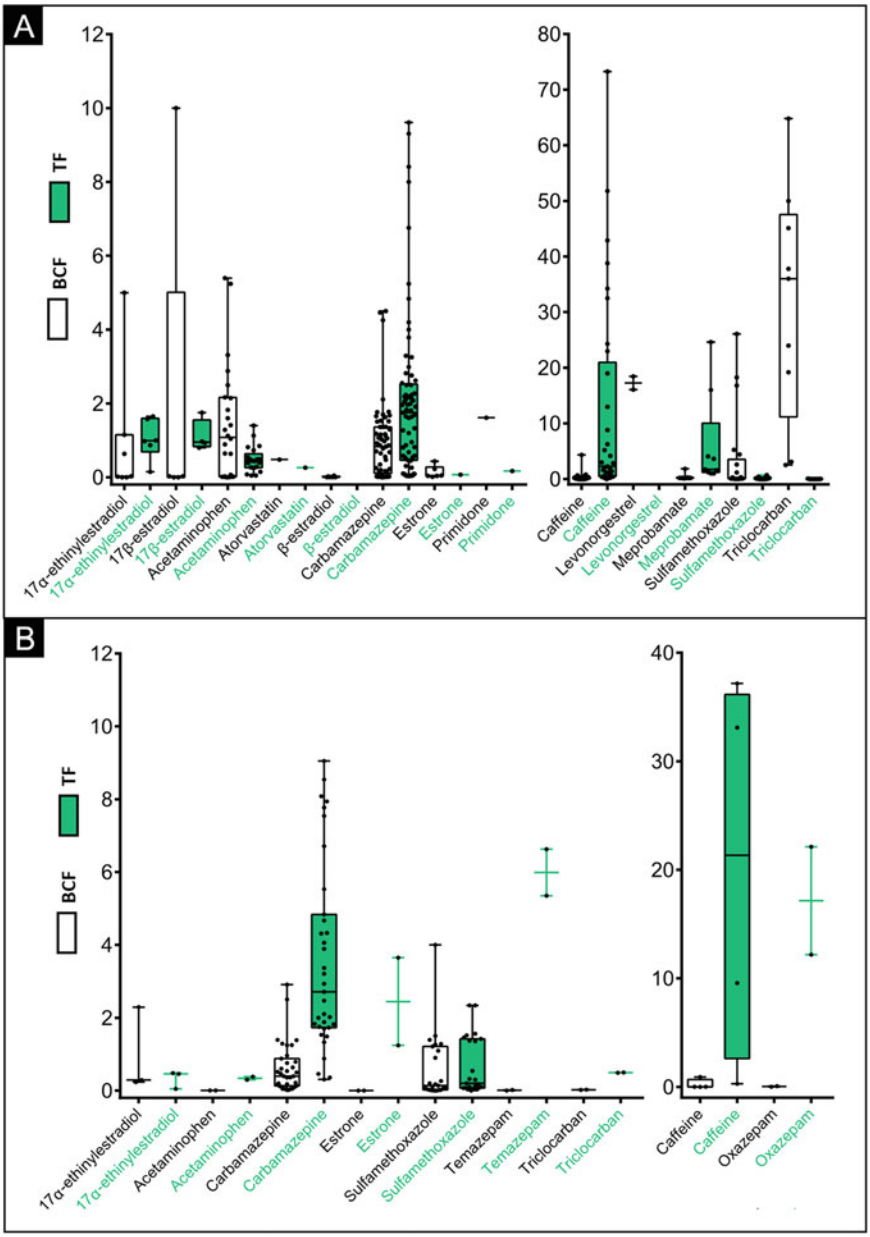

Fig. 4 Boxplot visualization of all BCF (black) and TF (green) values of several neutral compounds (every dot, represents an observation) from hydroponic (**a**) and soil studies (**b**); data references in Tables 1 and 2

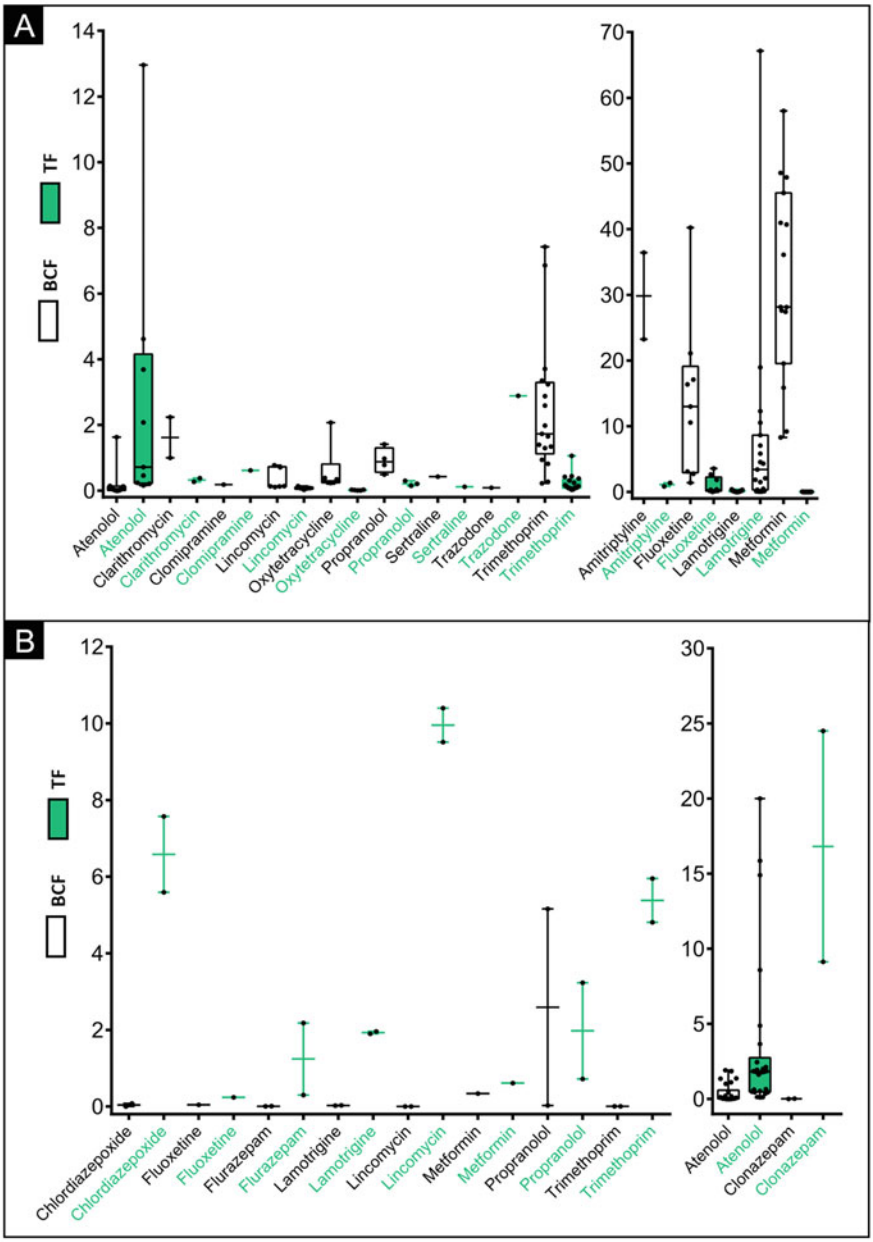

Fig. 5 Boxplot visualization of all BCF (black) and TF (green) values of several anionic compounds (every dot, represents an observation) from hydroponic (**a**) and soil studies (**b**); data references in Tables 3 and 4

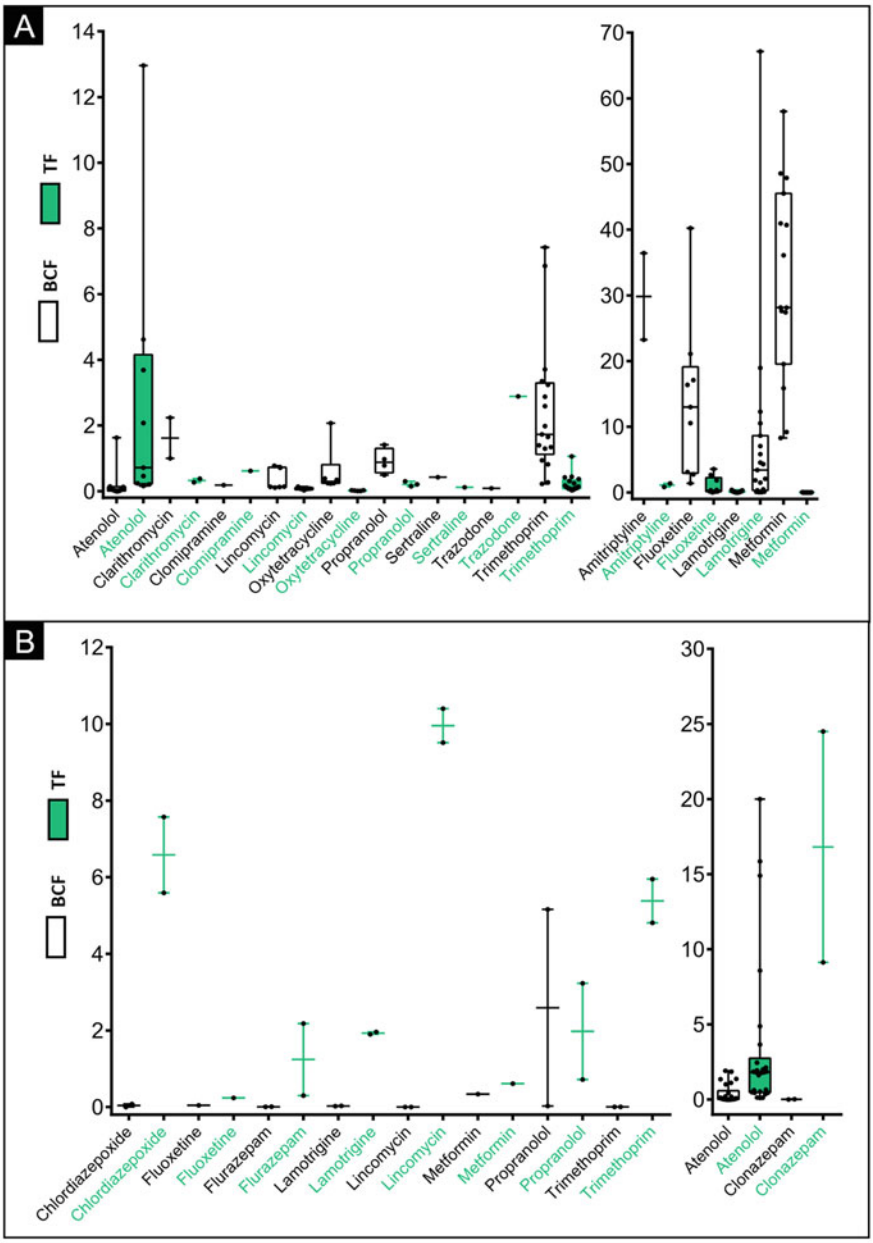

Fig. 6 Boxplot visualization of all BCF (black) and TF (green) values of several cationic compounds (every dot, represents an observation) from hydroponic (**a**) and soil studies (**b**); data references in Tables 5 and 6

(Table 3). These results are mainly represented by Wu and co-workers [110], where the highest translocations were observed for pepper plants (*Capsicum annuum*) even when exposed to different concentrations (0.5 and 5 μg/L), which might indicate a favoured translocation because of the plant species. Moreover, dilantin displays only a slightly negative p*K*a (−0.003), which could mean that its behaviour is more similar to a neutral compound, like carbamazepine, than to an anionic one.

The uptake of the psychotropic drug diazepam was studied in a radish experiment in soil [130]. As for all studied compounds on this crop, TF values were higher than the ones for BCF (Table 4, Fig. 5b), however according to its log D_{OW} (4.73), it would be expected the opposite, which might indicate the important role of this specific plant species [11, 102, 112, 121, 124]. This hypothesis is also supported by the higher BCF > TF values of diazepam in different other plants (cucumber, lettuce, pepper, spinach), which was tested in hydroponic experiments [109, 110].

Lastly, average TF values of ibuprofen (anti-inflammatory) were higher than average BCF values in hydroponic studies [109, 110, 126, 127, 135]. However, these differences are mainly caused by the presence of an outlier in TF observations (see Fig. 5a).

3.2.3 Cationic Compounds

In hydroponic studies with cationic compounds, generally higher BCFs>TFs were obtained (Tables 5 and 6, Fig. 6). The main reason behind this observation might be the fact that plant cell walls are negatively charged, due to their high concentration in uronic acids [142]. The electrostatic attraction between the root cell wall and the cationic compounds may facilitate adsorption to the root epidermis. Compounds that are positively charged at pH 4–6 can be trapped in the apoplast or root vacuoles (pH 5) [63]. Consequently, a reduced concentration can enter the vascular system for the translocation to aerial parts.

Among these cases, atenolol (beta-blocker) and trazodone (psychotropic drug) presented TFs > BCFs. For both compounds, this might be related to the high concentrations applied (830–1,000 and 10,000 μg/L, respectively) and to the plant species used [11, 139]. Kedosová and colleagues [11] registered higher atenolol concentrations in leaves of radish and spinach than in arugula and lamb's lettuce. Additionally, in the study of Reichl et al. [139], high amounts of trazodone in cress aerial tissues (*Lepidium sativum*) were registered, showing that uptake efficiency is dependent of the plant species used, and therefore, for studies of human health risk assessment, different plant species should be tested to estimate more reliable risks.

For soil data, when compared to BCFs values, higher TFs were calculated (Table 6). According to Miller and co-workers [9], some evidences were already demonstrated, that cationic compounds applied to soil have higher TF values than, for example, anionic ones. However, in our studies no correlation was found between TFs and the respective log D_{OW}, suggesting that other factors might be more relevant for the translocation of cationic compounds.

4 Recommendations and Outcomes from Data Analysis

When compiling data to do this analysis, it became obvious that some articles had to be omitted, because there was a lack of important information needed to compare data between studies. Basic guidelines for controlled uptake and translocation studies, including relevant properties of the compound, the plant and the environment, are crucial to produce valid results. Indeed, comparability and reliability of scientific data have become burning topics recently and therefore were discussed by many publishing and governmental agencies, which are concerned about data integrity and how data can be made "available" for all stakeholders. Accordingly, a resume of recommendations for future studies might be:

A crucial parameter is the *concentration applied* in water or soil at the beginning of each study as theoretical and analytical value. In several articles where both concentrations were provided, theoretical and practical concentrations varied significantly for specific compounds. In any case, similar *concentration units* (expressed in fresh weight or dry weight) should be provided, to better relate data expressed in the same units.

In case *additional irrigation or replenishment* of nutrient media is needed, during the time course of the experiment, authors should mention the volume of water added, frequency of occurrence and if irrigation water was previously spiked with pharmaceuticals. Additionally, the quantification of the spiked irrigation water is a crucial information to calculate the exact concentration to which the plant was exposed. This is very important when estimating the BCF, since the concentration in nutrient media/soil is always considered as a base. In many cases, authors only relate its value to the concentration at T0, which finally leads to an overestimation of BCFs. Also, if the nutrient media is completely renewed, the concentration before and after removal should be measured and mentioned. For *kinetic studies*, it is moreover important to quantify the concentration in the nutrient media/soil at each sampling time, in order to relate it to the concentration in the plant at that specific time point and avoid wrong BCF assumptions.

In all the cases, *pH* measurements – in nutrient media or in pore water and soil – are recommended at least for each time point of collection. Some *chemical properties* (i.e. p*K*a and log *D*ow) of selected compounds are dependent on the measured pH values; this is central when compounds change their ionic status easily in a very narrow pH range.

Moreover, authors should always consider using different *controls*, i.e. the inclusion of negative controls (where no plant is included in the spiked nutrient media/soil, which is used to evaluate the adsorption and potential degradation along the study) and the plant in a non-spiked situation (to evaluate the plant growth performance in normal conditions).

For soil studies, measuring soil properties besides pH, like *percentage of humidity* and *organic carbon content* plus the *soil porosity* and *texture*, is recommended to enable the comparison of studies and diminish the bias.

Another parameter influencing the uptake and translocation of pharmaceuticals is the *plant* per se. It is recommended to consider the plants' age (number of days after germination) and developmental stage (e.g. two-leaf stage, vegetative growth or flowering/fruiting) at the time point of exposure and during the study. The plant variety, the percentage of dry weight (root and aerial part) as well as the total lipid content should be provided, since this information is necessary to successfully indicate the differences on the uptake and translocation of especially lipophilic pharmaceuticals in different plant organs or varieties.

Analytically, the *extraction protocol* for target compounds in the different studied matrices should be provided along with the specific *limits of detection* and *quantification*. This is essential when authors cannot quantify a specific compound, so the readers can understand if this is due to an analytical limitation or if the compound is not present in that matrix. Furthermore, concentrations of pharmaceuticals in plant tissues can be easily underestimated when only parent compounds are quantified. As some pharmaceuticals can undergo a rapid metabolization within a few hours, it is recommended to consider the measurement of the main *metabolites*, if technically possible, to prove the uptake and translocation of such compounds.

4.1 Concluding Remarks

In many studies it became obvious that the concentration in nutrient media/soil does not correlate with the concentration in plants, and thus it is not easy to forecast transfer rates. Chemistry and plant physiology play important roles in the processes involved. Moreover, interactions with soil constituents, rhizosphere processes governed by microbes and the selective uptake mechanisms of several plant species may be decisive for the fate of PPCP as well. The concentration of pharmaceuticals applied in controlled experiments may affect in opposite way the BCF and TF ratio values, since in some studies higher uptake and translocation ratios were achieved with lower concentrations, which is highlighting the relevance of realistic environmental concentrations in uptake studies. Some plant species may also have special features, such as Cucurbitaceae, which is known to be the only family to take up and translocate hydrophobic PAHs. Interestingly, radish from the Brassicaceae family stands out with consistent higher translocations, for all pharmaceutical compounds in the analysed studies. Furthermore, it may hold true that most cationic pharmaceuticals show higher TFs in soil studies, but some will also undergo activation and metabolization on the way, which might change their behaviour and fate. As highlighted before, it is crucial to take all relevant plant and physicochemical properties into consideration through every step of the scientific process that starts with the experimental design and ends with data analysis and interpretation.

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Part II
Fate, Uptake and Metabolism of Drugs in Crops

Soil Sorption and Degradation Studies of Pharmaceutical Compounds Present in Recycled Wastewaters Based on Enantiomeric Fractionation

Monica Brienza, Belinda Huerta, Rayana Manasfi, and Serge Chiron

Contents

Abstract Wastewater (WW) reuse and biosolid application for vegetable crop culture is a practice applied worldwide. This strategy helps mitigate the pressure on water resources and improve the fertility of soil. Wastewater reuse is currently not included in chemical risk assessment, but its application has risk of potential accumulation of contaminants of emerging concern such as pharmaceutical active compounds (PhACs). In fact, this practice has caused the uptake of PhACs by plant and their subsequent entrance on the food chain. Residual quantities of contaminants may enter in soil, and they can be accumulated or percolated, consequently leading to contamination of groundwater. Herein, we report the main factors that play an important role on the accumulation of PhACs in soil after irrigation with treated wastewater. Limited data is actually available on the fate of PhACs in field studies because several processes are in competition for their dissipation including sorption

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and formation of non-extractable residues, leaching, as well as biotransformation. Consequently, an approach based on enantiomeric fractionation of chiral PhACs has been suggested to discriminate between biotic and abiotic dissipation processes.

Keywords Abiotic dissipation, Ameliorating, Biosolid, Biotransformation, Osmotic effects

1 Introduction

Water is fundamental for food production, and the increasing scarcity of this essential natural resource has significant repercussions for the ability of humanity to feed itself. The exponential world population growth and associated food consumption are exerting immense pressure on freshwater resources, resulting in groundwater withdrawal rate to increase 1% per year since the 1980s. Water for agriculture is at the core of any discussion of water and food security. Agriculture accounts for approximately 70% of all water withdrawals globally. Competition for water resources is expected to increase in the future due to food demand growth by at least 50% by 2050. A greater application of nonconventional, alternative sources of water, such as wastewater effluents, could mitigate this situation.

Wastewater is defined as a combination of one or more of black water (excreta, urine, fecal sludge), gray water (kitchen and bathing wastewater), commercial and industrial effluents (including hospitals), storm water and other urban runoff, as well as agricultural, horticultural, and aquaculture effluents. In fact, according to the UN (2003), about 200 million ha in more than 50 countries are irrigated with untreated and/or treated wastewater. In Israel, treated wastewater (TWW) has been used for crop irrigation since the early 1980s [1]. Countries such as Syria, Iraq, and Mexico use more than 40% of their municipal wastewater for this purpose [2]. Likewise, agricultural irrigation with TWW is a common practice in many other areas, including Greece, Italy, Spain, France, and China [3–5].

Wastewater treatment produces also large amounts of biosolids, which are considered a good source of organic matter and beneficial plant nutrients, especially N and P, therefore becoming good soil-ameliorating agents [6]. Approximately 4 billion tons of solid waste (municipal, industrial, and hazardous waste) is produced globally on an annual basis. For example, the generation of municipal solid waste (MSW) ranges from 1.6 to 2 billion tons [7]. The reuse of wastewater and biosolids in agriculture brings many social and economic benefits and contributes to agricultural and environmental sustainability.

However, there are some negative effects related to the application of treated wastewater on soil and crops, including (1) osmotic effects on the water potential of the soil and plants [8]; (2) toxic effects due to high concentration of ions, e.g., sodium, chloride, and boron [9, 10]; and (3) alteration on the physical properties of

soil such as increase of sodium adsorption ratio, hydraulic conductivity and aggregate stability due to high sodium adsorption ratio [SAR] and exchangeable sodium percentage [ESP], and consequences in the rooting zone [11–16]. In addition, wastewater irrigation results in continuous discharges of heavy metals, pathogens, resistant organic pollutants, and other "contaminants of emerging concern" into the agro-food system. The focus of human/environmental concern has now included pharmaceutical active compounds (PhACs) that enter the environment predominantly through domestic routes and ultimately end up in biosolids from wastewater treatment plants (WWTPs). Humans and animals are the main producers of pharmaceutical residues in the environment, although PhACs are also used to control the bacterial infections of plants through injection or soil drenching. Soil amendments, fertilizers made of contaminated animal manure, and biosolids also introduce pharmaceuticals into water cycle through drifting, surface runoff, irrigation, and leaching of residues deep into the soil layers [17, 18].

PhACs have hardly been studied in soil environmental matrices under real agricultural conditions, and the very limited data on their occurrence is associated with research on pharmaceutical removal following effluent irrigation onto land [19]. The chemical risk assessment frameworks only require testing in simulation soil studies, usually applying radioactively labelled compounds and often studying each individual compound separately. The results of these studies are actually poorly transferable to specific situations such soils irrigated with TWW with a high content of organic matter and a large diversity of PhACs, making the prediction of their fate still very difficult.

The aim of this chapter is to report the main factors that play an important role on the accumulation of PhACs in soil after irrigation with TWW, and special focus will be on a new tool based on enantiomeric fractionation of chiral PhACs to investigate their fate in field studies where the use of radioactively labelled compounds is not allowed.

2 Pharmaceuticals in Soil: Occurrence, Sources, and Fate

PhACs are considered as emerging contaminants as many of them are ubiquitous, persistent, and biologically active substances. The main route for them to enter the soil is through treated or untreated wastewater irrigation and biosolid amendments. The fate of PhACs in soil has been scarcely investigated due to complexity of the soil-water-plant system. The potential occurrence, sources, and fate of PhACs in agricultural soils irrigated with wastewater or amended with biosolids are serious causes for concern due to their potential uptake by crops and their potential introduction into the food chain [20].

2.1 Factors Affecting Pharmaceutical Concentrations in Wastewater Effluent and Irrigation Water

Kasprzyk-Hordern et al. [21] explained that the probability of soil and groundwater contaminations by PhACs as a result of the discharge of wastewater treatment effluents depends on factors such as the physicochemical properties of these pollutants, the type of wastewater treatment used, and climatic conditions (e.g., temperature, rainfall, and irradiation).

Irrigation wastewater quality must be compliant with FAO guidelines [22]. The characteristics of the wastewater used for irrigation can help in elucidating the potential transfer of these contaminants from water to soil. We can find an example in the following parameters: the concentrations of Biochemical Oxygen Demand (BOD_5) and Chemical Oxygen Demand (COD). COD is the amount of oxygen required to chemically oxidize organic matter in wastewater into inorganic matter, whereas BOD is the amount of oxygen required to biologically oxidize the organics, usually after 5 days or 21 days of incubation time, depending on the bioassay followed. The ratio of BOD/COD of wastewater is a good indicator of the concentrations of the total organic load (or oxygen demand) that is bioavailable for degradation. This organic load affects the bioavailability of weakly acid pharmaceuticals. Polar interactions between acidic pharmaceuticals and dissolved organic matter (DOM) create water-soluble complexes that are not available for uptake or sorption to the solid phases, which also reduces their concentrations in soil. Another important parameter is the water pH, because it determines the dissociation of ionic organic compounds. For instance, in slightly alkaline water, contaminants such as diclofenac and sulfamethoxazole are present mainly as ions, whereas trimethoprim is in its neutral form.

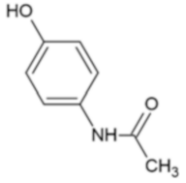
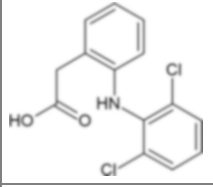
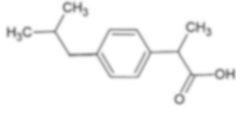
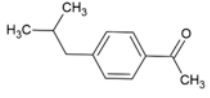
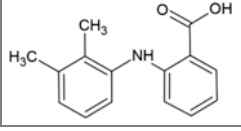
Wastewater treatment plants were not designed to remove PhACs. In fact, removal efficiencies drop to $<10\%$ for compounds such as carbamazepine, atenolol, mefenamic acid, and atenolol. The PhAC concentrations discharged into the environment vary according to time, space, season, and socioeconomic aspects, as they depend on usage patterns, location, input of manufacturing facilities, and the presence of hospitals. For example, antihypertensive, antibiotic, and nonsteroidal anti-inflammatory drug use increases during winter while sunscreens and antihistamines during summer. The concentrations found in effluents used for irrigation are in the range up to $\mu g\ L^{-1}$ [23, 24], and it depends on the season due to the dilution effect brought by the higher flow in January respect in spring/summer season. Biel-Maeso et al. [25] established the connection between presence, quantity, and seasonal distribution of several PhACs in wastewater and sewage-impacted receiving soils. Compounds in urban wastewater were detected at concentration from 73 to 372 $\mu g\ L^{-1}$ in the influent and from 3 to 41 $\mu g\ L^{-1}$ in the effluent. Removal efficiencies were $<50\%$ and only traces ($ng\ g^{-1}$) of PhACs such as diclofenac, acetaminophen, and caffeine were detected in soil irrigated with TWW.

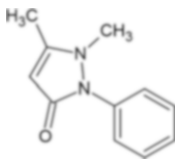
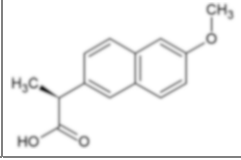
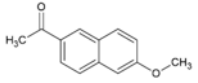
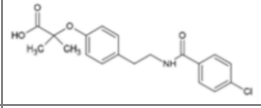
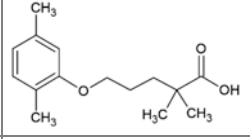
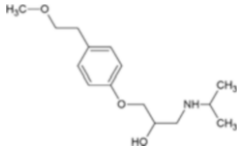
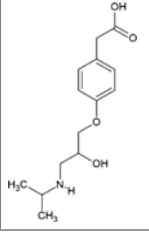
2.2 Occurrence in Soil

Data on the concentration of PhACs in agricultural soil is sparse. Table 1 shows the more prevalent contaminants detected in agricultural soils irrigated with TWW. On this topic, a recent study on the occurrence of pharmaceuticals in soils irrigated with reclaimed wastewater conducted by Biel-Maeso et al. [25] showed the prevalence of analgesic and anti-inflammatories, followed by antibiotics and psychiatric drugs on surface soils (0–20 cm). All the PhAC concentrations summed up in soils were between 2 and 15 ng g^{-1} d.w. The highest concentrations were detected for the compounds diclofenac and caffeine, followed by hydrochlorothiazide, mefenamic acid, flumequine, and carbamazepine. Biel-Maeso group [41] demonstrated the occurrence of a wide number of PhACs in vertical soil profiles up to 175 cm, demonstrating also potential leaching. Gielen et al. [42] reported the impact of soil type on the removal or leaching of PhACs such as carbamazepine, caffeine, ibuprofen, naproxen, and salicylic acid. Only carbamazepine and caffeine were discovered in the soil-water leachates. Carbamazepine behaved very conservatively in the sand soil, and it was largely removed in the investigated volcanic soil. Lamotrigine has similar pharmacological activity, while carbamazepine, however, has distinct chemical structure and properties that can affect its environmental behavior. In fact, Paz and co-workers [43] reported a high sorption affinity of lamotrigine to soil with respect to carbamazepine. The triazine ring and amino group give the ability of the molecules to form hydrogen bonds with functional groups on polar soil organic matter. Borgman and Chefetz [44] demonstrated the importance of organic matter content for lamotrigine sorption to soil, increasing it when biosoilds were added to sandy soil. The accumulation of carbamazepine, sulfamethoxazole, and ciprofloxacin after long-term irrigation of soils with untreated wastewater was also reported by Dalkmann and co-workers [28]. The accumulation of sulfamethoxazole and ciprofloxacin in soil was not accompanied by an increase of relative abundance of respective resistance genes. Also, Kinney et al. [19] found that carbamazepine, acetaminophen, fluoxetine, caffeine, and erythromycin accumulated in the upper 30-cm soil layer. In arid zones, wastewater irrigation is extensively applied, which can lead to accumulation of PhACs in soil and leaching to groundwater. However, not all compounds present in wastewater effluent accumulated in soil. For example, Durán-Alvarez et al. [31] reported the concentrations of acidic pharmaceuticals in soil irrigated by untreated wastewater for 90 years were less than 1 ng g^{-1}. In a study developed in Mexico by Gibson et al. [33], carbamazepine and triclosan had accumulated by a factor of 603–942% and 519–858%, respectively. In contrast, diclofenac, naproxen, and bezafibrate were not retained and therefore did not accumulate in soil. The persistence of PhACs can potentially enrich the reservoir of antimicrobial resistance genes in soils [45].

Besides the accumulation of PhACs, the possible formation of stable transformation products (TPs) in soil was reported in few studies (see Table 1). For instance, Koda et al. [35] reported the persistence of carbamazepine and its metabolites (10,11-epoxide; 10,11-dihydrocarbamazepine; and trans-10,11-dihydro-10,11-

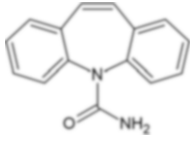
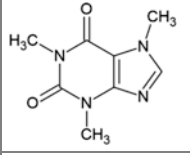
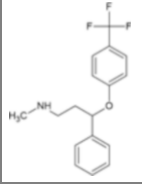
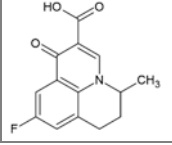
Table 1 Concentrations of prevalent PhACs and their transformation products (TPs) detected in agricultural soils irrigated with TWW

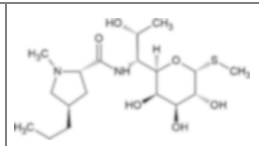
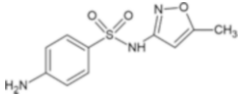
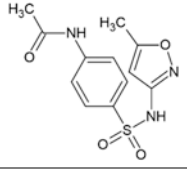
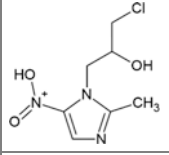
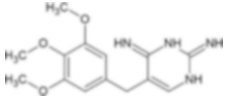
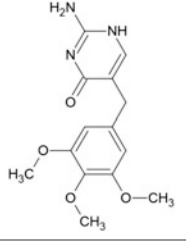
Compounds	Structure	TPs[a]	Conc. (ng g^{-1})	Ref.
Analgesic				
Acetaminophen		Hydroxy-acetaminophen Hydroquinone 1,4-benzoquinone N-acetyl-p-benzoquinone imine p-acetanisidide 4-methoxyphenol 2-hexenoic acid 1,4-dimethoxybenzene	*5.95*[b] *33.2*[c]	Biel-Maeso et al. [25] Kinney et al. [19] Li et al. [26][a]
Diclofenac		2,6-dichloroaniline carboxylated 2-hydroxy-phenylacetic acid 1-O-acylglucuronide Hydroxy-diclofenac di-hydroxy diclofenac	*5.06*[b] *0.3–0.1; 0.2–0.2;* *0.1–0.1*[d] *0.1–0.54*[e] *0.06–0.15*[f]	Biel-Maeso et al. [25] Corada-Fernández et al. [27] Dalkmann et al. [28] Christou et al. [29] Facey et al. [30][a]
Ibuprofen		4-Isobutyl-acetophenone 	*0.25 ± 0.04*[g]	Durán-Alvarez et al. [31] Vulava et al. [32][a]
Mefenamic acid			*0.08–1.97*[h]	Biel-Maeso et al. [25]

Phenazone			*0.36*[b]	Biel-Maeso et al. [25]
Naproxen		2-acetyl-6-methoxy-naphthalene 	*0.55 ± 0.01*[g] *0.73 ± 0.20*[g] *0.51–3.06*[e] *0.27–0.61*[i]	Durán-Alvarez et al. [31] Dalkmann et al. [28] Gibson et al. [33] Vulava et al. [32][a]
Lipid regulator – antihypertensive				
Bezafibrate			*0.38*[b] *<LOD–1.07*[e]	Biel-Maeso et al. [25] Dalkmann et al. [28]
Gemfibrozil			*0.02–0.06*[h]	Biel-Maeso et al. [25]
Metoprolol		Metoprolol acid 	*0.5–0.1, 0.6–0.6, 0.1–1.5–0.6*[d] *0.91, 1.33, 3.27*[j]	Corada-Fernández et al. [27] Grossberger et al. [34] Koda et al. [35][a]

(continued)

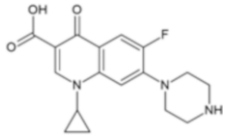
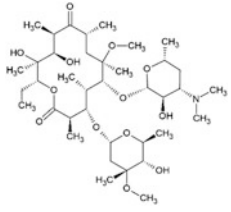
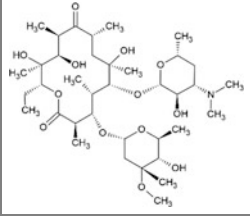
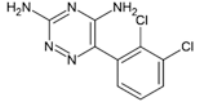
Table 1 (continued)

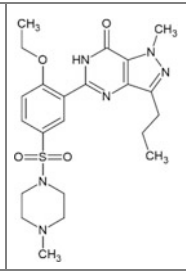
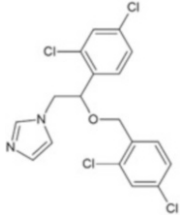
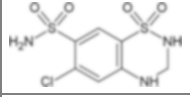
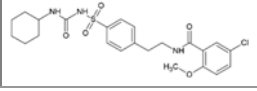
Compounds	Structure	TPs[a]	Conc. (ng g^{-1})	Ref.
Psychiatric drugs				
Carbamazepine		10,11-dihydro-10-hydroxy CBZ CBZ-10,11-epoxide acridone-N-carbaldehyde 4-aldehyde-9-acridone acridine	*0.08–1.36*[h] *17.6*[c] *0.1–0.1, 0.1*[d] *6.48 ± 0.59*[g], *5.14 ± 0.48*[g] *1.49–8.38*[e] *2.6–7.57*[i] *5.67, 2.33, 3.88*[j]	Biel-Maeso et al. [25] Kinney et al. [19] Corada-Fernández et al. [27] Durán-Alvarez et al. [31] Dalkmann et al. [28] Gibson et al. [33] Grossberger et al. [34] Li et al. [36][a]
Caffeine			*0.51–3.21*[h] *6.81*[c] *12.62, 6.51, 12.28*[j]	Biel-Maeso et al. [25] Kinney et al. [19] Grossberger et al. [34]
Fluoxetine			*8.8*[c]	Kinney et al. [19]
Antibiotics				
Flumequine			*5.31*[b]	Biel-Maeso et al. [25]

Lincomycin			*0.03*[b]	Biel-Maeso et al. [25]
Sulfamethoxazole		N_4-acetyl-sulfamethoxazole 	*0.251* *9.133* *0.98–5.966* *0.28, 0.25, 0.128* *0.38 to 0.989*	Biel-Maeso et al. [25] Kinney et al. [19] Dalkmann et al. [28] Grossberger et al. [34] Christou et al. [29] Martínez-Hernández et al. [37][a]
Ornidazole			*0.47*[h]	Biel-Maeso et al. [25]
Trimethoprim		Hydroxy- trimethoprim 	*0.04*[b] *1.22*[c] *0.13–2.44*[e] *0.15 to 0.62*[f]	Biel-Maeso et al. [25] Kinney et al. [19] Dalkmann et al. [28] Christou et al. [29] Koba et al. [38][a]

(continued)

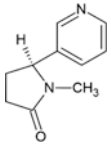
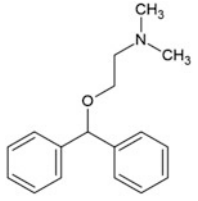
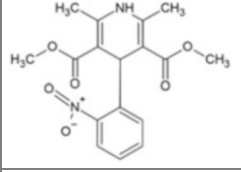
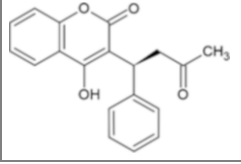
Table 1 (continued)

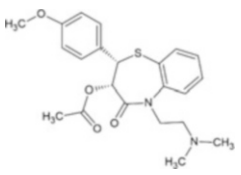
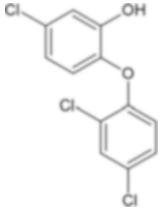
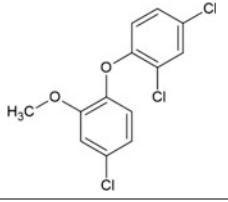
Compounds	Structure	TPs[a]	Conc. (ng g^{-1})	Ref.
Ciprofloxacin			*0.35–2.62*[e]	Dalkmann et al. [28] Girardi et al. [39][a]
Clarithromycin			*0.5–0.5, 1.3–0.5, 0.3–0.6–0.7*[d] *<3–5.43*[e]	Corada-Fernández et al. [27] Dalkmann et al. [28]
Erythromycin			*202*[c] *<LOD–1.21*	Kinney et al. [19] Dalkmann et al. [28]
Antiepileptic				
Lamotrigine			*4.43, 2.35, 2.2*[j]	Grossberger et al. [34]

Erectile dysfunction				
Sildenafil			*2.54, 2.16, 5.98*[j]	Grossberger et al. [34]
Antifungal				
Miconazole			*1.41*[c]	Kinney et al. [19]
Other PhACs				
Hydrochloro-thiazide			*0.38–1.20*[h]	Biel-Maeso et al. [25]
Glyburide			*0.02*[b]	Biel-Maeso et al. [25]

(continued)

Table 1 (continued)

Compounds	Structure	TPs[a]	Conc. (ng g^{-1})	Ref.
Cotinine			*3.4*[c]	Kinney et al. [25]
Diphen-hydramine			*1.56*[c]	Kinney et al. [19]
Dehydro-nifedipine			*1.39*[c]	Kinney et al. [19]
Warfarin			*23.9*[c]	Kinney et al. [19]

Diltiazem			*0.13*[c]	Kinney et al. [19]
Triclosan		Methyl triclosan 	*4.4 ± 0.1*[g] *18.6 ± 1.2*[i]	Durán-Alvarez et al. [31] Gibson et al. [33] Butler et al. [40]

[a]Citation refers to transformation product
[b]Max concentration detected
[c]Average pharmaceutical masses in soils normalized to soil organic carbon content [19]
[d]Concentrations of PhACs in three soil cores [27]
[e]Total extracted pharmaceutical concentration from soil [28]
[f]Range concentration in soil during the 3-year irrigation experiment [29]
[g]Concentrations in Phaeozem and Leptosol soils [31]
[h]Min-max concentration in superficial soil samples [25]
[i]Total extracted pharmaceutical concentration from soil [33]
[j]Average concentration in the three studied soils [34]

dihydroxycarbamazepine) on 13 different soils. This phenomenon with low carbamazepine sorption may cause adverse effects on the environment due the possibility to leaching to groundwater. Similar behavior was observed by Butler and co-workers [40] on the fate of triclosan and its major metabolite methyl-triclosan that is more lipophilic and potentially more persistent than the parent compound [46]. After 1 year, both triclosan and methyl-triclosan remained in the top 10 cm layer in all three investigated agricultural soils.

Sorption of PhACs and personal care products to soil is influenced by the soil pore water chemistry and the type of mineral and organic sorbents [47]. The retention of some analytes may reflect the interactions of PhAC physicochemical properties and soil characteristics with the different locations, and it is not only consequences of their high abundance in wastewater.

To understand the fate of PhACs in soil, it is necessary to investigate all processes that can be involved such as biodegradation, sorption, and the formation of NER, as well as to understand what parameters can influence these processes (e.g., pH, soil texture, particulate and dissolved organic matter, ion exchange capacity, hydrophobicity of PhACs, and their charge).

2.2.1 Biodegradation

Biotransformation of PhACs is the most important and effective way for their removal in soil. Microorganisms have the ability to interact with chemicals, both chemically and physically, leading to structural changes or to complete degradation of the target molecules. For example, after 45-d incubation time, the fraction of degraded clofibric acid and diclofenac in non-sterile and sterilized agricultural soils was 88–100% (non-sterile) and 33–43% (sterilized), respectively, indicating a significant role of microorganisms in degrading these pharmaceuticals. The degradation rate decreased at increasing initial chemical concentrations in soil, implying that the microbial activity was inhibited with high chemical loading levels [48]. In soil, microorganisms metabolize PhACs aerobically and/or anaerobically. For instance, Thelusmond et al. [49] reported the biodegradation of diclofenac, carbamazepine, and triclocarban in four agricultural soils. Rapid degradation of diclofenac was observed under aerobic conditions with respect to carbamazepine and triclocarban. Specific phylotypes were found to be associated with the biodegradation processes. Pan and Chu [50] observed anaerobic and aerobic adsorption and degradation of five antibiotics. All antibiotics presented higher degradation under aerobic conditions with half-lives ranging between 2.9 and 43.3 days in non-sterilized soil and 40.8 to 86.6 days in sterilized soil. This study highlighted that biodegradation depends on antibiotic physicochemical proprieties, soil texture, and microbial activity as well as oxygen content. This was also confirmed by Biel-Maeso [41], who demonstrated that nine PhACs (nadolol, sulfamethizole, sulfamethoxazole, sulfamethoxypyridazine, carbamazepine, ibuprofen, diclofenac, hydrochlorothiazide, and gemfibrozil) and four artificial sweeteners (acesulfame, saccharin, cyclamate and sucralose) in soil had a high degradation rate under aerobic conditions, but

they were relatively persistent under anaerobic conditions. For instance, over 90% of nadolol was degraded in aerobic soils after 4 days of incubation, while only 18–24% was lost in the absence of oxygen after 1 month, resulting in half-life values between 95 and 103 days. Biosolid amendments also tend to contribute to PhACs accumulation in soil. Biodegradation of PhACs is correlated with soil properties, and it is well known that the application of biosolids have effects on the soil properties [48]. It was reported that biosolid amendments could inhibit PhAC degradation due to an increase of the organic matter content in soil, leading to increase sorption of PhACs to soil and prolonging their persistence [36].

2.2.2 Sorption

Sorption has often been the most studied process because it determines the mobility of PhACs in the porous media. Doretto et al. [51] studied the behavior of sulfonamides (sulfadimethoxine, sulfaquinoxaline, and sulfamethazine) in the 0–20-cm upper layer of four different soils in Brazil. The PhAC adsorption/desorption data fitted the Freundlich isotherms well in the logarithmic form. The adsorption coefficients obtained suggesting that all target PhACs were weakly adsorbed on the soils. The Freundlich desorption coefficients suggest that the sulfonamides tend to leached from soil whit high sand and low organic carbon contents. These results suggest that there is potential groundwater contamination by sulfonamides. Biel-Maeso et al. [41] have also reported the sorption of PhACs and artificial sweeteners in two soils under aerobic and anaerobic conditions. Equilibrium sorption data fitted well to a Freundlich isotherm model [41, 48]. The higher K_f was determined for cyclamate (162 L kg^{-1}) and acesulfame (156 L kg^{-1}), while the lowest sorption coefficients were measured for ibuprofen (1–7 L kg^{-1}). Pan and Chu [50] evaluated the adsorption of five antibiotics in sterilized and non-sterilized agricultural soils under aerobic and anaerobic conditions. The five antibiotics exhibited adsorption affinities on soil in the descending order: tetracycline > norfloxacin > erythromycin > chloramphenicol > sulfamethazine. Sulfamethazine was the most mobile antibiotic in soil among the five compounds, while tetracycline was the least mobile. Mobility depends on the presence of soil organic matter (SOM) and chemical and environmental properties [47, 52]. There is limited information on how the physicochemical properties of different soils can influence the sorption of PhACs, and prediction is elusive due to the complexity of interactions specifically between polar ionic contaminants and mineral surfaces in the presence of DOM.

2.2.3 Non-extractable Residues (NER)

Contaminants entering the environment undergo various processes already described, such as sorption and biodegradation, but in addition, a proportion will be immobilized in soil by NER. Conforming to the IUPAC definition [53], NER in plants and soil are defined as chemical substances that remain in soil or sediment

matrix when extracted by methods that do not significantly change the chemical nature or the structure of the matrix. NER are the sum of three types of residues: (1) those strongly entrapped in the soil, (2) those covalently bound to soil and considered as irreversibly bound, and (3) those derived from biotic degradation [54, 55]. Type I NER are relevant for the environment because they are presumed to be reversible once degradation of the humic matter fraction occurs that could lead to contaminant release [56]. NER have to be quantified by isotope-labelled (either with radioactive or stable isotopes) chemicals at the most stable part of the molecule, and consequently their analysis is often not possible at environmental concentrations of contaminants. Nowadays, there are no standardized procedures for their determination due to the lack of a common regulation in EU [55]. The most studied NER contaminants in the last 50 years have been soil-bound residues of pesticides [56, 57]. In contrast, information on pharmaceutical NER is very scarce because obtaining experimental data requires significant investments in terms of time and money. In these conditions, knowledge on pesticide NER will be likely very useful to predict the formation of pharmaceutical NER. For instance, Li et al. [58] reported recently the phytotransformation and metabolic pathways of ^{14}C-carbamazepine in carrot and celery. This study highlighted that ^{14}C detected in bound residues was lower than in extractable residues (>85% of the uptake ^{14}C radioactivity) in plant tissues and a total of nine radioactive transformation products of carbamazepine were identified.

2.2.4 Soil pH

Many contaminants are ionizable under environmental conditions. Ionic compounds are more soluble in water than their neutral counterpart and typically nonvolatile. Strongly pH-dependent distributions were found for many chemicals, including basic aromatic amines [59], basic N-heterocyclic compounds [60], basic and acid pesticides [61], basic and acid pharmaceuticals [62]. These studies reported a decrease of sorption corresponding with an increase of pH. Normally, wastewater is slightly alkaline, which attenuates the acidic nature of the soil. Several equations to predict the correlation between pH and the sorption capacity have been developed, but unfortunately, the complexity of the interaction has not allowed for the application of a unique model. Theoretically, cationic pharmaceuticals should be able to sorb negatively charged soil components, such as clay and organic matter [63]. Vazquez-Roig et al. [64] showed the influence of pH on fluoroquinolone antibiotics due to their two p*K*a values. Ofloxacin has two p*K*a, 5.97 and 8.28, and at environmental pH tends to be zwitterionic but can also be cationic, anionic, or uncharged. The ciprofloxacin cation (p*K*a 6.18 and 8.76) dominant at pH $\leq$ 5 exhibited a greater potential for cation exchange than the net neutral zwitterion (relevant at pH > 6) [65]. Zhang et al. [66] demonstrated how low pH and a soil rich of organic matter had a positive impact on sorption of trimethoprim, sulfapyridine, sulfameter, and sulfadimethoxine. For example, the sorption of trimethoprim increased with decreasing pH. Based on its p*K*a, it is positively charged

at acid conditions and neutral at pH $\geq$ 7. The highest sorption affinity for this antibiotic was obtained in the range of pH 4 to 6. These results were similar to those reported in Bekçi et al. [67]. The pH can also influence the pH-dependent charge on organic matter and clay minerals which can have an effect on pharmaceutical sorption [68].

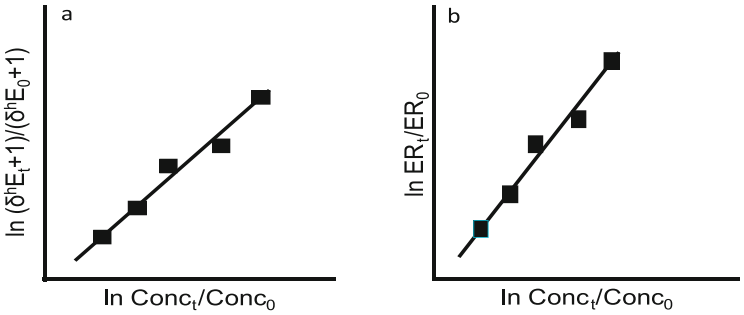
2.2.5 Cation Exchange Capacity (CEC)

Clay cation exchange charge (CEC) depends on the type of clay. The sorption of nonionic (neutral) PhACs is mainly driven by hydrophobic partitioning to the soil organic matter via van der Waals and electron donor-acceptor interactions and by hydrogen bonding with hydroxyl groups on the solid surfaces. Sorption of neutral compounds is therefore highly dependent on the soil organic matter content. In contrast, the mechanisms and effects of ionization on the behavior of PhACs in soil are less known and remain inconclusive. The sorption of cationic molecules is mainly governed by the attraction to negative charges of the solid surface (e.g., a clay mineral surface or organic matter). In an attempt to predict persistence and mobility of ionizable PhACs, the determination of the cation exchange capacity (CEC) of soils, which is a measure of the negatively charged site on the soil surface, has been suggested. Clay may exhibit an anion exchange capacity (AEC) due to the protonation of the surface hydroxyl groups. The ratio AEC/CEC gives an indication about the difference between the soil pore water pH and the pH of the net zero charge of the clay, as reported by Hyun and Lee [69]. The cation exchange charge of the clay has been reported to be important for the sorption of some antibiotics such as fluoroquinolones [65].

The larger the CEC value, the more important the sorption of the positively charged PhACs can be [70]. However, sorption of organic compounds and CEC evaluated on disintegrated soils (i.e., in soil slurry) could be greater than that measured on soil aggregates. The main reason is that a part of the sorption capacity of the soil components is not available due to their interactions with aggregated soil and acidic environments.

2.2.6 Soil Organic Matter

Soil organic matter is made up of particulate organic matter (POM) and, in pore water, colloidal dissolved organic matter (CDOM). In soils, organic matter (OM) is the most important sorbent for hydrophobic organic pollutants due to their colloidal properties that can increase the solubility and contribute to reduce their sorption to solid matrixes in soil and sediments. The mobility and the bioavailability of organic compounds may be influenced by the interactions with dissolved organic matter [71]. Leenheer [72] reported that DOM can be fractionated based on the hydrophobic-hydrophilic characteristics of its materials, and it was demonstrated that this fractionation scheme has provided important information on the interaction



$$\ln\frac{\delta\,{}^{h}E_t + 1}{\delta\,{}^{h}E_0 + 1} = \varepsilon_E \ln\frac{Conc_t}{Conc_0} \qquad \ln\frac{ER_t}{ER_0} = \varepsilon_{ER} \ln\frac{Conc_t}{Conc_0}$$

Fig. 1 Rayleigh equation model for the mathematical description of the relationship between the extent of degradation and (**a**) the isotopic composition of a targeted PhAC and (**b**) the enantiomeric composition of a chiral PhAC

of DOM with the environment and with organic pollutants. Ilani et al. [73] reported that sorption of triazine by DOM is correlated by the content of the hydrophobic acid and neutral fraction of DOM. This phenomenon was also confirmed by Moaz and Chefetz [74] on the sorption of naproxen and carbamazepine, but it was strongly ph-dependent. In fact it was efficient at pH near the p*K*a of the analytes. The hydrophilic fraction exhibited the highest sorption affinity with naproxen at pH 8.

The transport of pharmaceuticals may be enhanced by wastewater irrigation either in low or in high POM content soils. Since the 1980s, it has been reported the ability of surface water CDOM to bind contaminants even though the mechanisms have been poorly elucidated. For instance, Carmosini and Lee [75] reported that ciprofloxacin was partitioned to the humic material CDOM following a pH-dependent cation exchange mechanism.

3 Enantiomeric Fractionation as a Tool to Investigate the Fate of PhACs in Soil

The occurrence and potential accumulation of PhAC residues in soil irrigated with treated wastewater are scarce. In contrast, our knowledge on their fate in soil has been more limited. Several processes are in competition for PhAC removal in soil including sorption and formation of NER, phototransformation at the soil surface, plant uptake, and biotransformation [58]. Most of the studies dealing with the fate of PhACs in soil compartments have been carried out at lab-scale, under controlled conditions of temperature and moisture. In these conditions, compounds labelled with a carbon isotope (e.g., ^{14}C or ^{13}C) were used, and a mass balance could be established as well as major dissipation pathways elucidated. Kinetic profiles have

often been found biphasic due to nutrient and carbon limitations, typically with faster initial phases followed by slower declines. When switching to field studies, ^{14}C-labelled compounds are not allowed, and additional dissipation processes of compounds can occur simultaneously including leaching to deeper soil layers with irrigation water and photodegradation at the surface of soil due to an upward water flow movement by capillarity [76]. In field studies, the loss in microbial activities due to nutrient limitation is not an issue. However, biphasic degradation kinetics are still often observed. This is due to the decrease in compound bioavailability as the result of compound concentration decrease due to their biodegradation or plant uptake [77]. Biphasic degradation may also be found with chiral compounds because the individual stereoisomers are often degraded at different rates. If the degradation rates of two enantiomers in a racemic mixture are very different, the overall decline of the compound, when determined with a non-enantioselective analytical method, will be biphasic [78]. Consequently, the behavior and fate of PhACs in soil are very complex due to interconnected processes. It is often impossible to discriminate between abiotic and biotic processes, so the development of an in situ molecular marker of biodegradation would be desirable. This will be relevant because biodegradation is probably the most important dissipation pathway, which can lead to PhAC elimination.

In order to develop a molecular marker of biodegradation, several approaches have been considered. Isotopic fractionation (i.e., compound stable isotope analysis (CSIA)) is considered the best molecular marker of biodegradation. Stable isotope fractionation relies on the observation of ratio shifts of stable isotope mainly C, H, and N, prompted by the breaking or generation of chemical bonds during chemical transformations [79]. The mathematical description of the relationship between the extent of degradation and isotopic composition of a targeted compound can be expressed by the Rayleigh equation (see Fig. 1a).

E_t and E_0 represent the initial and conversion-dependent isotope ratio, $Conc_t/Conc_0$ is the residual fraction of the contaminant, and ε represents the isotope enrichment factor. ε can be obtained as the slope of the linear regression line of the natural log of the isotopic enrichment against the natural log of the extent of the degradation. It is usually expressed in per mill unit, requires very accurate measurements to obtain, and is very specific to a biotransformation reaction. This approach has already been applied to quantify in situ contaminant biodegradation and to investigate mechanisms of biodegradation [80]. However, CSIA require specific instrumentations (e.g., GC-IRMS), are still difficult to achieve in a routine way, and are very often limited to GC amenable compounds. Limits of detection (LODs) are not compatible with the occurrence and levels of compounds in soil (i.e., ng g^{-1} level), and analyte enrichment and purification steps are needed during which isotopic fractionation might be induced.

In this context, a novel approach applied to investigate biodegradation of PhACs in environmental compartments is enantiomeric fractionation, which measures the enantiomeric ratio (ER). Enantioselective process occurs when one enantiomer of a chiral compound is favored over the other during biotransformation. This approach makes sense because more than 50% of PhACs are actually commercialized as racemic mixtures, which are mixtures of two enantiomers at equal concentrations.

Enantioselective metabolism has been deeply investigated in pharmacokinetics and pharmacodynamics because biological activity and toxicity are very often largely dependent on chirality. Less is known about environmental enantioselective transformations because chirality has not been included in any regulatory risk assessment procedure yet. As enantioselectivity is mainly specific to biodegradation processes, PhAC chirality could be used to track the pollutant sources in treated and nontreated wastewater [81] as well as to better understand their environmental fate. Indeed, enantioselectivity reflects biological processes, since nonbiological enantioselective catalysis is rare in nature. Abiotic enantioselective processes such as sorption on environmental surfaces have been found in some cases [82] but usually of minor significance with respect to biodegradation-related enantioselectivity. The major advantage of the enantiomeric fractionation approach has been the relative simple analysis of ER using chiral chromatography coupled to conventional mass spectrometry (LC-MS) with appropriate LODs of ng L^{-1} in waters or of ng g^{-1} in solid matrices for environmental studies. The concept of applying the Rayleigh equation, which was developed for isotopic fractionation, to enantioselective processes was suggested by Jammer et al. [83, 84] for in vitro enzymatic transformations under laboratory controlled conditions. In this case, the isotope ratio was replaced by the enantiomeric enrichment (ERt), expressed as a ratio between two enantiomers. A linear relationship between the evolution of the log of enantiomeric ratio and the log of the evolution of a chiral compound concentration can be still obtained, and the slope of the straight line gives the enantiomeric enrichment factor ε_{ER} (see Fig. 1b). This factor is usually larger than the isotopic enrichment factor. Actually, it is expressed in percent unit and not in per mill unit and can be used as a characteristic tool for an enzymatic reaction. This extension of the Raleigh model to enantioselective processes is only valid providing that degradation kinetics of each enantiomer fit to a first-order kinetic model. Enzymatic reactions are frequently described by Michaelis−Menten kinetics (Eq. 1), which are nonlinear. However, the equation gives linear dependence when the concentration of the substrate C is much lower than the Michaelis–Menten constant, K_M:

$$\frac{dC}{dt} = \frac{-kC}{[K_M + C]} \tag{1}$$

This approximation is usually correct in environmental studies because contaminants are found in very low concentrations. However, the first-order kinetic model can be disturbed by sorption processes because sorption processes are nonlinear processes [85]. Indeed, when PhAC concentrations decrease, their bioavailability often decreases leading to slower declines. This can be a source of uncertainty in the determination of the biodegradation extent by using the enantiomeric fractionation making it less accurate than the CSIA approach. Till now, the parallel process of comparing the enrichment of one enantiomer relative to the other has been applied in enantiomeric analysis to prove the existence of biodegradation and to try to quantify the extent of this process exclusively in activated sludge treatments both at the lab-scale [86] and in biological wastewater treatment plants [87, 88]. The contribution of this work was to expand the current knowledge of enantioselective processes

in activated sludge to soil and to better know what chirality can teach about biodegradation significance in soil. For this purpose, two probe chiral compounds were selected: (1) metoprolol (MTP), a highly prescribed β-blocker, and (2) climbazole (CLB), an imidazole fungicide often used as an active ingredient in antidandruff shampoos removing dandruff at approximatively 15 g L^{-1} rate (see Fig. 3 for chemical structures). MTP and CLB are chiral pharmaceuticals due to the presence of an asymmetric carbon in their chemical structure, and both are marketed as racemic mixtures of two enantiomers. These compounds were selected because MTP undergoes biodegradation under aerobic conditions, while CLB undergoes biodegradation under anoxic/anaerobic conditions, covering then different soil conditions under irrigation with treated wastewater.

Transformation pathways have to be known because many significant environmental transformations are enantioselective and the possibility of overlapping of different enantioselective reactions may exist. The best scenario would be to find chiral PhACs that undergo a very predominant enantioselective biotransformation pathway. First, the transformation pathways of MTP and CBZ were determined under aerobic (nitrification) and under anoxic (denitrification) conditions, respectively. For MTP, biotransformation included two pathways. A major one was a O-dealkylation enantioselective reaction leading to metoprolol acid (MTPA) (see Fig. 2a). A very minor one was through benzylic hydroxylation, leading to α-hydroxymetoprolol (α-HMTP). Under anoxic conditions, the enantioselective reduction of the ketone function of CBZ into a secondary alcohol function occurred to give CBZ alcohol (CBZ-OH; see Fig. 2b). Moreover, some compounds have

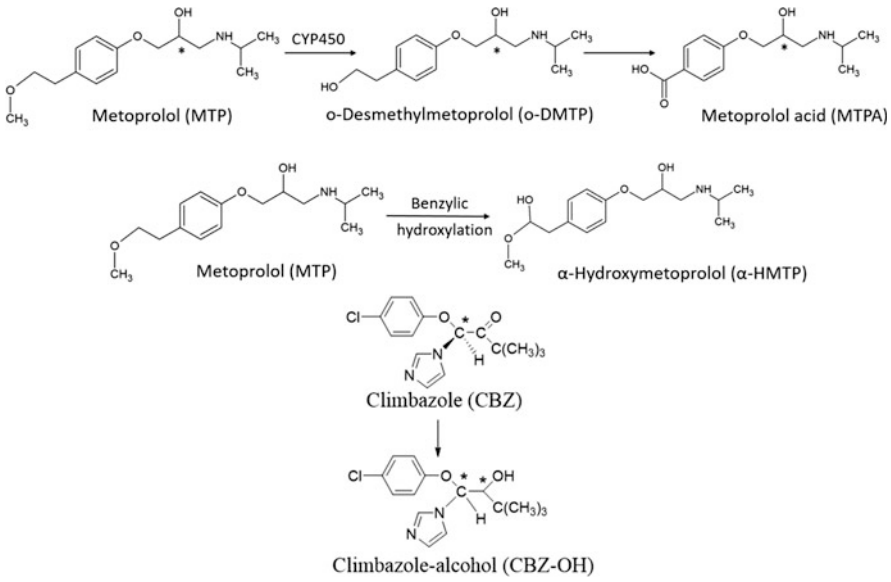


Fig. 2 Proposed transformation pathways of metoprolol (MTP) under aerobic conditions and climbazole (CBZ) under anoxic conditions

unstable stereo-configuration [89] and could undergo enantiomer interconversion during degradation but also during sample preparation and storage. This is a very sensitive step. For MTP, pure enantiomers were commercially available and separately incubated in soil slurry experiments. Enantiomer interconversion was never observed. Due to the lack of enantiopure standards of CBZ, potential CBZ enantiomerization was investigated in the presence of D_2O, where the formation of deuterated CBZ enantiomers was observed. However, the rate constants for deuteration reaction (K_{deut}) were found to be slightly higher for CBZ (E1) than for CBZ (E2) ($K_{deut} = 0.91$ and 1.12×10^{-5} min^{-1} ($T_{1/2} = 52.9$ days and 43.0 days) for CBZ (E1) and CBZ (E2), respectively). This result could be explained by the very weak hydrogen acidity of the α-carbonyl carbon precluding the C–H bond cleavage, the formation of a carbocation, and at the end the possibility of CBZ enantiomerization [87].

Chiral analytical methods were then developed for the determination of the ER of MTP and CBZ in soil slurries. In routine chiral analysis, several rules should be respected. The most important one is the removal as much dissolved organic matter as possible because humic and fulvic substances are good chiral selectors which usually degrade the quality of the enantiomer separation after a few injections. Consequently, a resolution >1 should be obtained for accurate ER calculation. Finally, quantification by isotopic dilution is compulsory because matrix effect causes different ion suppression of a pair of enantiomers in electrospray with negative implications in ER calculation. In practice, 10-mL supernatant of soil slurries were percolated through mixed-mode cation exchange cartridges after water acidification at pH 3 to remove as much dissolved organic matter as possible. The use of a sequential elution protocol allows for the removal of neutral and acidic interferences before analyte elution with methanol containing ammonia. This led to a significant reduction of matrix effects in LC-HRMS analysis [87]. Enantiomers of MTP and those of its major transformation products were separated using an ASTEC vancomycin-based analytical column (Chirobiotic V) using a reverse phase isocratic mode of elution with a mobile phase consisting of water +30 mM ammonium acetate/methanol, 10/90 (v/v) (see Fig. 3a). Enantiomers of CBZ were separated using a Phenomenex Lux Amylose-2 analytical column only using water/acetonitrile (35/65, v/v) as mobile phase in an isocratic mode of elution (see Fig. 3b). LODs down to 10 ng L^{-1} were obtained with both analytical methods, which made them suitable for the analysis of MTP and CBZ in soil slurries.

The validated chiral analytical methods were then applied to investigate the relationship between the evolution of the enantiomeric ratio of MTP and CLB and the extent of their biodegradation rates in soil slurry experiments under laboratory control conditions. Soil slurries were spiked with MTP or CBZ at concentrations close to environmental concentrations, that is, 20 μg L^{-1}. MTP incubations were carried under aerobic conditions by bubbling the reactor with air, and CBZ incubations were conducted under anoxic conditions in 100 mL serum bottles, sealed with butyl rubber stoppers and aluminum caps in which syringes were inserted for sample collection. Serum bottles were charged with 10 g of an agricultural soil and filled with secondary treated wastewater collected at a biological wastewater treatment

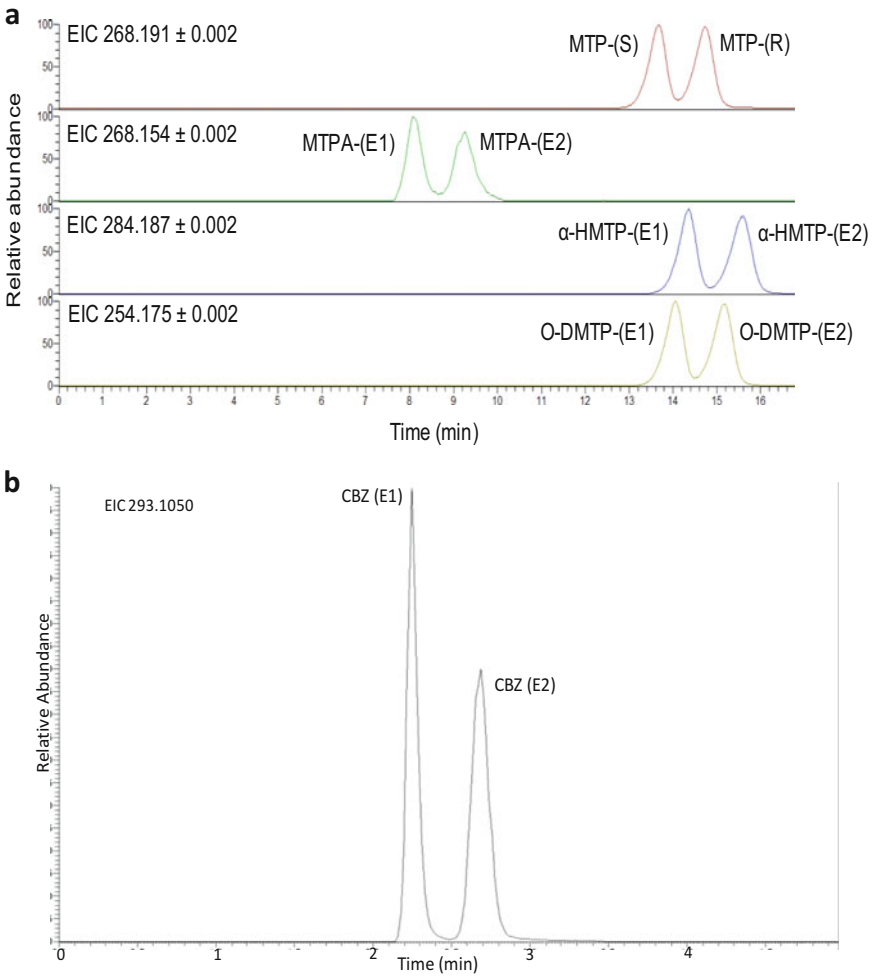


Fig. 3 Extracted ion chromatograms (EICs) corresponding to chiral analysis of (**a**) metoprolol (MTP) and its major transformation products, metoprolol acid (MTPA), O-desmethylmetoprolol (O-DMTP), and α-hydroxymetoprolol (α-HMTP), and (**b**) climbazole (CBZ) by LC-HRMS, both in positive mode of ionization

plant (WWTP). Prior to spiking probe compounds, the test systems were preconditioned for 2 days. The pH, redox potential, and dissolved oxygen were controlled by electrode measurements. Poisoned experiments with sodium azide (NaN_3) at 1 g L^{-1} were also added. Abiotic control was needed by using sterilized soil for estimating enantioselective sorption processes which are usually minor processes. Experiments were also carried out in dark and light conditions because MTP and CBZ might also undergo phototransformation upon natural light irradiation. At different time points, 10-mL supernatant were collected and filtered on 0.22-μM nylon filter before SPE and LC-HRMS for kinetic studies and TP identification.

As expected biodegradation was the main transformation route of MTP with metoprolol acid (MTPA) being the major identified TP. However, photodegradation also contributed to the degradation of MTP even though in a minor extent than biodegradation [88]. For this specific route of degradation, O-desmethyl-MTP and α-hydroxy-MTP were identified in addition to MTPA. The bioreduction of CBZ into CBZ alcohol was the unique biodegradation route identified for CBZ, and biodegradation was the major transformation route of CBZ, while phototransformation also occurred in light conditions but similarly to MTP in a much lesser extent than biodegradation. Reductive dechlorination, hydroxylation, and cleavage of the ether bond were the major transformation routes observed under photolysis [87]. Results for MTP biodegradation kinetic experiments in soil slurry experiment system are shown in Fig. 4a, b.

Experiments in the light degraded MTP quicker than their dark equivalents due to MTP photolysis. No stereoselectivity in MTP degradation was observed in any abiotic experiments. Stereoselective degradation of MTP leading to a (S)-enrichment was exclusively observed under biotic conditions, confirming the specificity of ER variations to biodegradation processes. Enantiomeric fractionation (ER_t/ER_0) was plotted against the MTP residual fraction (C_t/C_0) according to the Rayleigh equation for all biotic experiments. The linear fit to the Rayleigh approximation was obtained under both dark and light conditions ($R^2 > 0.99$ and > 0.98, respectively). Both enantiomers followed the same first-order kinetic and probably obey the same degradation mechanisms. In these conditions, the ER-conversion relationship does not depend on reaction conditions but mainly depends on reaction time. The enantiomeric enrichment factor was calculated to be 22% and 56% in light and dark conditions, respectively.

In anoxic soil slurry experiments, the transformation of CBZ into CBZ-OH was nearly quantitative, and biodegradation gave a first-order kinetic fit (see Fig. 5a, b). When the enantiomeric fractionation was plotted against the CBZ residual fraction, the linear fit to the Rayleigh equation was obtained with r squared above 0.98 as quality control parameter. The enantiomeric enrichment factor was given by the slope of linear regression line and was found to be 33% in dark conditions and 26% in light conditions. This lower value in light condition is due to the slight contribution of phototransformation to the whole degradation of CBZ.

The environmental significance of these results is that on the basis of the knowledge of the enantiomeric enrichment factors (ε_R), which are specific to one biotransformation reaction, and on the basis of the experimental measurement of ER of MTP and CBZ by chiral LC-MS, it is possible to derive the percentage of the dissipation of selected PhACs in soil which can be directly attributed to biodegradation processes without establishing a mass balance during the course of the degradation. This approach avoids to carry out analysis in soil leachates and in plant or to evaluate the formation of non-extractable residues to evaluate the biodegradation rate of a chiral pharmaceutical.

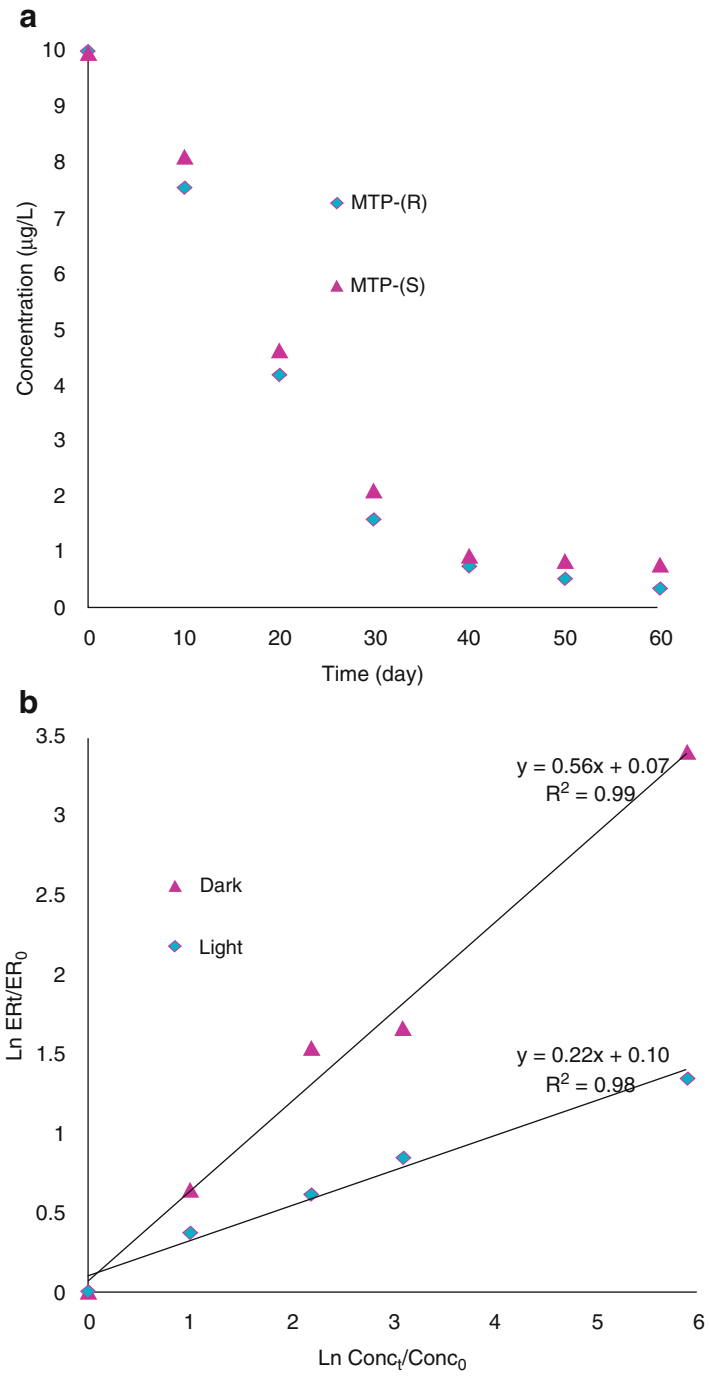


Fig. 4 (**a**) MTP-(S) and MTP-(R) concentration evolution against time under aerobic soil slurry conditions and in dark conditions, (**b**) Rayleigh representation of the enantiomeric enrichment of MTP versus MTP biodegradation in dark and light irradiation conditions

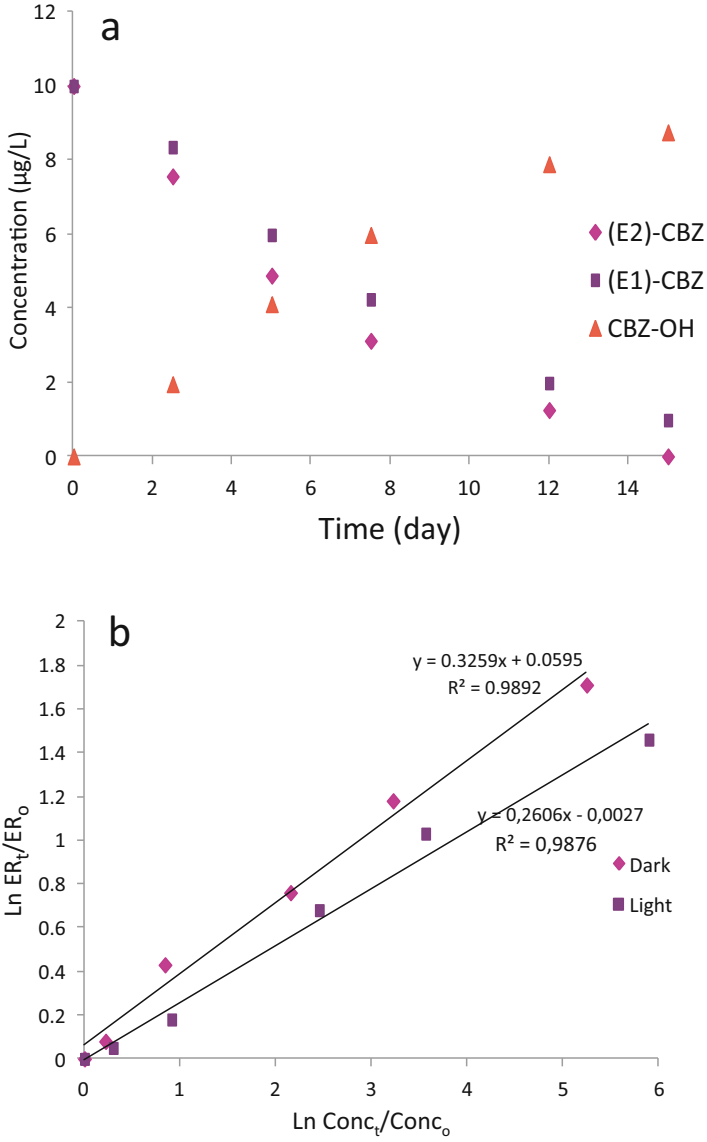


Fig. 5 (**a**) CBZ (E1), CBZ (E2), and CBZ-OH concentration evolutions against time during soil suspension incubation under anoxic conditions; (**b**) Rayleigh representation of the enantiomeric enrichment of CBZ versus CBZ biodegradation

4 Conclusion

The extensive use of treated wastewater in irrigation can be a significant source of PhACs into agricultural soils, which can be further accumulated in plants and food. There were only a few studies reporting their potential accumulation in irrigated soils. This chapter only analyzed studies conducted under agricultural growing and irrigation conditions because they enable an appropriate risk assessment of wastewater-derived contaminants to accumulate in soil as they integrate irrigation, soil, and plant processes.

The main conclusion highlights the lack of PhAC cumulative pattern in soil across time under long-term TWW irrigation scenarios. PhAC concentration levels in the 0.06–200 ng g^{-1} range were frequently observed. This lack of accumulation could be likely related to abiotic and biotic transformation processes and to the formation of NER. Chronic and repeated application of TWW on agricultural soils has implication on PhAC degradation because there is a potential for agricultural soil to develop accelerated biodegradation due to microorganism's adaptation processes, similar to what has been observed for pesticides [90].

Soil plays therefore a significant role as regulator of the pharmaceuticals available for plant uptake. The behavior and fate of PhACs in soil are very complex due to interconnected processes (e.g., sorption, formation of NER, biodegradation). These environmental processes were briefly discussed in this chapter.

However, the main challenge is related to the determination of the relative significance of these different abiotic and biotic processes. Enantiomeric fractionation of chiral PhACs has been discussed as a tool for discriminating abiotic and biotic degradation processes in field studies where the use of radioactively labelled compounds is not allowed and consequently when a mass balance is difficult to achieve. The enantiomeric fractionation of two chiral PhACs, namely, MET and CLB, has been investigated by using chiral LC-HRMS because the former one underwent aerobic biodegradation and the latter one under anaerobic conditions, thus covering different environmental conditions. As enantioselectivity reflects very predominantly biological processes, a shift in enantiomeric ratio of a chiral PhAC highlighted biodegradation and allowed for differentiating between photodegradation and biodegradation processes. This approach might be also quantitative by applying the Rayleigh model allowing for a quantitative assessment of biodegradation processes without establishing a mass balance. The main scientific gap remains the quantitative determination of the formation of NER of PhACs in soil because these studies require the use of isotope-labelled compounds (e.g., ^{14}C or ^{13}C) and are costly and suffer from a lack of standardized procedures in their implementation.

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Uptake and Effects of Pharmaceuticals in the Soil-Plant-Earthworm System

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Contents

Abstract The reuse of wastewater to meet increasing demands on freshwater resources coupled with the use of biosolids as soil amendments in agricultural landscapes provides many pathways for exposure to pharmaceuticals into the

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agroecosystem. Wastewater and both agricultural and municipal biosolids are known reservoirs for the potentially hundreds of pharmaceuticals that are in use throughout the world. Over the past 15 years, research has focussed on gaining an understanding of the extent of exposure, the fate and uptake of these compounds and the potential toxicological impacts these compounds may have once introduced. The agricultural system is a complex web of micro and macro fauna that includes microbes, fungi, invertebrates and plants which all may act as sinks for bioaccumulation and receptors for these biological active xenobiotic compounds. In this review, we describe how different experimental designs have been utilised to provide insights into the extent of uptake into plants and invertebrates, the mechanisms that govern this fate process and the evidence of biological effects that makes up our current understanding of pharmaceutical exposure in agricultural systems. We highlight the types of compounds as well as the model plant and invertebrate organisms that have been most studied. Furthermore, we discuss how geographical and economic drivers have influenced where research has been conducted and how this may bias our current understanding of pharmaceutical exposure risk as it relates to low- and middle-income countries.

Keywords Biosolid amendments, Earthworms, Pharmaceuticals, Plant uptake, Wastewater reuse

1 Introduction

The demonstrated persistence of pharmaceuticals in soils following land application of wastewaters, sludges and manures [1–3] spurred on a wealth of studies to evaluate the fate of pharmaceuticals in terrestrial systems. In particular, research efforts have centred on the uptake and accumulation of pharmaceuticals in plants and terrestrial invertebrates, including earthworms.

In the case of plant uptake studies, the majority has been carried out to consider edible crop accumulation of pharmaceuticals and related human health risks following ingestion (reviewed by [4]). Other studies have focussed on the ability of plants to remediate pharmaceutical-contaminated water bodies through the use of selected aquatic macrophytes, such as constructed wetlands. For example, several researchers have tried to evaluate the removal of carbamazepine by plants (e.g. *Lolium perenne*, *Typha* spp., *Typha latifolia*, *Iris sibirica*, *Zantedeschia aethiopica* and *Scirpus validus*) and their potential use in phytoremediation with removal efficiencies reported to range from 34 to 82% [5–9].

A suite of experimental set-ups in both the field and the laboratory have been used to evaluate the uptake and accumulation of pharmaceuticals from soils into terrestrial plants. In countries with a high demand for limited freshwater resources (e.g. Israel, Saudi Arabia), research has typically focussed on field studies coupled with

exposure via artificially spiked or natural wastewater, in an attempt to understand the impacts of wastewater reuse schemes increasingly used in agriculture [10, 11]. Other field experiments have studied the uptake of pharmaceuticals following the land application of organic fertilisers such as sewage sludge (biosolids) and manure (e.g. [12, 13]). Antibiotics are typically the most abundant pharmaceuticals detected in plants following soil amendments with manure, a result of their high usage in agricultural husbandry, which are commonly used as agricultural fertilisers.

A variety of crop types including roots, shoots, stems and fruits have been shown to accumulate a range of pharmaceuticals. Studies have reported highly variable rates of accumulation with concentrations ranging from no detection to low μg/kg concentrations in an environmentally relevant exposure scenario. As highlighted in Tables 1 and 2, there has been a focus on edible crops that require minimal processing, such as vegetables with fewer studies evaluating the uptake and accumulation in grain crops such as maize and wheat. In addition, studies have typically worked to define whole organ accumulation, such as leaf tissues, rather than demonstrating cellular accumulation [34, 35].

Short-term laboratory exposures are typically used to provide mechanistic insight into the uptake of pharmaceuticals (e.g. [5]) as well an evaluation of the formation of metabolites (e.g. [35, 36]) (see chapter "Impact of PhACs on Soil Microorganisms"). Mechanistic studies also typically use hydroponics, where plants are grown in a nutrient medium, thereby negating competitive sorption processes observed when soil is present. Laboratory exposures allow for the evaluation of specific end points following exposure in well-controlled conditions (e.g. growth chamber) where temperature, light and humidity can be regulated. However, it has been argued that this exposure lacks environmental relevance as it does not replicate natural environmental fluctuations. Generally, plant uptake studies are relatively short term consisting of one crop cycle (i.e. fruiting or maturation) and have seldom considered accumulation or toxicity resulting from a multigenerational exposure (i.e. from contaminated seed).

To date, research has demonstrated that physiochemical properties of the pharmaceuticals, such as ionisable functional groups, have a profound impact on the uptake, accumulation, translocation and transformation of pharmaceuticals in plants. In addition, the plant species traits, soil properties which control the fate of the chemical, water quality and experimental set-up (exposure duration, concentration and pharmaceutical application) also affect the uptake and accumulation of these chemicals.

The following discussion will give an overview of the uptake of pharmaceuticals in plants and invertebrates through various exposure pathways, including from spiked soils, wastewater irrigation and application of wastewater sludges, or biosolids. Factors that affect the uptake of pharmaceuticals, such as plant species or the physicochemical properties of the pharmaceutical and the environment, are discussed along with implications of uptake, including biological transformation and toxicity of pharmaceuticals. Finally, the geographic location of these studies is considered throughout this chapter to identify where our current understanding is applicable and where knowledge gaps need to be addressed.

Table 1 Pharmaceutical compounds detected in edible plant tissues irrigated with treated wastewater

Compound	Experiment type	Concentration (ng/g or ng/L)[a]	Plant species	Tissue concentration (ng/g)	Fresh weight (FW) or dry weight (DW	BCF	Location	Reference
Acetaminophen	Field	200	Maize	27.5	DW	NA	Jarama River subbasin, Spain	[14]
Amitriptyline	Greenhouse	70	Lettuce	1.5	FW	NA	Spain	[15]
Atenolol	Field	850	Cabbage	55.0	FW	1.4	Al Hayer, Saudi Arabia	[11]
	Semi-field	492	Maize	1.2	FW	2.67	Madrid, Spain	[16]
	Semi-field	492	Lettuce	2.0	FW	0.67	Madrid, Spain	[16]
	Semi-field	492	Radish	1.9	FW	1.58	Madrid, Spain	[16]
	Greenhouse	1,629	Lettuce	0.1	FW	NA	Spain	[15]
Azithromycin	Field	302	Bermuda grass	90.0	FW	NA	Arizona, USA	[17]
Caffeine	Greenhouse	N/M	Tomato fruit	1.0	FW	NA	Almeria, Spain	[18]
	Field	1,548	Cabbage	125.0	FW	1.3	Al Hayer, Saudi Arabia	[11]
	Field	17,000	Cabbage	21.0	DW	NA	Jordan Valley, Jordan	[19]
	Field	17,000	Potato	30.0	DW	NA	Jordan Valley, Jordan	[19]
	Lysimeter	1,003	Sweet potato	0.3	FW	NA	Kiryat Gat, Israel	[20]
	Lysimeter	1,003	Carrot	0.3	FW	NA	Kiryat Gat, Israel	[20]
	Greenhouse	590	Cucumber	3.7	DW	NA	Rehovot, Israel	[10]
	Greenhouse	590	Tomato	0.5	DW	NA	Rehovot, Israel	[10]
	Greenhouse	756	Radish	1.2	FW	NA	Spain	[15]
	Greenhouse	756	Lettuce	1.3	FW	NA	Spain	[15]

Carbamazepine	Field	23	Wheat grain	1.9	DW	NA	Pennsylvania, USA	[21]
	Greenhouse	N/M	Tomato	0.2	FW	NA	Almeria, Spain	[18]
	Field	2,400	Eggplant	32.0	FW	NA	Jordan Valley, Jordan	[19]
	Field	2,400	Cabbage	10.0	DW	NA	Jordan Valley, Jordan	[19]
	Field	2,400	Zucchini	7.0	DW	NA	Jordan Valley, Jordan	[19]
	Field	2,400	Pepper	9.0	DW	NA	Jordan Valley, Jordan	[19]
	Field	2,400	Tomato	5.0	DW	NA	Jordan Valley, Jordan	[19]
	Field	2,400	Parsley	90.0	DW	NA	Jordan Valley, Jordan	[19]
	Field	2,400	Rucola	60.0	DW	NA	Jordan Valley, Jordan	[19]
	Field	2,400	Lettuce	230.0	DW	NA	Jordan Valley, Jordan	[19]
	Field	2,400	Potato	75.0	DW	NA	Jordan Valley, Jordan	[19]
	Field	2,400	Carrot	15.0	DW	NA	Jordan Valley, Jordan	[19]
	Field	130	Maize	0.4	DW	0.05	Jarama River subbasin, Spain	[14]
	Semi-field	114	Maize	–	FW	0.22	Madrid, Spain	[16]
	Semi-field	114	Lettuce	1.8	FW	2.12	Madrid, Spain	[16]
	Semi-field	114	Radish	–	FW	1.15	Madrid, Spain	[16]
	Lysimeter	810	Sweet potatoes	0.1	FW	NA	Kiryat Gat, Israel	[20]

(continued)

Table 1 (continued)

Compound	Experiment type	Concentration (ng/g or ng/L)[a]	Plant species	Tissue concentration (ng/g)	Fresh weight (FW) or dry weight (DW	BCF	Location	Reference
	Lysimeter	810	Carrot	0.8	FW	NA	Kiryat Gat, Israel	[20]
	Greenhouse	369	Carrot	60.0	FW	NA	Caldes de Montbui, Spain	[22]
	Greenhouse	1,060	Cucumber	13.0	DW	NA	Rehovot, Israel	[10]
	Greenhouse	1,060	Tomato	1.5	DW	NA	Rehovot, Israel	[10]
	Greenhouse	156	Radish	0.2	FW	NA	Spain	[15]
	Greenhouse	156	Lettuce	0.4	FW	NA	Spain	[15]
	Greenhouse	2,990	Cucumber	1.0	FW	18	Rehovot, Israel	[23]
Ciprofloxacin	Field	600	Cabbage	7.0	DW	NA	Jordan Valley, Jordan	[19]
	Field	600	Carrot	12.0	DW	NA	Jordan Valley, Jordan	[19]
Clarithromycin	Field	ND	Bermuda grass	135.0	FW	NA	Arizona, USA	[17]
Clofibric acid	Greenhouse	18	Lettuce	18.0	FW	NA	Caldes de Montbui, Spain	[22]
Diclofenac	Field	49.67	Tomato fruit	11.6	FW	132	Nicosia, Cyprus	[24]
	Field	1,600	Eggplant	18.0	DW	NA	Jordan Valley, Jordan	[19]
	Greenhouse	22,410	Lettuce	19.0	FW	NA	Caldes de Montbui, Spain	[22]
Flunixin	Greenhouse	367	Lettuce	83.0	FW	NA	Caldes de Montbui, Spain	[22]
	Greenhouse	367	Carrot	17.0	FW	NA	Caldes de Montbui, Spain	[22]

Gabapentin	Field	2,100	Parsley	20.0	DW	NA	Jordan Valley, Jordan	[19]
	Field	2,100	Rucola	38.0	DW	NA	Jordan Valley, Jordan	[19]
	Field	2,100	Carrot	9.0	DW	NA	Jordan Valley, Jordan	[19]
	Field	452	Cabbage	75.0	FW	1.3	Al Hayer, Saudi Arabia	[11]
	Field	452	Green beans	45.0	FW	1.2	Al Hayer, Saudi Arabia	[11]
	Field	452	Eggplant	34.0	FW	0.9	Al Hayer, Saudi Arabia	[11]
Hydrochlorothiazide	Greenhouse	N/M	Tomato fruit	0.2	FW	NA	Almeria, Spain	[18]
	Field	2,100	Parsley	10.0	DW	NA	Jordan Valley, Jordan	[19]
	Greenhouse	1927	Radish	2.0	FW	NA	Spain	[15]
	Greenhouse	1927	Lettuce	0.5	FW	NA	Spain	[15]
Ibuprofen	Field	220	Maize	1.1	DW	0.05	Jarama River subbasin, Spain	[14]
	Greenhouse	350	Lettuce	30.0	FW	NA	Caldes de Montbui, Spain	[22]
	Greenhouse	350	Carrot	16.0	FW	NA	Caldes de Montbui, Spain	[22]
Lamotrigine	Lysimeter	1910	Carrot	1.0	FW	NA	Kiryat Gat, Israel	[20]
	Greenhouse	1,490	Cucumber	2.0	DW	NA	Rehovot, Israel	[10]
Mepivacaine	Commercial greenhouse	N/M	Tomato	0.1	FW	NA	Almeria, Spain	[18]
	Greenhouse	39	Lettuce	0.2	FW	NA	Spain	[15]

(continued)

Table 1 (continued)

Compound	Experiment type	Concentration (ng/g or ng/L)[a]	Plant species	Tissue concentration (ng/g)	Fresh weight (FW) or dry weight (DW	BCF	Location	Reference
Naproxen	Field	252	Cabbage	38.0	FW	NA	Al Hayer, Saudi Arabia	[11]
	Greenhouse	576	Lettuce	113.0	FW	NA	Caldes de Montbui, Spain	[22]
	Greenhouse	576	Carrot	17.0	FW	NA	Caldes de Montbui, Spain	[22]
Nicotine	Field	220	Maize	1.2	DW	NA	Jarama River subbasin, Spain	[14]
Nicotinic acid	Greenhouse	4,738	Lettuce	3.1	FW	NA	Spain	[15]
Ofloxacin	Field	2,200	Wheat grain	2.3	DW	NA	Pennsylvania, USA	[21]
Sildenafil	Greenhouse	30	Tomato	0.2	DW	NA	Rehovot, Israel	[10]
Sulfamethoxazole	Field	55.23	Tomato fruit	0.6	DW	5.4	Nicosia, Cyprus	[24]
	Field	22,000	Wheat grain	0.6	DW	NA	Pennsylvania, USA	[21]
Tramadol	Greenhouse	N/M	Tomato	0.7	FW	NA	Almeria, Spain	[18]
Triclosan	Semi-field	40	Maize	3.1	FW	4.24	Madrid, Spain	[16]
	Semi-field	40	Lettuce	1.0	FW	0.13	Madrid, Spain	[16]
	Semi-field	40	Radish	1.8	FW	0.03	Madrid, Spain	[16]
Trimethoprim	Field	73.23	Tomato	3.4	DW	6.4	Nicosia, Cyprus	[24]
Venlafaxine	Greenhouse	649	Lettuce	2.3	FW	NA	Spain	[15]
	Greenhouse	N/M	Tomato	0.1	FW	NA	Almeria, Spain	[18]

Table 2 Pharmaceutical compounds detected in edible plant tissues following application of biosolids

Location	Experiment type	Compound	Concentration (ng/g)[a]	Plant species	Biosolids application rate	Findings	Reference
Canada	Field	44 pharmaceuticals	0.8–5,870	Sweet corn Carrot Tomato Potato	8 t/ha (nine WWTPs)	Uptake of atenolol, ciprofloxacin, 4, epianhydrotetracycline, glyburide, naproxen, triamterene (all <6 ng/g) relative to control	[25]
Chile	Greenhouse	17α-ethynylestradiol (EE2)	500 (10,000[a])	Wheat	90 t/ha (one WWTP)	BCF greater in unspiked biosolid; biosolids increased sorption and decreased desorption of EE2	[26]
China	Field	Climbazole Clotrimazole Miconazole	165–492	Wheat Corn	5–40 t/ha (one WWTP)	No plant uptake occurred	[27]
	Greenhouse	Carbamazepine	7,600[a]	Celery Carrot Pak choi	5 and 10% w/w (one WWTP)	Biosolids significantly reduced uptake for all plant species over 60d growth compared with no biosolid; biosolid rate had smaller effect	[28]
Czech Republic	Greenhouse	45 pharmaceuticals	Up to 920	Spinach	3–4% w/w (two WWTPs)	13 pharmaceuticals accumulated in leaves and roots; large bioaccumulation for sertraline, tramadol and carbamazepine	[29]
Israel	Lysimeter	Carbamazepine (and metabolites)	29 ± 2	Tomato Wheat Lettuce	60 m^3/ha (one WWTP)	Biosolids significantly reduced BCF for tomato, lettuce and wheat ears	[30]
Sweden	Greenhouse	Ketoprofen (KET) Naproxen (NAP) Diclofenac (DCF) Ibuprofen (IBU)	22 (DCF), 217 (IBU)	Soybean Wheat	1.5% w/w (one WWTP)	No plant uptake occurred	[31]

(continued)

Table 2 (continued)

Location	Experiment type	Compound	Concentration (ng/g)[a]	Plant species	Biosolids application rate	Findings	Reference
UK		50 pharmaceuticals (mainly anti-microbial) screened	ND	Sugar beet Oilseed rape Wheat	ND	No plant residues of 50 pharmaceuticals detected; biosolid application rate and pharmaceutical loads not done	[32]
USA	Greenhouse	Carbamazepine (CBZ), Salbutamol (SBT), Sulfamethoxazole (SFM), Trimethoprim (TRM)	93 30 67 25	Cabbage	2% w/w (one WWTP)	CBZ, SFM (not aerials), SBT detected in cabbage. Biosolids decreased frequency of SFM uptake but not CBZ, SBT	[33]
	Greenhouse	Carbamazepine (CBZ) Diphenhydramine (DPH) Fluoxetine (FLX)	10,000[a]	Soybean	5 t/ha (one WWTP)	Biosolids led to greater uptake (compared with pharmaceutical-irrigated soil) due to greater load but reduced bioconcentration factor	[13]

[a]Artificially spiked

2 Plant Uptake from Hydroponic Solutions

Hydroponic exposure, where pharmaceuticals are dissolved in nutrient solutions and plants grow suspended either in the solution or in non-reactive media like glass beads, can offer a mechanistic understanding of plant uptake without the complexities of soil-plant-pharmaceutical interactions. In addition to being ideal systems for uptake studies, hydroponic studies maintain environmental relevance especially as it relates to phytoremediation of contaminated water systems.

2.1 Mechanistic Uptake

One of the earliest mechanistic uptake studies was conducted with the tetracycline antibiotic oxytetracycline in alfalfa (*Medicago sativa*) [37]. This study revealed that the uptake into alfalfa from solution followed the Michaelis-Menten equation which relates the rate of uptake and substrate concentration with a measure of substrate-binding affinity called the Michaelis constant (K_M). The uptake of a structurally different sulphonamide antibiotic, sulfamethazine, in alfalfa was investigated by Kurwadkar et al. [38], which revealed that the greatest concentrations were detected in the roots, whilst there was some translocation to other plant portions including shoots and sap. Interestingly, they found that higher concentrations were measured in the upper plant tissues compared to lower shoots. Mathews et al. [39] investigated the uptake and translocation of the antimicrobials triclocarban and triclosan in 11 food crops. Their study revealed that translocation of these two compounds was limited with maximum translocation from root to shoot of 1.9% and 3.7% for triclocarban and triclosan, respectively. Concentrations in tubers were also less than the concentrations in roots.

The use of chemical inhibition of various plant processes has also provided insights into the uptake mechanisms of antibiotics. Kong et al. [37] followed up their initial oxytetracycline experiments by showing that decreased uptake corresponded to plant metabolic inhibition using 2,4-dinitrophenol and that aquaporin competition with glycerol and silver ions (Ag^+) had no impact on oxytetracycline uptake. Furthermore, cellular stress resulting from mercury (Hg^{2+}) exposure also reduced uptake. These results suggest that uptake of oxytetracycline is not passive, rather an energy-dependent process. More recently, Zhang et al. [40] used the respiration inhibitors salicylhydroxamic acid and sodium azide (NaN_3) and the aquaporin blocker mercuric chloride ($HgCl_2$) to show that the uptake of three veterinary antibiotics (chlortetracycline, sulphamethoxazole, sulfathiazole) was also an active process and that the uptake of chlortetracycline and sulfamethoxazole was associated with aquaporin activity.

^{14}C radiolabelled compounds have been used to provide detailed insights into the distribution of pharmaceuticals in hydroponic systems. The benefits of using radiolabelled compounds include the ability to overcome poor ionisation of some

compounds as well as matrix effects inherent in traditional mass spectrometric instruments [41]. Dodgen et al. [42] investigated the uptake of diclofenac and naproxen and found limited translocation of these anionic compounds in plant edible portions of lettuce (*Lactuca sativa*) and collards (*Brassica oleracea*). Interestingly, they found that the vast majority of ^{14}C was not extractable from plant tissues.

Meanwhile, the development of multi-residue methods for the analysis of pharmaceuticals with varied physiochemical properties was used to elicit associations between compound properties and distribution within plants. In experiments using 20 frequently occurring pharmaceuticals and personal care products (PPCPs), the root uptake of neutral compounds was shown to positively correlate with the pH-adjusted octanol-water partition coefficient (K_{OW}) adjusted for pH (D_{ow}), whilst the inverse correlation governed translocation from roots to leaves [43]. These trends suggest the importance of fat solubility (lipophilicity) in root uptake and water solubility (hydrophilicity) in xylem-mediated translocation. This work builds upon the results of Tanoue et al. [44] who associated plant uptake with octanol-water partitioning and translocation with chemical polarity using a suite of 13 pharmaceutical compounds.

Recently, the uptake of 13 other commonly used pharmaceuticals selected to represent the wide range of physicochemical properties inherent to these compounds was investigated in lettuce [34]. This study investigated the multiple inter- and intracellular pathways by which pharmaceuticals can enter and translocate within plants. These were the symplast pathway, utilising either passive diffusion across the lipid bilayer membranes or transport utilising integral protein transport in cell walls, and the apoplast pathway as well as the role of the casparian strip in controlling which compounds enter the xylem. The study suggests that there is a molecular weight cut-off around 300 g/mol where physical limitation controlled the pathway that a compound could utilise. Furthermore, using a traditional sorption isotherm system with freeze-dried plants roots, the sorption affinity of these compounds was shown to be a strong indicator of root accumulation and predicted limited translocation to other plant parts [34].

Protein-mediated transport was demonstrated for the psychoactive pharmaceutical amitriptyline by Nason et al. [45]. Investigating the uptake of four psychoactive drugs (carbamazepine, amitriptyline, fluoxetine and lamotrigine), the authors found that the uptake strongly followed transpiration-based accumulation and suggested that underestimation by the model could be the result of a lack of consideration of transporter protein-facilitated uptake. Furthermore, this work also showed that the co-occurrence of psychoactive compounds affected both uptake and metabolism of these compounds and highlights the need for more studies investigating mixture effects. Nason et al.'s study confirmed the earlier work of Dodgen et al. [46], whereby the hydroponic system was used to show that increased transpiration resulted in increased uptake for ionised compounds.

2.2 *Plant Metabolism*

Hydroponic studies have also been used to reveal the mechanisms used by plants to metabolise pharmaceuticals and identify the metabolites. Emhofer et al. [47] used high-resolution mass spectrometry to reveal the metabolites of four non-steroidal anti-inflammatory drugs (ketoprofen, mefenamic acid, naproxen and diclofenac). Using the model species cress (*Lepidium sativum*), they identified 16 metabolites formed by hydroxylation of the parent compound or conjugation with polar plant metabolites like glucose, amino acids and small organic acids. They then followed this up by studying the in-plant metabolism of lipid-lowering statin drugs, including atorvastatin, fluvastatin and simvastatin. These studies revealed nearly 40 metabolites and confirmed hydroxylation and conjugation with sugars and amino acids as major metabolic pathways [48]. Importantly, at near environmentally relevant exposures (1–10 μg/L), 50% of the metabolites identified could be detected. Hydroxylation of diclofenac has been shown to occur within 3 h of exposure, whereby the resulting 4′OH-diclofenac metabolite is conjugated with glucose [49].

Recently, Chuang et al. [34] investigated the metabolism of caffeine in lettuce. Their studies revealed the significance of demethylation reactions in the metabolism of caffeine in lettuce roots with oxidation and hydroxylation providing other metabolic pathways. Using authentic standards for eight metabolites, this study revealed that 20% of the initially applied caffeine was transformed into demethylated metabolites.

2.3 *Environmental Phytoremediation: Uptake in Aquatic Plants*

The environmentally relevant implications of hydroponic exposure are demonstrated by studies investigating the suitability of aquatic plants, often referred to as hydrophytes or macrophytes, for phytoremediation in wetlands. Whilst addressed in detail in chapter "Conclusions and Future Perspectives", it should be noted that an extensive amount of work has been done investigating the plant uptake of pharmaceuticals in these systems including veterinary medicines [50, 51], triclosan [52], metformin [53], ibuprofen [54] and diclofenac [55]. More generally, the efficacy of constructed wetlands in the removal of 137 pharmaceuticals was reviewed by Verlicci and Zambello [56].

3 Plant Uptake from Spiked Soil

Following identification of potential pathways by which pharmaceuticals, from both human and veterinary origin, can accumulate in soils, work started to evaluate the potential for uptake and accumulation of these chemicals in plants. This was

primarily led from a human health perspective with a need to assess the risks from consuming edible food crops contaminated with pharmaceutical residues [57].

Some of the earliest published research, from mid-2000 onwards, started by assessing the uptake and accumulation of pharmaceuticals in plants, from soils spiked directly with chemical residues. Boxall et al. [57] provided some of the first experimental evidence that veterinary medicines persist in soils and can accumulate in carrot (*Daucus carota*) roots (tubers) and lettuce leaves. Results suggested that a combination of chemical properties and the crop species influenced the degree of uptake, with only two of the ten target analytes detected both in the carrot and the lettuce leaves (florfenicol and trimethoprim) with levamisole also observed in lettuce leaves and diazinon and enrofloxacin in the carrot roots. The lack of uptake of some pharmaceuticals can be well explained by their degradation in soils, with >90% of dissipation of amoxicillin, sulfadiazine and tylosin observed by the time the lettuce plants were harvested.

Building on the results from the Boxall et al. [57] study, Carter et al. [5] carried out a series experiments to elucidate relationships between the fate of pharmaceuticals in soils and potential uptake by plants. The consistently high carbamazepine uptake into both radish (*Raphanus sativus*) and ryegrass (*Lolium perenne*) (<52 μg/g, dry weight) was suggested to result from a combination of persistence in the soil (DT50 > 40 days), a high degree of bioavailability in soil pore water and being a moderately hydrophobic (log K_{ow} 2.25) and unionised compound. Meanwhile, when the pharmaceutical demonstrated fast dissipation in the soil (e.g. sulfamethazine), this results in diminishing concentrations in the soil matrix and thus smaller fractions are available for uptake. Carbamazepine is a neutral compound with a K_{ow} value similar to where maximum uptake of neutral organics is observed according to the Gaussian distribution proposed by Briggs et al. [58]. Similar results were recently published by Li et al. [28] who also observed that out of a suite of 15 pharmaceuticals spiked into a sandy loam soil, carbamazepine accumulated to the greatest extent in radish leaves and roots, which was up to 738 times greater than the accumulation of the least accumulated compound estrone in the roots. Carbamazepine was weakly sorbed to soil, as well as being highly persistent, making it highly favourable for plant uptake.

Comparatively, Carter et al. [5] observed more hydrophobic pharmaceuticals (e.g. fluoxetine and diclofenac) accumulated to a greater extent in the roots with low translocation capacity to aerial plant organs. As these chemicals were extensively ionised at test soil pH, relationships between log D_{ow} and accumulation in the plants revealed a general increase in log D_{ow} corresponded to an increase in plant uptake factors. Meanwhile, [28] demonstrated, with a strong positive correlation (R^2 0.94), that log D_{ow} was a good predictor of plant bioconcentration from pore water ($BCF_{pore\ water}$) for non-ionised pharmaceuticals, with similar relationships not evident for ionised pharmaceuticals. Interestingly, the relationship with log D_{ow} for non-ionised pharmaceuticals could not be replicated for bioconcentration factors (BCFs) based on bulk soil concentrations (BCF_{soil}) implying that pharmaceuticals present in soil pore water represent the major bioavailable fractions for plant uptake. Li et al. [28] concluded that BCFs calculated on the basis of pharmaceutical

concentration in bulk soil are not comparable amongst the studies using different soils because of the varying affinities of pharmaceuticals to soils. For example, the differences in soil-based BCFs between three soils for caffeine, carbamazepine and lamotrigine in tomato (*Solanum lycopersicum*) or cucumber (*Cucumis sativus*) were found to be 20, 7.8 and 245 times, respectively [10]. To follow this up, Li et al. [28] applied the quasi-equilibrium partition model, first developed by Chiou et al. [59], with extensive literature data and found that the root concentration factors (RCF) correlated strongly with chemical-root lipophilic coefficients ($f_{lip}K_{ow}$).

3.1 Distribution Amongst Different Plant Organs

Accumulation in different plant organs of the same species can be explained by movement of the chemicals in the xylem from the roots, upwards towards aerial tissues which is driven by the transpiration stream [28]. Research to date underpins that this is largely driven by chemical properties of the pharmaceuticals.

For example, ionisation of functional groups was shown to play a significant role in the distribution of pharmaceuticals in the radish experiment by Li et al. [28]. Both carbamazepine and lamotrigine have similar log D_{ow} values (2.45 and 2.57, respectively) and molecular weights (236.27 and 256.10 g/mol, respectively) and based on their p*K*a values were known to exist in their neutral form in the pore water. However, the magnitude of pharmaceutical uptake towards the aerial tissues, measured by a translocation factor, was approximately four times larger for carbamazepine (~8.0) than lamotrigine (~2.0). This was explained by the fact that 41% of lamotrigine became positively charged in the vacuoles, meaning that it became trapped in the negatively charged cell walls and thus reduced the translocation of lamotrigine to the leaf material. In comparison, carbamazepine has no ionisable functional groups and would have had no ionic interaction with the cell walls. Ion trapping has been previously shown to enhance the accumulation of organic chemicals in plants due to the alteration of chemical speciation in cell organelles, whereby cationic chemicals become attracted to negatively charged plant root cell membranes [60].

Recently, Li et al. [61] used radioautographic analysis to understand the distribution patterns of ^{14}C labelled carbamazepine uptake by three edible plant species, celery (*Apium graveolens*), pak choi (*Brassica rapa* subsp. *chinensis*) and carrot. Whilst ^{14}C-carbamazepine was taken up by all three plants, a lower amount of ^{14}C was visualised in the stems in comparison to the roots and leaves supporting earlier findings that the movement of carbamazepine towards fruits and leaves is driven by transpiration processes with stems serving as a pathway for transport of carbamazepine by mass flow [5]. Movement of carbamazepine via mass flow towards the aerial parts of the plant has also been suggested to be responsible for the accumulation of this pharmaceutical in the nectar and pollen of flowering plants [62]. In this study, residues of carbamazepine up to 371 ng/mL and 30 μg/g were detected in nectar and pollen sampled from zucchini flowers (*Cucurbita pepo*) grown in

carbamazepine spiked soil (0.5–20 μg/g). Under realistic exposure conditions from the use of recycled wastewater, carbamazepine concentrations were estimated to be 0.37 ng/L and 30 ng/kg in nectar and pollen, respectively. These findings were then used to simulate pharmaceutical exposure to honeybees via contaminated nectar and pollen at a landscape scale. This work illustrates a fundamental first step in assessing the risk of pharmaceuticals to bees, although more work is needed to assess the accumulation of a wider range of pharmaceuticals in nectar and pollen in fruiting plants. Given the biological potency of pharmaceuticals, accumulation of these chemicals in nectar and pollen suggests potential implications for honeybee health, with unknown ecosystem consequences.

Plant uptake of the antidiabetic compound metformin, the antibiotic agent ciprofloxacin and the anti-coccidial narasin was investigated in a spiked soil exposure with barley (*Hordeum vulgare*) (root, leaf, seed) and carrot Napoli (root, leaf) [63]. Whilst metformin was the only pharmaceutical to be detected in all plant compartments, all pharmaceuticals were measured at higher concentrations in plant roots compared to their aboveground compartments in barley and carrot. Higher concentrations in roots than aboveground compartments were similarly observed for all pharmaceuticals tested where chemicals were spiked into a growth medium by means of exposure [64, 65]. Interestingly, within the carrot root itself, [57] observed that a majority of the veterinary medicines that were taken up were associated with the outer layer of the carrot, with the exception of trimethoprim. Similar results were found by Eggen et al. [63] where metformin BCFs for carrot and potato (*Solanum tuberosum*) were higher in the peels than for the cores. These results demonstrate that even within the root itself, there is variation in pharmaceutical accumulation between the various plant organs.

However, other studies have observed that pharmaceutical distribution between different plant organs is dependent on the plant species in question and the pharmaceutical itself. In spiked soil studies, differences in accumulation of pharmaceuticals between radish leaf and radish root were also observed by [5], with higher concentrations in the roots reported for fluoxetine, triclosan and propranolol which translated into larger uptake factors (based on soil concentrations) for these chemicals in the roots. Comparatively, higher total concentrations and larger uptake factors (based on soil concentrations) for carbamazepine and diclofenac were observed in the radish leaf in comparison to the root. Studies showing higher concentrations of pharmaceuticals in aboveground parts compared to roots have been reported previously, when pharmaceuticals were added to soil-plant systems either via the addition of biosolids or reclaimed wastewater [8, 13, 23]. The difference in accumulation amongst pharmaceuticals clearly demonstrates the significant role of chemical properties play in the distribution of pharmaceuticals within a single plant species.

Karnjanapiboonwong et al. [66] evaluated the accumulation of triclosan and 17α-ethynylestradiol (EE2) in the pinto bean (*Phaseolus vulgaris*) in both sand and soil exposures. Plants accumulated EE2 at higher concentrations than triclosan which was thought to be related to the sorption of these compounds in soil, with less triclosan chemical available for plant uptake in comparison to the more bioavailable EE2. In the soil exposure, both EE2 and triclosan accumulated to a greater extent in

the roots than the leaves of the pinto bean, up to 31 μg/g and 6.4 μg/g, respectively. Unlike most published research where plant samples are taken on the final day of harvest for chemical analysis, samples in this experiment were taken every 7 days for a period of 28 days which enabled the kinetics of plant uptake to be evaluated. In the soil exposure, maximum accumulation in the roots was reached after 14 days for both chemicals, after which there was little to no further increase. In fact, the distribution of EE2 to roots decreased over time from 1.5% in the first week to 0.8% in the fourth week. Conversely, for the sand exposure, accumulation was much larger on the whole and continued to increase in a linear fashion in the roots for both EE2 and triclosan. Comparatively, the distribution of EE2 in leaves from the sand exposure was very low (0–0.7%) during the first 3 weeks, although it did increase over time, whereas it was very low in leaves (0.1–0.7%) over the entire study period.

3.2 Differences in Accumulation Amongst Different Plant Species

Studies previously discussed highlight differences in the uptake and accumulation of pharmaceuticals not only within various plant organs but also across a wide range of plant species. Differences may be explained by factors such as degree of root growth, transpiration rates and the size and shape of the leaf material. Differences in plant lipid contents may also be important as this can affect the sorption of hydrophobic chemicals [67]. For example, the lipid content of perennial ryegrass is higher than for radish bulbs, which only contain trace amounts of lipid. Carter et al. [5] suggested this may, in part, explain the lower uptake of carbamazepine, diclofenac and propranolol in radish. Differences in plant uptake behaviour, however, could not be solely attributable to differences in lipid content between plants, which was supported by findings in a study by Wu et al. [68].

The role of species traits in plant uptake has been comprehensively addressed in a greenhouse study by Eggen et al. [63] who investigated the accumulation of the antidiabetic medication, metformin, in nine edible plant species encompassing fruits, cereals, leaves and roots. The species included barley (*Hordeum vulgare* cv. Edel), wheat (*Triticum aestivum* cv. Bjarne), oat (*Avena sativa* cv. Berlinda), carrot (*Daucus carota* cvs. Napoli and Amagar), potato (*Solanum tuberosum* cv. Astrix), tomato (*Solanum lycopersicum* cv. Suzanne), zucchini (*Cucurbita pepo* cv. Black Beauty), bean (*Vicia faba* cv. Red Epicure) and rape (*Brassica rapa* cv. Valo, *Brassica napus* cv. Sheik and *Brassica napus* cv. Sola). High uptake and translocation of metformin in oily seeds of rape *B. napus* and *B. rapa* were measured with BCFs up to 21.72. Comparatively, the BCFs for the cereals were 15–70 times lower, 0.29, 0.91 and 1.35 for wheat, barley and oat, respectively, and accumulation factors of metformin in tomato and squash fruits were even less. The authors suggested this might be a result of metformin being able to mimic natural nitrogen compounds which are easily carried across membranes via transporters in high allocation

nitrogen crops such as *B. napus*. Meanwhile, translocation rates of metformin to fruits were observed to be influenced by distance from the root, with BCFs for the first truss of mature tomato fruits (0.024) significantly lower than the fourth truss (0.058). A corresponding, but not significant, trend for BCF for the first (0.122) and fourth (0.182) fruits of squash was found.

3.3 Metabolism

A study by Carter et al. [36] on uptake of benzodiazepines, which also highlighted the importance of the relationship between soil partitioning, ionisation of benzodiazepines and plant uptake, included an analysis of known benzodiazepine metabolites. Results revealed active in-plant metabolism of benzodiazepines, potentially analogous to the known metabolic transformation pathway of benzodiazepines in humans. Interestingly, the metabolites detected in the diazepam, temazepam and chlordiazepoxide treatments were benzodiazepine parent compounds in their own right. Significant concentrations of nordiazepam were detected in the diazepam- and chlordiazepoxide-exposed plants, in both soil types, which were in excess of the concentration reported for the parent compound [36].

Eggen et al. [63] also analysed plant samples for guanylurea, a known metabolite of metformin. It was only detected in barley grains, bean pods, potato peel and small potatoes in the range of 2.6–5.7 mg/kg with no relationship between high plant metformin concentration and the detection of guanylurea. As with the benzodiazepine study, as guanylurea was not detected in the soil, this supports active in-plant metabolism processes rather than root uptake of guanylurea from the soil. With a predominant focus of assessing plant uptake and accumulation of pharmaceutical parent compounds, these studies highlight the need for further research to elucidate the metabolic pathways pharmaceuticals in plants and to determine whether the resulting metabolite products retain their bioactive nature and thus pose a risk to human and ecosystem health.

The last 15 years have generated a wealth of research from both greenhouse and growth chamber studies where soils have been spiked with a range of pharmaceuticals. Such studies have shown clear differences in accumulation between plant species and amongst various plant organs. However, where soils have been spiked to replicate exposure in the environment, the concentrations used in these studies are often in excess of measured environmental concentrations which have been reported in the μg – low mg/kg range. For example, Boxall et al. [57], Li et al. [28], Karnjanapiboonwong et al. [66] and Carter et al. [5, 36] all spiked soils at concentrations of approximately 1 mg/kg, whilst Eggen et al. [63], Eggen and Lillo [69] and Ahmed et al. [70] reported nominal concentrations for their studies to range between 5 and 20 mg/kg. However, it is important to consider these studies are generally designed to understand kinetic uptake mechanisms and explore potential fate processes such as metabolism, and therefore spiking at higher concentrations is essential to ensure uptake into the plant to observe these changes.

4 Plant Uptake from Wastewater Irrigation

Increasingly, wastewater, treated and untreated, has become an alternative for diminishing freshwater reserves in water-stressed regions throughout the world [62]. As wastewater is a known reservoir for human-use and veterinary pharmaceuticals, the reuse of wastewater represents an important pathway for these biologically active compounds to enter the environment. As such, literature has increasingly investigated how routine irrigation with wastewater may impact the levels of pharmaceuticals and their metabolites in plants, with a particular focus on the edible portions included in the human diet.

4.1 Fortified Wastewater Exposure

Wastewater represents a complex matrix that is theoretically composed of a mixture of the residues of potentially hundreds of pharmaceuticals that are consumed by the population serviced by a particular wastewater treatment plant or WWTP [71]. This complexity makes the study of the fate and uptake of pharmaceuticals in soil-plant systems challenging. Therefore, studies have been undertaken using water fortified with known concentrations of specific pharmaceuticals as a means to simplify experiments. There have even been some efforts to develop synthetic wastewater which provides consistent exposure of study compounds whilst increasing environmental relevance as it approaches wastewater and wastewater effluent [72, 73].

4.1.1 Impacts of Plant Species

As has been shown in soil-spiked systems, different plant species accumulate pharmaceuticals at different rates and in various tissue compartments (e.g. root, shoot and fruit). Studies utilising pharmaceutical-fortified irrigation water have also demonstrated these trends. One such study fortified water to irrigate arugula (*Eruca sativa*) and corn (*Zea mays*) with eight pharmaceutical compounds at concentrations measured in Italian wastewater ranging from 4.6 ng/L (salbutamol) to 249 ng/L (lincomycin) as well as mixtures 10x and 100x these measured concentrations [74]. Lincomycin and ofloxacin were the only compounds detected above the limits of detection in corn grain at the environmentally relevant exposures, whereas all eight compounds were detected in the arugula leaves. Arugula uptake of three pharmaceuticals (atenolol, sulfamethoxazole and carbamazepine) was investigated by Kodesova et al. [29] in addition to three other vegetables (lettuce, spinach and radish). Through their analysis of carbamazepine and its metabolism, they showed that the two Brassicaceae species (radish and arugula) were far less efficient in metabolising the parent compound than lettuce and spinach. Sallach et al. [75] showed a sub-species level difference in the uptake of two antibiotic compounds

(lincomycin, sulfamethoxazole) by two different lettuce cultivars. By sampling at three points throughout the growth stage, they also demonstrated how the rate of plant growth during the maturation process can exceed the rate of uptake, resulting in decreasing concentrations in lettuce leaves. Azanu et al. [76] showed similar relative concentrations of amoxicillin and tetracycline uptake in the edible portions of lettuce and carrot with average concentrations of amoxicillin 27.1 ng/g and tetracycline 20.2 ng/g.

Such results have contributed to prioritisation efforts, like that of Christou et al. [77], who ranked different crop species based on potential risk resulting from the accumulation of pharmaceuticals. They show that leafy green vegetables and root edible vegetables pose the most significant human exposure risk due to the consumption of pharmaceuticals in fresh produce.

4.1.2 Impacts of Soil Type

Unlike hydroponic systems, the soil-plant system introduces both the relevance and complexities of chemical-soil-plant interactions. A few studies using pharmaceutical-fortified irrigation water have been conducted using soils of differing properties in an effort to determine how soil-pharmaceutical interactions impact uptake. In addition to four plant species, Kodesova et al. [29] also investigated three soil types (loess, paragneiss and sand). Interestingly, they showed that differences in transformation processes of the compounds in the different soils had a more significant impact on uptake than the sorption affinity of the compounds to the different soils. Sallach et al. [78] utilised a manipulated soil system, whereby sand and Sharpsburg silt clay were mixed at varying proportions to provide a low organic sand, sandy loam and loam soil. Uptake into lettuce of three antibiotic compounds (lincomycin, oxytetracycline and sulfamethoxazole) was compared with their sorption coefficient (K_d) values in each of the three soils. Results showed that only the uptake of sulfamethoxazole in lettuce shoots followed the expected trend of increasing uptake resulting from decreasing soil sorption (K_d), whereas oxytetracycline, with a K_d value three orders of magnitude greater than sulfamethoxazole, was not detected in lettuce shoots.

4.1.3 Impacts of Environmental Conditions

Whereas the hydroponic system has been used to show the significance of transpiration rate on pharmaceutical uptake [45, 46], the fortified irrigation system has been used to evaluate how environmental processes, namely, soil moisture conditions, impact the uptake of pharmaceuticals. Santiago et al. [79] used varied volumetric soil moisture depletion thresholds of 14% (−4.26 kPa), 10% (−8.66 kPa) and 7% (−18.37 kPa) to investigate the uptake of atenolol, diclofenac and ofloxacin in cowpea (*Vigna unguiculata*), Swiss chard (*Beta vulgaris* var. *cicla*), turnip (*Brassica rapa* var. *rapa*), whole collards, basil (*Ocimum basilicum*), lettuce and cilantro

(*Coriandrum sativum*). They found that, consistently, uptake potential followed ofloxacin > atenolol > diclofenac and that there was a minor influence on uptake resulting from soil moisture depletion, whereby higher concentrations of the compounds were highest in plants grown under the higher soil moisture condition of 14% (−4.26 kPa).

Similarly, the effect of soil moisture, as evaluated by drought stress in lettuce plants, on the uptake of antibiotics and antibiotic-resistant *Salmonella* by lettuce was investigated by Zhang et al. [80]. Using an experimental design where lettuce was grown to maturity using clean freshwater then allowed to reach one of the three wilting conditions (no wilting, mild wilting and severe wilting) prior to irrigation with freshwater fortified with *Salmonella* and sulfamethoxazole, lincomycin and oxytetracycline. Upon irrigation after wilting, all lettuce plants were able to recover, confirming no permanent wilting damage. However, drought conditions did affect concentrations with increasing concentration of lincomycin and oxytetracycline corresponding to increased drought and the inverse results for sulfamethoxazole.

4.1.4 Impact of Irrigation System

Whilst the majority of pharmaceutical irrigation studies utilise or simulate drip irrigation systems, only a few studies have looked at the impact that different irrigation systems have on the accumulation of pharmaceuticals in plants irrigated with reclaimed water. Bhalsod et al. [81] investigated how root and shoot concentrations of 11 pharmaceutical compounds differed between exposure via an overhead irrigation and a drip irrigation system. Their study showed that overhead irrigation significantly increased pharmaceutical concentrations in lettuce shoots even after washing for tylosin, monensin and trimethoprim and highlights foliar sorption as a potentially important mechanism for pharmaceutical incorporation. Concentrations on a fresh weight basis ranging from 0.05 ± 0.04 μg/kg for sulfadiazine to 345 ± 139 μg/kg for carbamazepine, like other studies which have included carbamazepine, showed high levels of uptake and translocation into plant shoots.

Within subsurface drip irrigation systems, the potential to inject air has emerged as a technique to increase crop yields and overcome root zone wetting issues. D'Alessio et al. [82] investigated the potential impact of subsurface air injection on pharmaceutical uptake by lettuce when drip irrigation of water fortified with caffeine, carbamazepine and gemfibrozil. The added aeration increased lettuce plant mass and root length and altered soil microbial communities. It also affected the uptake of pharmaceuticals with increased uptake of carbamazepine and decreased gemfibrozil observed with air injection.

4.2 Wastewater Exposure

Hydroponic, spiked soil and fortified water irrigation studies have provided significant insights into the processes that underlie the uptake and translocation of pharmaceuticals in plants. However, these systems only simulate the agricultural practices by which pharmaceuticals enter the agroecosystem. These studies simplify the issue by removing the complexity of finished wastewater that is also composed of levels of organics, salts and nutrients as well as microbiological activity. Whilst some studies have made efforts to artificially include these complexities with abiotic synthetic wastewater [75], work over the past 10 years has sought to approach and even evaluate pharmaceutical uptake from actual reclaimed wastewater in greenhouse, semi-field and field studies.

One of the challenges of using treated wastewater is that the levels of particular pharmaceuticals fluctuate day-to-day and even intra-day depending on usage practices [83]. Therefore, gaining insights into the mechanisms of uptake is challenging. However, fortifying wastewater with a known concentration of one or more pharmaceuticals ensures a minimum exposure concentration, in addition to the background levels in the wastewater, and allows for the study of uptake with all the additional complexity of the wastewater matrix. Although not field-based studies, studies using actual wastewater described below are limited in number of studies, region of the world studied and compounds investigated (Table 1).

4.2.1 Greenhouse Studies: Israel

Shenker et al. [23] were one of the first to apply this approach of fortifying wastewater to study the uptake and accumulation of carbamazepine in cucumber plants. Greenhouse studies with wastewater fortified with carbamazepine at 1 μg/L showed that BCFs in plant leaves ranged from 17 to 20, whilst root BCFs were far less at 0.8–1. This approach has been used to show the distribution of both neutral compounds (carbamazepine, lamotrigine and caffeine) and ionic compounds (metoprolol, bezafibrate, clofibric acid, diclofenac, gemfibrozil, ibuprofen, ketoprofen, naproxen, sulfamethoxazole and sildenafil) in plant portions of two root vegetables, carrots and sweet potatoes (*Ipomoea batatas*) [20]. Using treated wastewater from a conventional activated sludge system in Israel fortified with the select pharmaceuticals at environmentally measured concentrations, it was shown that neutral compounds lamotrigine, carbamazepine and the carbamazepine metabolite 10,11-epoxycarbamazepine were accumulated at the highest concentrations, as high as 25 ng/g fresh weight. Using a threshold of toxicological concern (TTC) approach, lamotrigine in carrots consumed by children could exceed the TTC threshold and thus be characterised as an emerging concern. Building on this work, Paz et al. [84] showed that sorption of lamotrigine and carbamazepine and its major metabolites in wastewater to soils was governed by soil organic matter content. When the soils produced by the experiments in Malchi et al. [20] were then used for wheat

production with rain-fed water, the uptake of carbamazepine and its metabolite in wheat tissues confirmed that the sorption was reversible and compounds sorbed to soil remained available for plant uptake.

This approach was expanded by Goldstein et al. [10] using cucumber and tomato as model crop species. By comparing freshwater and treated wastewater from an Israeli wastewater treatment facility fortified with a cocktail of pharmaceuticals representing a range of physicochemical properties, they showed that the wastewater matrix helped reduce the bioavailability of pharmaceuticals acting as acids and weak acids in solution.

4.2.2 Greenhouse Studies: Mediterranean Region

Whilst the situation in Israel has received much of the attention, due in part to the country's widespread utilisation of recycled wastewater in agricultural production for nearly 40 years, other regional scenarios have also been studied. The uptake of atenolol, carbamazepine and triclosan in three model crops (lettuce, maize and radish) has been studied in the Mediterranean region [16]. Treatments that included freshwater, treated wastewater and wastewater fortified with the three compounds at two different levels (10× and 100×) were investigated in outdoor conditions. The study revealed that uptake and translocation occurred and were dependent not only on the pharmaceutical physicochemical properties but also on soil properties as well as plant-specific physiological properties of the model crops. Calderon-Preciado et al. [22] investigated the uptake of microcontaminants from secondary wastewater effluent from a wastewater treatment facility in Spain which included six pharmaceuticals (clofibric acid, ibuprofen, carbamazepine, flunixin, naproxen and diclofenac). Using lettuce and carrots as the model crop species, they tested how different tertiary treatment options (chlorination and UV disinfection) impacted uptake. Their results, as demonstrated by principal component analysis, showed that tertiary treatments were effective in reducing the uptake of non-ionisable compounds into lettuce. Exposure and uptake from Spanish wastewater-derived pharmaceuticals was also investigated by Martinez-Piernas et al. [15] who applied their analytical method of 74 micro-pollutants in numerous plant tissue matrices to investigate the potential accumulation of these analytes in radish and lettuce following routine irrigation with an urban wastewater from a treatment facility operating secondary treatment with a conventional activated sludge system. This study revealed the uptake of nine pharmaceuticals and one metabolite in plant tissues resulting from irrigation at concentrations as high as 57.6 ng/g with the treated wastewater.

All previous studies have utilised simplified and controlled systems to evaluate the uptake and potential food chain transfer of pharmaceuticals in soil-plant systems. These studies have revealed valuable insights to determine the factors that contribute to plant uptake as well as to help prioritise compounds that represent the most significant risk to human exposure. However, comparatively few studies have

evaluated the uptake of pharmaceuticals from agricultural field production systems. The work described below is summarised in Table 1.

4.2.3 Field Studies: USA

The Southwest of the USA is composed of arid and semi-arid climates and large population centres putting massive pressure on scarce freshwater resources. Increasingly, the reuse of wastewater has been adopted as an alternative for irrigation. One of the earliest field trials in the USA was conducted by Jones-Lepp et al. [17] where a range of crops that included bell peppers (*Capsicum annuum*), cantaloupe (*Cucumis melo* var. *cantalupensis*), watermelons (*Citrullus lanatus*), carrots and spinach (*Spinacia oleracea*) were irrigated under field conditions with treated municipal wastewater from the city of Tucson, Arizona. Root and shoot portions of plant tissues were analysed for the detection of eight emerging contaminants that included four antibiotics (azithromycin, roxithromycin, clarithromycin and clindamycin) as well as methamphetamine, MDMA, pseudoephedrine and the industrial flavouring agent dimethylphenethylamine (DMPEA). Only DMPEA was detected in any of the edible plant tissues, with the highest accumulation at 180 ng/g in watermelon. Additional study in the USA was carried out by Franklin et al. [21] using the "Living Filter" water reuse site in University Park, Pennsylvania. In this study, wheat was grown under a treated wastewater spray irrigation system on approximately 516 acres of mixed-use land. Four pharmaceutical compounds including sulfamethoxazole, trimethoprim, ofloxacin and carbamazepine were studied both on the uptake and surface deposition of wheat grain and straw. Low-level residues of these pharmaceuticals were both detected on the surface and concentrated within the plant tissues with ofloxacin concentration as high as 10.2 ng/g in straw and 2.28 ng/g in the grain.

4.2.4 Field Studies: Mediterranean Region

The Mediterranean region of Europe is characterised by its limited freshwater resources and intense summer light intensity making wastewater reuse an appealing option. Tertiary treated wastewater from two wastewater treatment facilities in Cyprus provided irrigation water for 3 years of study investigating the uptake of diclofenac, sulfamethoxazole and trimethoprim in tomatoes grown under field conditions [24]. The results of this study demonstrate a key research gap that is not accounted for in typical short-term laboratory exposure experiments. Accumulation of all three pharmaceuticals increased in tomato fruits over the course of the three growing seasons, with highest concentrations of all three found in tomatoes harvested in the final year at 11.6, 5.3 and 3.4 ng/g for diclofenac, sulfamethoxazole and trimethoprim, respectively. This is noteworthy because in real wastewater reuse applications, the infrastructure investments and lack of alternative irrigation sources

will necessarily result in continued wastewater applications on the scale of multiple growing seasons.

Whereas the studies described above utilised greenhouse laboratory experimental designs, Martinez-Piernas et al. [18] investigated the uptake of 60 compounds in tomatoes grown in industrial greenhouse production systems with a 10-year history of wastewater irrigation using effluent from a municipal wastewater treatment plant in Spain. Soil in these systems had not been replaced over the course of the 10-year history. Results showed the highest concentrations of most pharmaceuticals in tomato leaves as compared to tomato fruits. Concentrations detected did not pose a human health concern following the toxicological threshold concern approach. Whilst most studies have considered the intentional use of treated wastewater for irrigation, Santiago-Martin and colleagues identified an unintentional exposure route with surface water highly impacted by wastewater treatment effluent upstream is used for crop irrigation [14]. Though their results showed insignificant risk to human health, acetaminophen, ibuprofen, carbamazepine and nicotine were all detected in maize grown from this impacted water source.

4.2.5 Field Studies: North Africa and the Middle East (MENA)

It is not surprising that the MENA region has become an early adopter of wastewater due in large part to the lack of freshwater resources and its arid climate. As previously discussed, Israel has made use of treated wastewater on a large scale now with over 50% of irrigation water coming from treated wastewater. Other countries in the region are utilising this alternative to precious freshwater resources. Pico et al. [11] investigated the accumulation of pharmaceuticals and pesticides in seven vegetable types (cabbage (*Brassica oleracea*), barley, green beans, eggplants (*Solanum melongena*), chilli (*Capsicum annuum*), tomato and zucchini) from fields irrigated with treated wastewater in Saudi Arabia. Their analysis revealed the presence of six pharmaceutical compounds (atenolol, caffeine, carbamazepine, 10,11-epoxycarbamazepine, gemfibrozil and naproxen) in samples taken from fields. Importantly, they also detected a number of pesticide residues in plant tissues demonstrating that chemical exposome is an increasingly complex mixture of compounds from multiple sources. In Jordan, the uptake of 28 micro-pollutants, including many pharmaceuticals as well as the metabolites of carbamazepine, was studied in ten different field-grown vegetable species [19]. The vegetables sampled included carrot, lettuce, potato, zucchini, tomato, pepper, cabbage, parsley, rucola and eggplant all grown in a drip irrigation system. Pharmaceuticals including caffeine, lamotrigine, gabapentin, ciprofloxacin and gemfibrozil, along with carbamazepine and six carbamazepine metabolites, were all detected in plant tissues. Based on this analysis, it was shown that accumulation in edible plants tissues followed leafy shoot > root > fruit edible vegetables. In terms of human health implications, the antibiotic ciprofloxacin and the carbamazepine metabolite, 10,11-epoxycarbamazepine, posed the most potential risk to human health.

5 Plant Uptake from Biosolid Amendment

A significant pathway for pharmaceutical exposure to terrestrial organisms is through application of sludge derived from wastewater to terrestrial environments. During wastewater treatment, especially biological treatment processes, large quantities of waste sludge are produced from biomass and inorganic matter. Wastewater sludges are typically high in organic carbon that can enhance their association through sorption with pharmaceuticals, especially pharmaceuticals with physicochemical properties that can promote this interaction. Physicochemical properties that can lead to enhanced sorption include a high (e.g. $>10^3$) octanol-water partition coefficient (K_{ow}) and ionisation of functional groups, especially where cationic or zwitterionic species are formed [85]. A broad range of pharmaceuticals have been detected in wastewater sludges, with antibiotics being particularly prominent, as well as NSAIDs, β-blockers and carbamazepine, with concentrations typically in the μg/kg to low mg/kg concentrations [86–89]. In the case of some classes of antibiotics that contain cationic functional groups at ambient pH, such as fluoroquinolones and tetracyclines, association with wastewater sludge can be substantial. This is despite relatively low K_{ow} (or D_{ow}, which represents the ionised K_{ow} value) values, which can occur for the fluoroquinolones [85]. In contrast, pharmaceuticals such as NSAIDs with primarily anionic functional groups at ambient pH are much less likely to associate with negatively charged surfaces of biosolids [88]. In the case of the antimicrobial triclosan, it is also likely to be at least partially negatively charged at ambient pH ($pK_a \sim 8$), but the presence of unionised triclosan, which has a high K_{ow} value, leads to its common detection in wastewater sludges [87, 88, 90].

Following wastewater treatment, sludges are then further stabilised to produce biosolids that can be beneficially reused in terrestrial environments for improving soil condition in agriculture and landscape rehabilitation. The amount of biosolid production and reuse varies globally. For example, in China, there are more than 3,500 WWTPs producing more than 6 million tonnes (dry weight) of sludge, of which the majority is appropriately landfilled, incinerated or reused in construction, whilst ~40% is applied to land [91–93]. Land application is likely to include agricultural use, although the amount diverted for this purpose varies considerably between regions. Although the overall land application of sludge as fertiliser in China is <40%, this can vary from none to all depending on the city or region where the sludge is produced [91]. Similarly, in the USA, reuse of biosolids can vary greatly depending on the state where the biosolids are produced, with an overall production of ~7 million tonnes/year with ~50% reuse in agriculture [94]. In the EU, around 10 million tonnes/year are produced, with the UK producing ~3.5 million tonnes/year of this for 78% agricultural use [95, 96]. Reuse of biosolids is reasonably consistent across regions in other countries such as Australia, which produces around 370,000 tonnes/year, of which 70% is reused for agriculture [97]. In contrast, New Zealand produces relatively low amount of biosolids (~70, 000 tonnes/year) but only uses ~6% for agriculture [97].

Biosolids are generally required to meet certain guidelines with respect to nutrient and contaminant content, although pharmaceutical limits are not applicable [98]. This may in part be related to the relatively low concentrations of pharmaceuticals in biosolids and the subsequent low risk for exposure. Whilst concentrations are generally low, the amount of biosolids applied to land in some jurisdictions can equate to large quantities of pharmaceuticals being transferred each year to agricultural land and being exposed to crops and soil organisms that can be essential for soil health. It is therefore important to assess the potential exposure and risk of pharmaceuticals in these systems to ensure the ongoing beneficial reuse of biosolids can be demonstrated to have little impact.

Comparatively few studies, however, have been undertaken to assess the potential for exposure and uptake of terrestrial organisms in agricultural systems where biosolids have been applied. Of these studies, there have been relatively few pharmaceuticals assessed for uptake into crops (Table 2 summarising pharmaceutical uptake in biosolid amended soils). A notable exception to this was a study by Sabourin et al. [25] who assessed the uptake of ~50 pharmaceuticals in tomato (*Solanum lycopersicon*), potatoes (*Solanum tuberosum*), carrots (*Daucus carota*) and sweet corn (*Zea mays*) grown in soils amended with biosolids at a rate of 8 t/ha, collected from nine Canadian WWTPs. Of the pharmaceuticals detected in the biosolids only ten (atenolol, caffeine, ciprofloxacin, cocaine, epianhydrotetracycline, glibenclamide, minocycline, naproxen, triamterene, trimethoprim) were detected in any of the plants following biosolid addition. Furthermore, uptake into plants was inconsistent amongst replicates, with very low (maximum 6.25 μg/kg, dry weight) concentrations detected in respective plants, suggesting a very low potential for uptake from recommended biosolid applications [25]. A similar trend is also apparent from a number of other studies, where relatively low uptake of pharmaceuticals occurs through application of biosolids in uptake studies or predicted through modelling [13, 27, 30–33, 61, 99–101]. Furthermore, the presence of biosolids was found to reduce the uptake factors of pharmaceuticals (including carbamazepine, 17α-ethinylestradiol and salbutamol) following their addition to fortified soils, despite their natural loads of pharmaceuticals [30, 33]. This is also consistent with studies where biosolids were fortified with pharmaceuticals. For example, the addition of radiolabelled carbamazepine to biosolids (equivalent to 7.6 mg/kg) and applied to soils at 10% w/w addition rates reduced its BCF in celery (*Apium graveolens*) roots, stems and leaves [61]. Specifically, the bioavailability of ^{14}C-carbamazepine was reduced at the end of the celery growing period which resulted in a reduction of 38.6 ± 18.5%, 36.5 ± 15.9% and 63.3 ± 6.3% of ^{14}C in the roots, stems and leaves, respectively, in the 10% biosolid-amended soil. Another study used similarly high concentrations (relative to concentrations measured in unfortified biosolids) of carbamazepine, diphenhydramine and fluoxetine (~10 mg/kg) in spiked biosolids to assess the uptake in soybean (*Glycine max*) following a relatively high application rate (30% w/w) of biosolids to soils [13]. In this study, the total amount of pharmaceuticals taken up was greater in the biosolid-amended soils, although the BCF was lower for carbamazepine and diphenhydramine. These studies highlight the ability of biosolids to decrease the bioavailability of pharmaceuticals to plants

despite the presence of pharmaceuticals in biosolids, due to the enhanced irreversible sorption of pharmaceuticals to biosolids and subsequent decrease in bioavailability to terrestrial organisms.

6 Uptake into Soil Invertebrates

As well as considering the uptake of pharmaceuticals into plants following land application of pharmaceuticals, work has also evaluated the uptake of pharmaceuticals in soil invertebrates, albeit to a lesser extent. A majority of this work has focussed on assessing uptake and accumulation in earthworms from spiked soil under laboratory exposure conditions, although a small number of studies have sampled earthworms from natural soils and have identified the presence of pharmaceuticals following land application of organic wastes. Earthworms represent an ideal sentinel organism for assessing soil contamination, as they are in contact with soil and soil solution, tend to migrate over only short distances and are widely distributed in soils around the globe.

Pharmaceuticals were amongst a suite of 77 anthropogenic waste indicators evaluated by Kinney et al. [102] across three agricultural fields in the Midwest USA. The antibiotic trimethoprim was detected in earthworms sampled from the biosolid- and manure-amended fields, at concentrations of 127 and 61 μg/kg, respectively. Trimethoprim was notably not detected in the soils from which the earthworms were sampled, thereby prohibiting the calculation of a bioaccumulation factor (BAF). The largest BAF for all anthropogenic waste indicators evaluated in this study was calculated at 27 for the personal care product, triclosan. Nevertheless, this study documents that when pharmaceuticals are present in biosolids and swine manures that are applied to agricultural land, these chemicals can be transferred to, and accumulate in, earthworms, under realistic exposure scenarios.

More recently, Bergé and Vulliet [103] determined levels of pharmaceuticals in earthworm samples collected from various soils around Lyon, France, to test their recently developed simple, rapid and effective multi-residue method (QuEChERS approach) for the determination of veterinary antibiotics and human contaminants in earthworm tissue. Concentrations ranging between a few ng/g and 73.5 ng/g (florfenicol) were observed for veterinary antibiotics, whilst concentrations for the human pharmaceuticals paracetamol and fluvoxamine ranged from below the limit of quantitation to 8.8 and 46.8 ng/g, respectively. Earthworms are in contact with the soil surface and are therefore exposed to a variety of organic pollutants from human activities, as evidenced by the results from the field sampling campaigns detailed above. These results demonstrate exposure to a large number of organic micropollutants, including pharmaceuticals, in the wider environment following commonly adopted sustainable agricultural practices such as amendment with manure and compost. For example, the earthworms analysed in the Bergé and Vulliet [103] study were sampled from kitchen garden soils in receipt of widely available compost.

Earthworms can therefore be considered as a terrestrial organism of choice in risk assessment for identifying sources of pollution or to better understanding the input of contaminants in food chains. Concerning the environmental fate and behaviour of chemicals, the Organisation for Economic Co-operation and Development (OECD) proposed guidelines to assess the bioaccumulation of chemicals in soil oligochaetes *Test No. 317* [104]. Recommended test species in this guideline include *Eisenia fetida* and *Eisenia andrei* (Lumbricidae), or white worms *Enchytraeus albidus*, *Enchytraeus crypticus* or *Enchytraeus luxuriosus* (Enchytraeidae). The test consists of two phases: an uptake phase, where test organisms are exposed to the test substance incorporated directly into the soil, and an elimination (post-exposure) phase. Following analysis, parameters which characterise the bioaccumulation of a chemical substance can be determined including the bioaccumulation factor (BAF), the uptake rate constant and the elimination rate constant.

Based on the recommendations outlined in the OECD guideline, the kinetics of pharmaceutical uptake in earthworms were evaluated by Carter et al. [105] in a series of radiolabelled laboratory experiments. Variability in pharmaceutical accumulation between chemicals was observed, with calculated pore water-based bioconcentration factors (BCFs) increasing in the order of carbamazepine < diclofenac < fluoxetine < orlistat. The relatively large BCF of 51.5 for orlistat was suggested to be attributable to the minimal elimination of this chemical in the depuration phase, whereas for carbamazepine, the fast elimination rate of 0.14 d^{-1} could account for the smaller BCF of 2.21. Differences in key physiochemical properties known to control the fate of chemicals, such as hydrophobicity, were also suggested to be responsible for the observed differences in accumulation between chemicals. For example, BCFs increased in a similar order to the increase in octanol-water partition coefficients (log K_{ow}) for the respective compounds, supporting previous research that has suggested that the degree of hydrophobicity has a key role to play in the uptake of pharmaceuticals into organisms. However, unlike neutral organic compounds, the uptake of ionisable pharmaceuticals was found to not be driven solely by the hydrophobicity of the chemical.

It has been widely published in scientific literature that pharmaceuticals can behave very differently in different soil types [106]. For example, distribution coefficients (K_d) between soil particles and soil pore waters are known to vary by several orders of magnitude for a range of pharmaceuticals in soils with varying properties [107]. Such differences in pharmaceutical fate would strongly influence the bioavailable fraction of pharmaceuticals available for uptake by soil dwelling species, and indeed, differences in uptake between soil types were observed by Carter et al. [108] in a later study assessing uptake of four chemically distinct pharmaceuticals in five soil types. BCFs of the individual compounds were found to differ across soil types, with greatest variability observed for diclofenac (7.02–69.57) and orlistat (30.50–115.88), whereas smaller variability of the BCFs was noted for fluoxetine (14.09–20.42) and carbamazepine (1.05–1.61). However, further analysis by Carter et al. [108] to understand the relationship between soil and pore water properties and earthworm uptake failed to highlight any key parameters which may be responsible for pharmaceutical uptake into earthworms. Ultimately,

this suggests that earthworm uptake is a complex interaction of a variety of factors and processes and does not exclusively rely on a single soil parameter.

Research to date has primarily been carried out using the model species in soil ecotoxicology, *Eisenia fetida*, which is a suggested test species in TGD guidelines. Whilst this is not a native soil-dwelling species, preferring to instead occupy high organic content manures, it is easy to maintain in laboratory cultures. However, biological attributes such as species size, feeding habits and reproduction have been widely reported to play a key role in the uptake and bioconcentration of a range of chemicals including metals and DDE [109–111]. Differences in the uptake of pharmaceuticals between *E. fetida* and the larger deep-burrowing earthworm, *Lumbricus terrestris*, have also been observed [112]. In a single soil type, BCFs for carbamazepine and diclofenac were similar between species, whereas for fluoxetine and orlistat, BCFs in *E. fetida* were more than double those seen in *L. terrestris*. Differences in rates of accumulation between species were also observed, with uptake rates faster in *E. fetida*, with the exception of carbamazepine. Observed differences between species such as this raise concerns around the use of a single test organism in risk assessments and bring into question if the current selected species, *E. fetida*, is representative of the diverse array of earthworm species that co-exist in the soil environment.

Given that earthworms occupy a low trophic level in terrestrial food webs, uptake and accumulation of pharmaceuticals by earthworms might serve as the entry point for these chemicals into terrestrial food webs and a route of exposure for higher trophic organisms. To date, wildlife exposure to pharmaceuticals remains poorly characterised with only a handful of published studies on this topic. Whitlock et al. [113] detected residues of the antidepressant fluoxetine in wild-grown Eurasian starlings (*Sturnus vulgaris*) feathers at concentrations up to 27.0 ng/g, providing some first evidence of pharmaceutical exposure in the wild. Nevertheless, accumulation of pharmaceuticals in the food chain has the potential to result in secondary toxicity. For example, [114] observed effects in *S. vulgaris* movement after ingestion of wax worms contaminated with fluoxetine and exposure to xenobiotic estrogenic compounds at concentrations similar to that observed in earthworms collected from trickling filter beds resulted in significant enlargement of the high vocal centre (portion of the brain controlling song production), increased song production and complexity and a decrease in immune function in *S. vulgaris* [115, 116].

Nevertheless, the BCFs calculated in laboratory exposures as well as calculated from earthworms sampled from the field are all relatively small (<100) and would suggest the potential for food chain transfer and secondary toxicity is minimal. However, more research is clearly needed to investigate a wider suite of pharmaceuticals, in a broader range of soil types to fully assess the environmental risk of earthworm exposure to these bioactive chemicals. In addition, whilst we know very little about the uptake of pharmaceuticals by earthworms, we know even less about the accumulation of pharmaceuticals by other soil organisms (e.g. springtails and enchytraeids), which also occupy the soil environment and present a risk via food chain transfer.

7 Effects of Pharmaceuticals in Terrestrial Organisms

7.1 Pharmaceutical-Induced Effects in Plants

Once in the environment, pharmaceuticals can maintain their biological potency which has the potential to elicit effects in plants following their uptake and accumulation. Some of the earliest research documenting effects on plants observed phytotoxicity in response to antibiotic exposure, with studies dating back to as early as the 1980s [117].

For example, the antibiotic sulphadimethoxine was found to suppress normal post-germinative development and growth of barley (*Hordeum distichum* L.) roots and leaves growing both on synthetic medium and on soils with different organic content (0–10%) [118]. At harvest, on day 45 of the soil test, barley wet weight in sulphadimethoxine exposures was reduced (pooled treated: 571.10 mg) in comparison to the controls (pooled control: 772.43 mg). Effects were observed at exposure concentration of 300 mg/L and were dependent on the bioaccumulation rate. Sulphadimethoxine dissolved in a nutrient solution was also found to induce a 45% decrease in root growth of barley seedlings (*Hordeum vulgare* L.) at a concentration of 40 μM [119]. These results were in agreement with previous experimental data on millet (*Panicum miliaceum*), pea (*Pisum sativum*) and maize (*Zea mays)* demonstrating a reduction in root, stalk and leaf growth in response to sulphadimethoxine exposure [65].

In a later study, Migliore et al. [120] evaluated the phytotoxicity of enrofloxacin (50, 100 and 5,000 ug/L) on crop plants *Cucumis sativus*, *Lactuca sativa*, *Phaseolus vulgaris* and *Raphanus sativus*. Concentrations between 50 and 5,000 μg/L induced both toxic effects and hormesis in plants, by significantly modifying both length of primary root, hypocotyl and cotyledons and the number/length of leaves. Enrofloxacin altered all the examined plant organs; however, interestingly, this did not occur in a linear dose-dependent manner: 50 μg/L exposure increased the length/number of *Cucumis sativus* leaves, 5,000 μg/L exposure decreased in comparison to control, whilst at 100 μg/L, an intermediate response was seen. A similar biphasic dose response was also observed in *Raphanus sativus* and *Lactuca sativa*, whereby a slight increase in leaf length was measured at lower concentrations.

Both shoot and root biomass of alfalfa (*Medicago sativa* L.) decreased sharply with increasing concentrations of another antibiotic, oxytetracycline, in a growth solution [37]. Whilst oxytetracycline had no effect on alfalfa shoot biomass at 0.002 mM concentrations, the root biomass decreased significantly ($p < 0.05$) when the OTC concentration was above 0.002 mM. In addition, oxytetracycline decreased shoot and root fresh weight by a maximum of 61% and 85%, respectively, with the shoot/root ratio increasing significantly at lower exposure concentrations, which suggested that the roots are more sensitive to oxytetracycline than shoots.

Similar results were also found by Hillis et al. [121] who observed that roots (carrots) were more sensitive to a suite of ten antibiotics, often by an order of magnitude or more, in comparison to shoots such as lettuce and alfalfa. The response

of the three plant species to antibiotics was highly variable; however, compared with shoot and total length measurements, root elongation was consistently the most sensitive end point. The range of phytotoxicity of the antibiotics was large, with effect concentrations (EC_{25}) ranging from 3.9 to 10,000 μg/L. Chlortetracycline, levofloxacin and sulfamethoxazole were the most phytotoxic antibiotics, with significant effects of chlortetracycline observed as low as 1,000 μg/L and EC_{25}s ranging from 33 to 193 μg/L which the authors noted are lower than reported environmental concentrations of chlortetracycline in swine manure [122]. Interestingly, whilst plant growth was affected, plant germination was insensitive to the antibiotics, with no significant decreases up to the highest treatment concentration (10,000 μg/L).

Phytotoxicity studies have also been carried out using classes of pharmaceuticals other than antibiotics. For example, significant effects ($P \leq 0.01$) on plant dry weight across a suite of maize hybrids have been observed in response to exposure to paracetamol, ibuprofen and diclofenac by Hammad et al. [123]. Exposure to paracetamol in particular resulted in significant effects, with plant dry weight decreasing in a linear fashion with increasing paracetamol concentrations. Interestingly, effects were observed to vary amongst the different maize hybrids with plant dry weight decreasing from 204.5 to 172.0 g plant^{-1} in hybrid ICI 339, whereas a smaller decrease in dry weight was observed in the Syngenta 7720 hybrid from 169.0 to 166.3 g plant^{-1}. The application of paracetamol was also observed to significantly ($p < 0.05$) decrease grains' yield by up to 50% (75.8 vs. 37.5) in the sensitive maize hybrid (Syngenta 7720), with the smallest decrease in grain weight 34% (81.0 vs. 53.3) found in the resistant maize hybrids.

Detectable carbamazepine concentrations were found in edible zucchini (*Cucurbita pepo*) fruit from 1 to 20 μg/kg by Knight et al. [124], but this study also revealed novel insights into the effect of carbamazepine accumulation on *C. pepo* fruiting. Female *C. pepo* flowers were also unable to set fruit when translocation of carbamazepine resulted in leaf concentrations $\geq$14 mg/kg. These findings may have implications for future agricultural productivity in areas where reclaimed wastewater containing pharmaceuticals is a source of irrigation.

In the plant uptake studies by Boxall et al. [57], a decline in plant growth was observed for the veterinary medicine exposures, phenylbutazone, oxytetracycline and enrofloxacin. However, from a review of published literature, the extent to which a pharmaceutical impact on plant growth appears to be dependent on both the plant and physiochemical properties of the pharmaceutical compound. As such, Boxall et al. [57] observed that, whilst effects were seen in three exposures, there is was no effect of florfenicol, levamisole, trimethoprim and diazinon on plant weight data at the time of harvest.

In fact, positive effects on plant growth parameters in response to pharmaceutical exposure have been reported in recent scientific literature. Effects upon germination, development, growth and physiology of radish and lettuce, after exposure to non-steroidal anti-inflammatory drugs (NSAIDs) at environmentally relevant concentrations, were evaluated by Schmidt and Redshaw [125]. An increase in *R. sativus* root length was observed upon exposure to tolfenamic acid, and although not statistically significant, meclofenamic acid sodium-exposed *R. sativus* had longer median root lengths than observed under mefenamic acid and the solvent

control treatments. In addition, ibuprofen treatment resulted in statistically significant enhancement of lettuce primary root development in comparison to control. These results support earlier findings that impacts upon higher plants not only are compound specific but also differ between plant species.

It is important to highlight that the majority of studies which have elucidated the effect of antibiotics on plants have used much higher exposure concentrations into a growth medium, which do not resemble in vivo situations occurring in the natural soil environment (e.g. Liu et al. [126] (100–500,000 μg/ L), Michelini et al. [127] (11,500 μg/L), Migliore et al. [128] (5–50,000 μg L), Michelini et al. [129] (10000–200,000 μg/kg)). More recently, research has started to explore the potential for sublethal effects, or changes in key plant parameters at lower, environmentally relevant concentrations [130, 131].

7.2 Soil Invertebrate Toxicity

There is a general paucity of published scientific literature concerning the ecotoxicity of pharmaceuticals in soil invertebrates, with a majority of the data that is available focussed on earthworm species. Research has primarily centred on evaluating the effects of veterinary pharmaceuticals (antibiotics) with the most common end points considering survival, reproduction and alterations in behaviour such as avoidance and surfacing in response to pharmaceutical exposure. As with uptake in terrestrial invertebrates, studies have also tended to focus on earthworm species. Research published by Litskas et al. [132] revealed amoxicillin had no significant effects on reproduction or weight gain of earthworms (*E. fetida*) across the concentration range of 0.64–78.89 mg/kg soil. The effects of another antibiotic, doxycycline, on earthworm reproduction were later published by Litskas et al. [133]. Whilst no effects on earthworm weight were observed following 28 days of exposure to doxycycline, negative effects on the total number of juvenile earthworms were observed at the concentration level of 30 mg/kg soil. Comparatively, doxycycline did not induce effects on earthworm reproduction or mortality following the application of doxycycline spiked pig slurry (75 and 7,500 μg/mL) to a series of soil columns [134]. Low toxicity of antibiotics (sulphamethoxazole, trimethoprim and tetracycline) was also observed by Pino et al. [135] as none of the selected antibiotics were toxic to *E. fetida* below 2000 mg/kg. Similarly no mortality of earthworms (*E .fetida*) was observed in a multispecies-soil system at the highest oxytetracycline concentration used (100 mg/kg) [136] However, other tetracycline antibiotics have been shown to significantly impact on earthworms. For example, Lin et al. [137] showed that the total number of juveniles was reduced after exposure to chlortetracycline, with effect concentrations (EC_{50} values) for juveniles reported at 96.1 mg/kg. Chlortetracycline also reduced cocoon counts (EC_{50} 120.3 mg/kg) and induced physiological responses and genotoxicity in earthworms.

The effects of antibiotics on earthworms reported by Pino et al. [135] were part of a wider study which evaluated the ecotoxicity of 18 pharmaceuticals which included

non-steroidal anti-inflammatory drugs (NSAIDs), lipid regulators, β-blockers as well as antibiotics. Whilst 14 days of exposure to some of the blood lipid regulators, the β-blockers and antibiotics did not result in observed acute toxicity, the NSAIDs and some blood lipid-regulating pharmaceuticals were acutely toxic (lethality) to earthworms (*E. fetida*) [135]. The greatest toxicity was reported for ibuprofen (lethal concentration, $LC_{50} = 64.80$ mg/kg) followed by diclofenac ($LC_{50} = 90.49$ mg/kg) and simvastatin ($LC_{50} = 92.70$ mg/kg). The lethal concentrations reported for the pharmaceuticals in this study far exceed environmentally relevant concentrations reported for soils irrigated with reclaimed wastewater or amended with biosolids. Similar conclusions were reached in a study of the ecotoxicity of biosolids-borne triclocarban (disinfectant) in earthworms (*E. fetida*, [138]). The LC_{50} for triclocarban was greater than 40 mg/kg for biosolid-amended soils, which far exceeds the concentration of triclocarban previously reported in soils amended with biosolids. In the same study, the researchers determined that triclocarban in biosolids, even at concentrations exceeding the native concentration, did not affect common measures of microbial activity in soil, soil respiration and ammonification.

Based on published research to date, exposure to individual pharmaceuticals at environmentally relevant concentrations is unlikely to result in earthworm toxicity. However, reclaimed wastewater and biosolids contain a complex mixture of pharmaceuticals together with other organic and inorganic contaminants [2, 3]. A recent study of the ecotoxicity of land-applied biosolids found biosolids to be acutely toxic to earthworms (*E. fetida*) even at environmental relevant application rates to agricultural soils [139]. End points measured in the study included earthworm survival and measures of earthworm reproduction. Biosolids at application rates as low as 1% by mass in soil resulted in a significant reduction in adult earthworm survival and complete lethality at 3 and 4% biosolids in soil. Similar results have been reported in by others [140]. The production of juvenile earthworms and cocoons (measures of reproductive success) was also reduced with increasing exposure to biosolids from 1% up to 4% in soil [139]. More work is needed to elucidate if the toxic effects observed in these studies were the result of synergistic interactions of pharmaceuticals and other contaminants.

A small number of field studies have evaluated the effects of pharmaceuticals on soil invertebrates, with research largely focussed on laboratory tests. In one such study, the abundance of earthworms and springtails in soil beneath the dung from cattle treated with ivermectin was evaluated over 12 months [141]. Whilst ivermectin was detected in the soil beneath the dung pats (<0.006 mg/kg in months 5–7), earthworms (Lumbricidae) and springtails (Collembola) were found to be abundant and generally species rich across the evaluated sites, leading the authors to conclude that ivermectin had little effect on the soil invertebrate populations. Like earthworms, springtails are common small arthropod widely distributed in soils around the globe. However, information concerning the toxicity of pharmaceuticals to springtails is even more limited than for earthworms. One of the most comprehensive multispecies studies reported effects of ivermectin (survival and reproduction) across three species, the earthworm *Eisenia fetida*, the springtail *Folsomia candida* and the predatory mite *Hypoaspis aculeifer* [142]. Survival and reproduction of

collembolans were clearly affected with an LC_{50} of 8.4 mg/kg soil. Predatory mites and earthworms were less sensitive to ivermectin with LC_{50}s calculated to be greater than or equal to 31.6 and 10 mg/kg soil, respectively. Meanwhile, climbazole, an antifungal agent used in some antidandruff shampoos, was found to have no effect on reproduction in springtails (*Folsomia candida*) at concentrations as high as 1,000 mg/kg soil dry mass [143]. However, climbazole did disrupt dehydrogenase enzyme activity in the soil bacterium *Arthrobacter globiformis* with an EC_{50} of 456 mg/kg soil dry mass.

8 Implications of Pharmaceutical Uptake in Terrestrial Systems and Future Research Needs

Exposure to pharmaceuticals from both an ecosystem perspective (i.e. food chain transfer) and an agricultural (i.e. human exposure) perspective has the potential to result in a suite of unintended consequences. We are starting to drive more towards increased water reuse and biosolid amendment practices. When coupled with the observed effects of pharmaceuticals in terrestrial plants and invertebrates, at environmentally relevant concentrations, this suggests terrestrial systems are at risk from pharmaceutical exposure. This is primarily a result of the bioactive nature of these chemicals, where the chemical potency of pharmaceuticals is retained upon release into the environment. However, as detailed above, contrasting findings exist between the small number of phytotoxicity studies which have been currently published. Differences between toxicological responses exist between test species and experimental conditions for the same pharmaceutical. It is also important to note that observed effects on a whole plant organ that consider a single end point (e.g. germination) do not necessarily reflect effects on other important plant processes that ultimately regulate plant growth and development. More research, characterising this risk using a wider variety of pharmaceuticals under environmentally relevant exposure scenarios, is urgently needed.

To date, pharmaceutical exposure in agricultural systems has been largely ignored when it comes to developing wastewater reuse policy frameworks to support the increasing adoption of sustainable agricultural practices, with a focus typically on more traditional pollutants such as nutrients and metals. As highlighted by [144], more research is needed to quantify the current risk of pharmaceuticals in wastewater reuse systems, in particular where multiple receptors are considered, such as exposure to wildlife and the soil microbial community. This new knowledge will enable the development of thresholds for safe reuse of wastewater treatment by-products.

The above discussion has drawn on research undertaken in countries that are defined as high income (e.g. USA, Canada, Sweden, UK, Israel, Chile) or upper middle income (e.g. China). However, the number of people living in low- and middle-income countries (LMIC), excluding China, was 5 billion in 2018 [145]. There is a considerable difference in how wastewater is collected and treated

in various countries, depending on income status. For example, whilst 70% of wastewater produced from municipal and industrial sources is treated in high-income countries (HIC), this value falls to between 28 and 38% in middle-income countries (MIC), whilst less than 10% of wastewater is treated in low-income countries (LIC) [146]. Furthermore, even where sewerage networks or on-site wastewater systems are available, a high proportion of wastewater that is collected for treatment is not adequately treated, if at all. Treatment of wastewater is a mean of significantly reducing not only the burden of pathogens and excess nutrients but also contaminants such as pharmaceuticals. Whilst deliberate (or direct) reuse of highly treated wastewater for agricultural applications is increasingly seen as a valuable commodity in HICs, wastewater treated to a considerably lower degree can also be reused directly or through contamination of surface and groundwater with wastewater (indirectly) in LMICs [146].

Of the 2.75 million km^2 of agricultural land that requires irrigation, up to 7% (or 200,000 km^2) of this total has been estimated to be irrigated with wastewater, at varying degrees of treatment, with around 1 billion people estimated to be consuming agricultural products irrigated in such a manner [147]. Aside from the potential risks this entails for exposure to pathogens and nutrient pollution, this also represents a scenario where minimal mitigation of pharmaceuticals present in wastewater occurs. For example, biodegradation and sorption of pharmaceuticals, without the conditions to support enhanced biological removal of pharmaceuticals found in a WWTP, will be substantially reduced for many pharmaceuticals commonly found in wastewater [148]. As discussed previously, biosolids, generated during effective biological wastewater treatment, can accumulate many pharmaceuticals through hydrophobic and ionic interactions but can also play a protective role in uptake of pharmaceuticals in plants and terrestrial organisms. Additional treatments, including filtration, reverse osmosis and disinfection, are also effective in removing pharmaceuticals from wastewater, but these are more commonly used in HICs because of the initial and ongoing maintenance and cost requirements for their use.

It should be noted, however, that concentrations of pharmaceuticals measured in wastewater produced in HICs are not necessarily representative of wastewater in LMICs. For example, the use of pharmaceuticals in HICs is more prevalent due to the ability to access healthcare and also the higher proportion of chronic diseases that require long-term pharmaceutical therapy [149]. Conversely, the use of pharmaceuticals in human health and agricultural applications (e.g. aquaculture and livestock) is typically poorly regulated or used contrary to regulations in LMICs and pharmaceuticals [149, 150]. The direct and indirect use of wastewater from these agricultural applications can also therefore contribute to pharmaceutical loads in irrigation water for crops [146]. In addition to this, manufacturing of pharmaceuticals is increasing significantly in LMICs (e.g. China and India), especially for generic pharmaceuticals. This has led to additional burdens of pharmaceuticals in wastewater being released by these manufacturing facilities, which in some instances can be substantial [151, 152]. These sources of wastewater are likely to contribute to indirect wastewater irrigation of crops, albeit at potential high concentrations [153]. Furthermore, as shown in Fig. 1, few studies have investigated uptake from

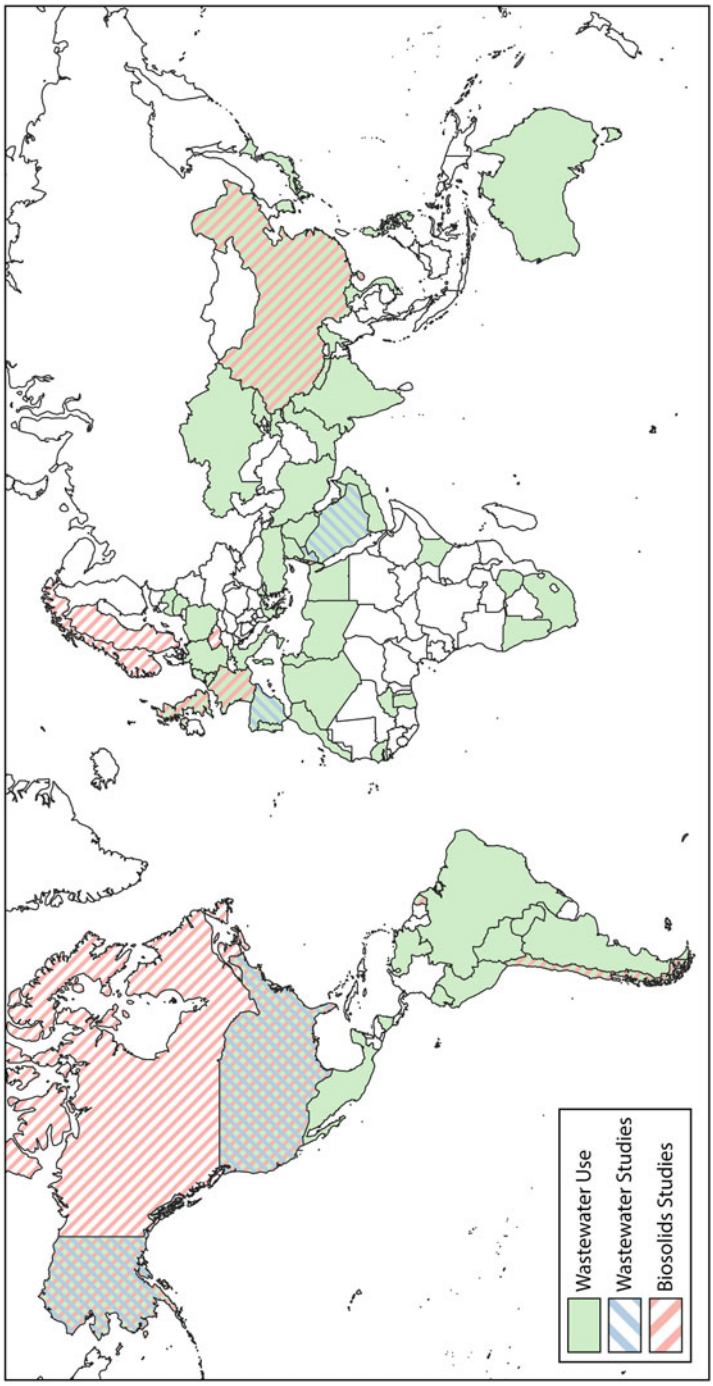

Fig. 1 Global wastewater use [154] and locations of pharmaceutical uptake studies that used actual wastewater or biosolids as described in Tables 1 and 2

actual wastewater or biosolid amendments. Those that have been conducted are largely from HICs even though the use of these wastewater products is used extensively in LMICs.

Significant knowledge gaps relating to the exposure and risks of pharmaceuticals to crops, terrestrial organisms and humans and livestock consuming these crops in LMICs clearly exist. These knowledge gaps include the classes and concentrations of pharmaceuticals in wastewater (either directly or indirectly) that are used to irrigate crops, the sources of wastewater used for irrigation (to inform mitigation strategies), the crops irrigated by pharmaceutical-contaminated wastewater (to determine exposure pathways and risks to crops and consumers of crops) and the stability of pharmaceuticals or uptake of pharmaceuticals in crops under different agricultural scenarios and climatic conditions that occur in LMICs. Figure 1 highlights this disparity showing the widespread geographical usage of wastewater for irrigation and the relatively few studies that have been conducted and their focus in HICs. This is not trivial, in that the majority of the global population lives in LMICs and relies on agriculture that abstracts the majority of the world's water, increasingly through wastewater reuse, for production.

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Metabolism of Pharmaceuticals in Plants and Their Associated Microbiota

Andrés Sauvêtre, Peter Eichhorn, and Sandra Pérez

Contents

Abstract With the increasing use of wastewater for irrigation of farmland, and thus the potential uptake and translocation of pharmaceuticals and their metabolites in crops, concerns about food safety are growing. After their uptake, plants are able to metabolize drugs to phase I, phase II, and phase III metabolites. Phase I reactions closely resemble those encountered in human drug metabolism, including

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oxidations, reductions, and hydrolysis. Phase II reactions, in turn, encompass conjugations with glutathione, carbohydrates, malonic acid, and amino acids. In phase III, these conjugates are transported and stored in the vacuole or bound to the cell wall. Pharmaceutical metabolism in plants has been investigated by using different approaches, namely, the use of whole plants grown in soil or hydroponic cultures, the use of plant tissues, and the incubation of specific plant cell suspensions. While studies relying on whole plants require long growth periods and more complex analytical procedures to isolate and detect metabolites, they constitute more realistic scenarios with the ability to determine site-specific metabolism and the translocation within the plant. The advantage of in vitro studies lies in their rapid setup. Recent advances in plant-microbiota investigations have shown that the plant microbiome modulates the response of the plant towards pharmaceuticals. Rhizospheric and endophytic bacteria can directly contribute to pharmaceutical metabolism and influence plant uptake and translocation of pharmaceuticals and their metabolites. Additionally, they can have beneficial properties for the host, contributing to plant health and fitness. This chapter gives an overview of human and plant drug metabolism followed by a comparison of different models used to identify pharmaceutical metabolites and their metabolic pathways in plants. A description of the mechanisms and reactions originating these metabolites is concisely presented. Finally, the role of the microbiome is critically discussed with examples of synergies between plants and their associated microbiota for pharmaceutical degradation.

Keywords Hairy roots, Human drug metabolism, Pharmaceuticals, Plant metabolism, Plant microbiome

Abbreviations

AAP	Acetaminophen
ABC	ATP-binding cassette
ACC	1-Aminocylcopropane-1-carboxylic acid
ALD	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
AO	Aldehyde oxidase
BChE	Butyrylcholinesterase
CBZ	Carbamazepine
CES	Carboxylesterase
CIP	Ciprofloxacin
CMP	Clomipramine
CW	Constructed wetland
CYP	Cytochrome P450
Cys	Cysteine
DCF	Diclofenac
DME	Drug-metabolizing enzyme
DZP	Diazepam

FMO	Flavin-containing monooxygenase
Gln	Glutamine
Glu	Glutamic acid
Gluc	Glucose
GSH	Glutathione
GST	Glutathione S-transferase
Hex	Hexose
HR	Hairy root
IBU	Ibuprofen
IOP	Iopromide
KPF	Ketoprofen
MACC	N-malonyl-ACC
MFA	Mefenamic acid
MFM	Metformin
MS	Mass spectrometry
NAPQI	N-acetyl-p-benzoquinoneimine
NAT	N-acetyltransferase
NBS	Nature-based solutions
NPX	Naproxen
OBZ	Oxybenzone
OFL	Ofloxacin
PAHs	Polycyclic aromatic hydrocarbons
PON	Paraoxonase
SRT	Sertraline
SULT	Sulfotransferase
TCS	Triclosan
T-DNA	Transfer DNA
TMP	Trimethoprim
TZD	Trazodone
UGT	Uridine 5′-diphosphoglucuronosyltransferase
UV	Ultraviolet

1 Introduction

Although legislation on wastewater reuse is based on water physicochemical parameters and indicator pathogen microorganisms, part of the scientific community agrees with the need to monitor and set evaluation procedures for some substances including some widely used drugs for the improvement of guidelines on the reuse of treated wastewater [1]. Pharmaceuticals are biologically active molecules that have been designed to interact with physiological processes and pathways in humans and animals or to be toxic for infectious bacteria, fungi, or parasites. But organisms others than humans and animals can be affected when they are exposed to these

molecules. Plants, algae, and other lower animals from our agroecosystems have similar receptor and metabolic enzymatic systems and can be affected by the release of pharmaceuticals in reclaimed wastewater.

Plants have evolved very sophisticated detoxification systems including a battery of xenobiotic-metabolizing enzymes to form metabolites of different nature. Some of them are similar to those in humans and animals, but there are several plant-specific ones. Therefore, it is necessary to address the metabolism in crop plants and soil because the parent compounds along their metabolites can enter the food chain. In some cases, these may have higher toxicity than the original molecule, thereby threatening ecosystems [2]. Identification of harmful metabolites in edible vegetables (formed or not in plants) should be addressed to prevent exposure of humans to hazardous compounds derived from drugs. In this context, information obtained by mass spectrometry (MS) is of special relevance. New available equipment and techniques have increased the sensitivity of non-target analyses, resulting in a relevant number of new molecules and metabolites identified in our agroecosystems. This information is necessary if we want to provide safe and healthy food whose production involves the use of reclaimed wastewater. Phytoremediation is another field where the identification of metabolites is important. Nature-based solutions (NBS) are commonly implemented to treat secondary wastewater effluents as a polishing step [3]. Even small communities may have only one wastewater treatment plant based on lagoon systems before finally disposing of the effluent into the environment. In these cases, monitoring of pharmaceuticals and their transformation products from the raw wastewater entering the treatment plant to the effluent discharged back into nature is necessary. Therefore, knowing what happens between these two points, i.e., in the rhizosphere and in the plant, can help determine whether a treatment is efficient or not and to implement corrective actions. Here, the aim is to check that plants can absorb, accumulate, and immobilize drugs and their transformation products, eliminating the risk to the environment and the trophic chain.

This chapter attempts to give a description of the mechanisms of pharmaceutical metabolism in plants which depend strongly on their physicochemical properties and the interactions with plant-associated bacteria. Similarities between human and plant metabolism are discussed, following some examples of metabolites recently identified in plant tissues. The use of models to study metabolism of pharmaceuticals in plants is also discussed. As the plant microbiome plays an important role in xenobiotic metabolism, degradation of pharmaceuticals can be modulated using techniques based on holobiontic approaches. Hence, the impact of several pharmaceuticals on plant microbial communities and the cooperative metabolism of pharmaceuticals by plants and their microbiome are presented.

2 Human Drug-Metabolizing Enzymes

As an innate defense mechanism against potentially harmful agents that may have entered the organism through ingestion, inhalation, or dermal exposure, enzymatic detoxification pathways are in place to aid in removing undesired substances from

the human body. The physicochemical properties of a large number of small molecule drugs are in a range where such enzymes are able to bind them as substrates at their active site and to convert them into metabolites with generally enhanced susceptibility to excretion and thus irreversible removal from the organism. As described in more detail in chapter "General Introduction on Pharmaceuticals", the chemical structures of most orally dosed pharmaceuticals are optimized for high metabolic stability with the objective of reducing dose size and dosing frequency. Nonetheless, enzyme-mediated biotransformations eventually constitute the principal clearance mechanism. In view of the exceptional role of drug-metabolizing enzymes (DME) in the compound optimization strategies applied in rational drug design, the catalyzed reactions, their tissue-specific expression and subcellular localization, their substrate selectivity, and their inducibility and polymorphism have been characterized in a very comprehensive fashion.

Although all organs and tissues in the human body exhibit DME activity to some degree, the most important site of drug metabolism is the liver followed by notable contributions from enzymes expressed in the intestine, the kidneys, and plasma. The fundamental importance of hepatic DMEs arises from the anatomical position of the liver acting as the port of entry into systemic circulation of organic compounds previously absorbed in the intestinal tract and delivered through the portal vein to the liver (see chapter "General Introduction on Pharmaceuticals").

At the highest level, human DMEs are classified into one of two categories: phase I enzymes catalyze oxidative, reductive, and hydrolytic reactions, whereas phase II enzymes mediate the transfer of larger moieties from a cofactor to the substrate thereby generating conjugates. In most instances, the increase in polarity induced by phase I reactions is modest and may produce metabolites being sufficiently permeable to passively diffuse from the liver back into the blood stream. Phase II metabolites, in contrast, are usually of substantially lower lipophilic nature than their parent compound. Despite the detrimental effect of reduced lipophilicity on passive membrane permeability, transmembrane proteins located on the apical side of the hepatocyte help transport conjugates against a concentration gradient into bile and thereby facilitate excretion. It is not uncommon to observe metabolites in human wastes, originating from biliary or renal excretion, that have been formed by a sequence or combination of phase I and phase II reactions.

2.1 Phase I Reactions

Among the phase I enzymes, the superfamily of the highly versatile monooxygenase cytochrome P450 (CYP) is the single most prominent one [4, 5]. CYP enzymes are divided into families based on their amino acid homology; members of the same family (indicated by a letter) share 40% homology, while those of the subfamily (indicated by a number) overlap by at least 55% of their amino acid sequence. Of the CYPs recognizing and transforming synthetic drug molecules, the human isoforms CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 are the most important

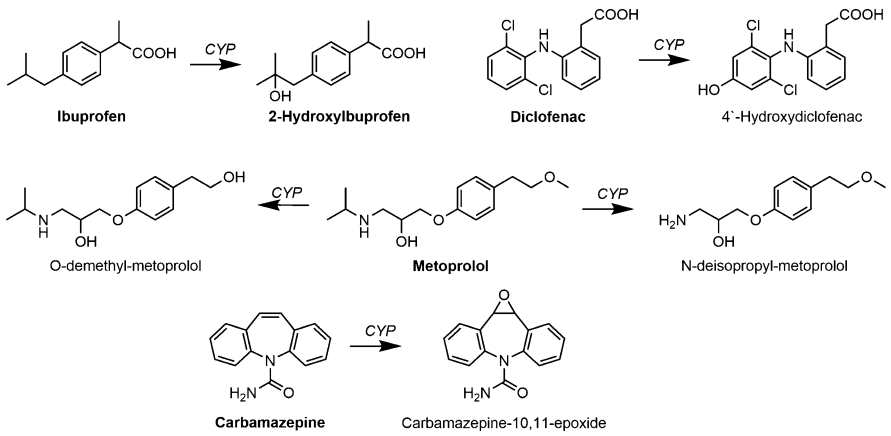

Fig. 1 CYP-mediated carbon oxidations: aliphatic hydroxylation of ibuprofen, aromatic hydroxylation of diclofenac, heteroatom dealkylations of metoprolol, and epoxidation of carbamazepine

members with some minor contributions from CYP1A1, CYP2B6, and CYP2C8. At a quantitative level, the affinity towards the substrate, and hence the rate of the reaction, is positively correlated with its lipophilicity. In other words, very polar drugs (logP <0) are rarely recognized by CYPs and therefore frequently escape oxidative metabolism in the human body. Of the aforementioned isoforms, CYP3A4 is the most promiscuous one exhibiting broad substrate selectivity. Despite some overlap in the selectivity between the isoforms, structural features of the substrate, such as size, planarity, and presence of charge center, result in preference for a specific enzyme [6].

As far as type of CYP-mediated reactions are concerned, the most prominent transformations include hydroxylation of aliphatic and aromatic carbon atoms, e.g., hydroxylation of in the isobutyl group of ibuprofen (IBU) (yielding a mixture of primary, secondary, and tertiary alcohols) or the hydroxylation of the dichlorophenyl ring in diclofenac (DCF) (Fig. 1). A subtype of the former reaction is the carbon hydroxylation at the benzylic position being favored due to stabilization of an intermediate in the catalytic cycle. When the oxygenation of the carbon atom occurs on the α-carbon of the alkyl group attached to a heteroatom, the chemically unstable intermediate (a hemiacetal in case of O-alkyl; a hemiaminal in case of N-alkyl) decomposes to yield the dealkylated metabolite (metoprolol in Fig. 1). In the former case, this unmasks an alcohol resulting a considerably more polar compound. A further CYP-mediated reaction at a carbon center is the epoxidation of double bonds. Although in most instances the epoxides are chemically reactive and undergo subsequent reactions, the formation of the symmetrical epoxide of carbamazepine (CBZ) is an example of a metabolite being sufficiently stable to be excreted into human urine (Fig. 1).

Apart from oxidizing carbon in various structural environments, CYPs are also capable of oxygenating heteroatoms such as nitrogen and sulfur. As such, aromatic

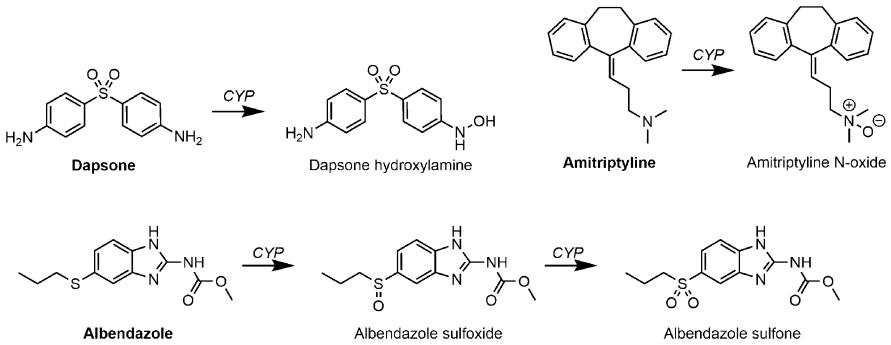

Fig. 2 CYP-mediated heteroatom oxidations: N-hydroxylation of dapsone, N-oxidation of amitriptyline, and S-oxidation of albendazole

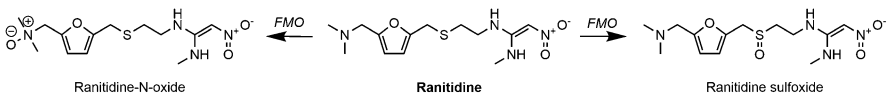

Fig. 3 FMO-mediated nitrogen and sulfur oxidation of ranitidine

amines can be converted into the corresponding hydroxyl amines, whereas tertiary amines may produce N-oxides. Oxidation of sulfur, on the other hand, converts thioethers into sulfoxides and sulfoxides into sulfones (Fig. 2). These few examples highlight the remarkable versatility of the isozymes belonging to the CYP superfamily.

Nonetheless, a number of DMEs with a narrower substrate selectivity exist typically recognizing specific functional groups such as alcohol dehydrogenase (ALD) and aldehyde dehydrogenase (ALDH) that transform primary or secondary alcohols into aldehyde and ketone and aldehydes into the corresponding carboxylic acids, respectively [7, 8]. A further relevant phase I enzyme is the heteroatom-targeting flavin-containing monooxygenase (FMO) with its capability of oxidizing soft nucleophiles containing nitrogen or sulfur atoms (Fig. 3) [9]. Aldehyde oxidase (AO), as its name indicates, oxidizes aldehydes to their corresponding carboxylic acid but also electron-deficient N-heterocycles at a carbon atom adjacent to the nitrogen atom (Fig. 4) [10].

Taken together, the above enzymes offer a plethora of mechanisms for attacking potential sites within a drug molecule. Although it is possible to propose plausible sites of oxidation by simply examining its chemical structure, the predominant pathways under in vivo conditions are difficult to predict. For any biotransformation to occur to a measurable extent, the substrate has to bind tightly to the active site of the enzyme through molecular interactions with amino acids, i.e., exhibit a high association constant, but the proper orientation within the catalytic cavity is also crucial for an efficient conversion of the substrate.

Unlike oxidation reactions, the site of hydrolytic cleavages is much easier to pinpoint with a limited number of functional groups being liable to hydrolysis,

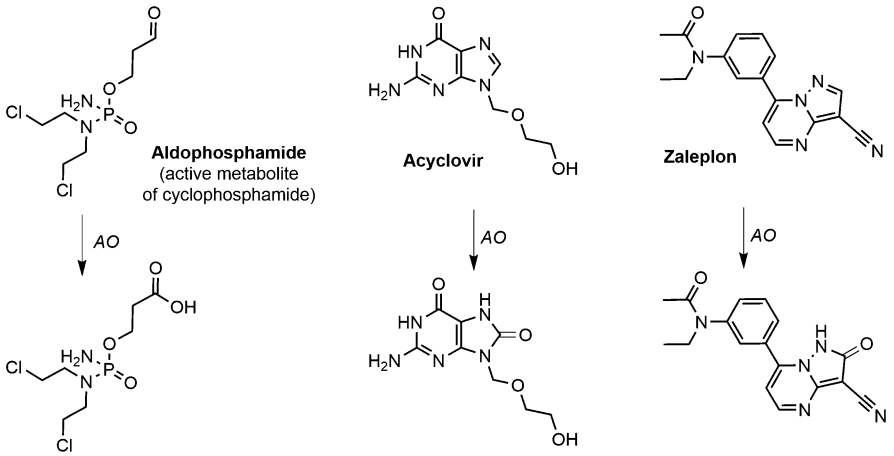

Fig. 4 AO-mediated oxidation of aldophosphamide, acyclovir, and zaleplon

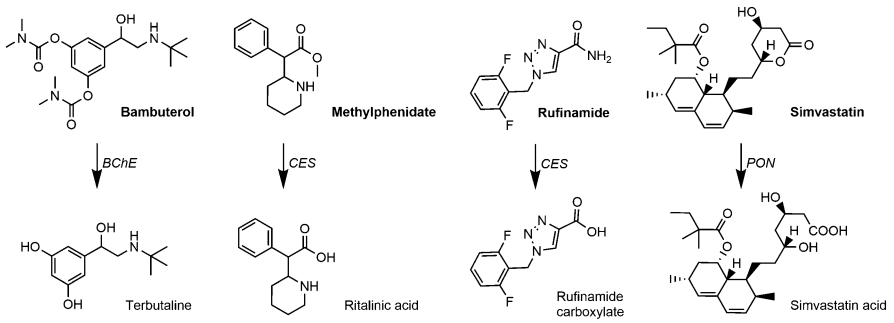

Fig. 5 Esterase-mediated reactions: bambuterol (carbamate), methylphenidate (methyl ester), rufinamide (amide), and simvastatin (lactone). With the exception of rufinamide, the shown compounds are pharmacologically inactive prodrugs releasing upon hydrolysis the active metabolite

mainly (carbonyl) esters, amides, and carbamates. The spectrum of relevant enzymes in this category comprises paraoxonase (PON), butyrylcholinesterase (BChE), and carboxylesterases (CES) [11, 12]. While the human serum esterase PON affords quite selective recognition of drugs bearing lactones and carbonyl esters, the structure-affinity relationships of BChE and CES are less well understood (Fig. 5). When it comes to designing metabolically stable molecules, avoiding the above groups as building blocks is a straightforward strategy.

As mentioned above, the third class of phase I reactions is reductive reactions. However, owing to the high positive redox potential of an aerobic organism like the human being, such processes are far less common than oxidative biotransformations. Known examples comprise reductive dehalogenation, stepwise conversion of nitro into amino group, and carbonyl reduction to the corresponding alcohol [13]. In

contrast to truly human DMEs, bacterial enzymes originating from the intestinal microflora possess a higher inherent ability to catalyzing reductive reactions of drugs. The outcome of this drug-gut bacteria interaction can manifest in various ways and can have significant effects on pharmacokinetic and safety profiles: metabolism of the active may reduce its oral bioavailability (e.g., nizatidine), enzymatic breakdown may release a pharmacologically active metabolite from the inactive parent compound (e.g., sulfasalazine), ester hydrolysis of acyl glucuronides may recycle the acidic drug and facilitate enterohepatic recirculation (e.g., DCF) thereby extending its elimination half-life, and formation of reactive metabolites may cause toxicity (e.g., metronidazole) [14].

2.2 Phase II Reactions

As for conjugative phase II reactions, they rely on the presence of specific functional groups to accommodate the moiety transferred from the cofactor to the substrate. By far the most prominent DME in this class is uridine 5′-diphospho-glucuronosyltransferase (UGT) which in a nucleophilic substitution attaches glucuronic acid to carboxyl, aliphatic and aromatic hydroxyl, and amino groups or to N-heterocycles to yield the glucuronide conjugate [15]. Given the critical function of glucuronidation in human drug metabolism, the UGT family has been characterized to an extent comparable to that of CYPs with respect to identification of the human isoforms (belonging to the families UGTA1 and UGT2B), tissue expression, substrate selectivity, and polymorphic variants. It is worth stressing that certain UGTs involved in the conjugation of drugs are exclusively expressed in extrahepatic tissues [16]. Consequently, a liver-centered approach in assessing the extent of glucuronidation is likely to fail in accurately predicting the overall contribution of this phase II reaction to the overall metabolic clearance [17]. One aspect of particular interest to the environmental scientist dealing with the fate of glucuronide conjugates of acidic drugs carrying a carboxyl group (e.g., DCF and IBU) resides in the susceptibility of the resulting ester towards enzymatic hydrolysis (Fig. 6). This reaction, which can already be catalyzed prior to excretion by intestinal bacteria exposed to bile secretions, may regenerate the parent drug if no preceding phase I reaction took place.

Second to glucuronidation is the sulfotransferase-mediated (SULT) conjugation of a sulfo group (SO_3) to an alcohol, phenol, or amine [18]. In this sulfation reaction, the resulting metabolite is a sulfate and a sulfamate, respectively. Although human cytosolic SULTs are high-affinity enzymes, their quantitative relevance is limited owing to their low capacity. Accordingly, sulfation is usually a minor pathway in the metabolic scheme of synthetic drugs. Sulfate and glucuronide conjugations share the pharmacokinetically relevant characteristic of greatly lowering the lipophilicity of drug molecules and thus are usually the final metabolic step before efficient metabolite secretion into bile or excretion into urine takes place.

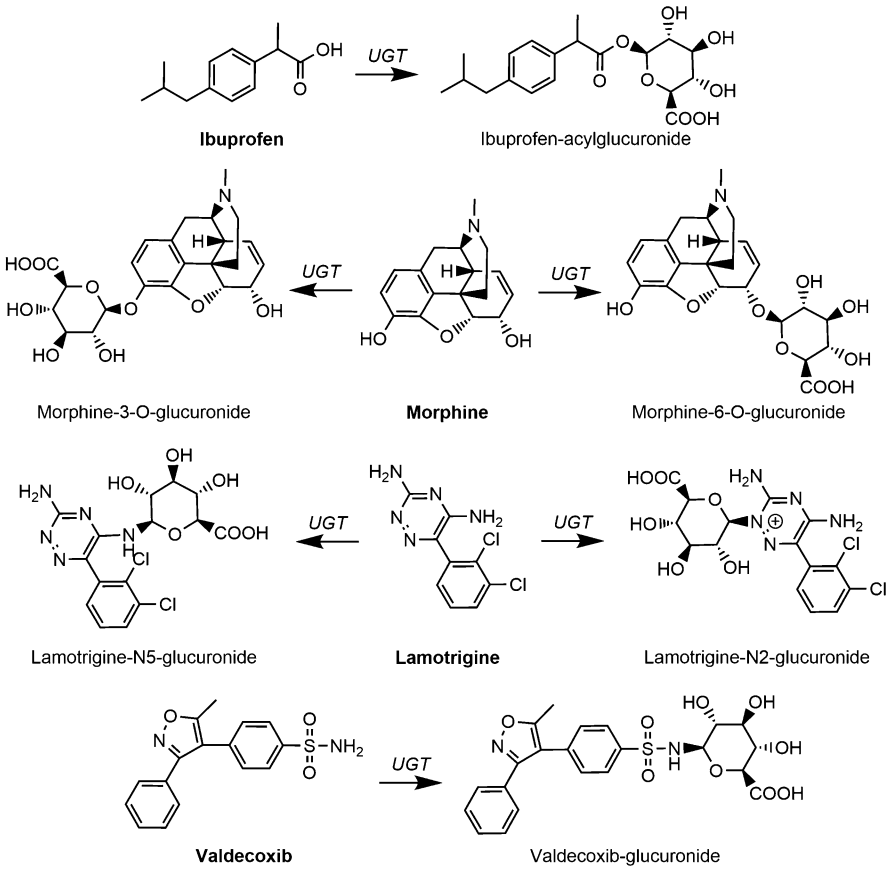

Fig. 6 UGT-mediated reactions: acyl glucuronide formation of ibuprofen, O-glucuronidation of aromatic and aliphatic (allylic) hydroxyl group in morphine, N-glucuronidation of amino group and N-heterocycle in lamotrigine, glucuronidation of sulfonamide in valdecoxib

Apart from the UGT-mediated conjugation of drugs containing carboxyl groups, an alternative pathway, though of negligible significance in the human body, is the formation of amino acid conjugates in the mitochondria via a multi-stage reaction in which the substrate is first activated by conversion into a high-energy CoA thioester [19]. In the subsequent nucleophilic reaction, it then establishes an amide bond with the amino group of the amino acid. The very few reported cases in humans involve glycine as the reaction partner. whereas animal species have been shown to form carnitine conjugates as well as conjugates with taurine. A further, quite specific phase II reaction is the N-acetylation of arylamines, arylhydroxylamines, and arylhydrazines (Fig. 7). It is catalyzed by the cytosolic conjugating enzyme N-acetyltransferase (NAT) which transfers an acetyl group from acetyl-CoA to the drug acceptor substrate.

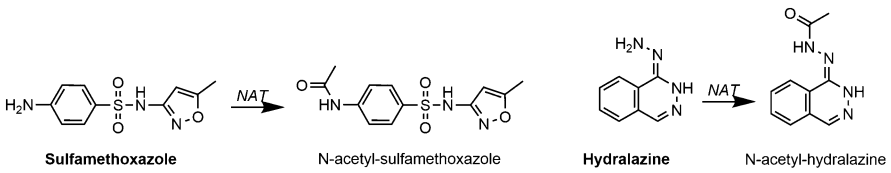

Fig. 7 N-acetylation of the arylamine sulfamethoxazole and the arylhydrazine hydralazine

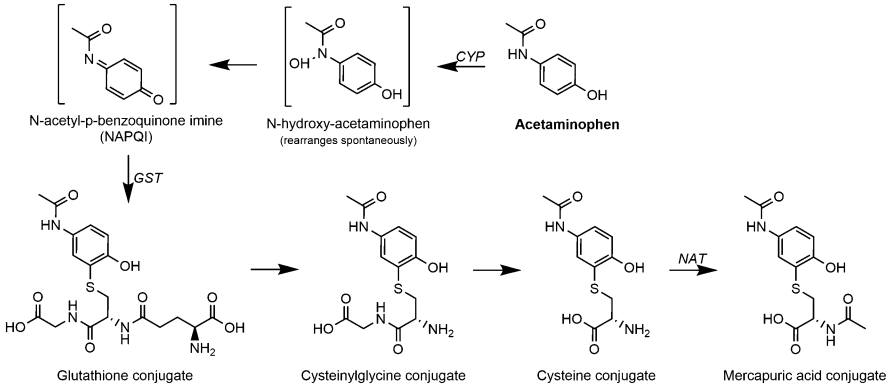

Fig. 8 CYP-mediated bioactivation of acetaminophen to NAPQI, scavenging of the reactive metabolites by GSH, sequential hydrolytic cleavage of glutamic acid and glycine from GSH, and N-acetylation of the resulting cysteine conjugate to yield the mercapturic acid conjugate

Viewing the metabolism of drugs as a detoxification mechanism of the human body, the glutathione S-transferases (GST) truly play a pivotal role in the protection against chemically reactive species [20]. This cytosolic enzyme class catalyzes the transfer of the nucleophilic tripeptide glutathione (GSH), composed of γ-glutamic acid-cysteine-glycine, to an electrophilic substrate by taking advantage of the nucleophilic character of the thiol group in the central amino acid. GSH conjugation is most frequently observed following phase I-induced bioactivation that has led to the formation of a strongly electron-deficient intermediate. In the absence of GSH as scavenger, such activated drug metabolite may otherwise react with functional groups of macromolecules, potentially compromising their cellular functions. In fact, depletion of the cofactor GSH at the cellular level due to generation of high amounts of reactive metabolite can result in acute liver failure. The classical example of bioactivation is the CYP-mediated conversion of acetaminophen into N-acetyl-p-benzoquinone imine (NAPQI; Fig. 8). Hepatic formation of GSH conjugates is usually followed by sequential cleavage of first glutamate by γ-glutamyltranspeptidase and then glycine through cysteinyl glycinase. N-acetylation of the cysteine amino group can then complete the reaction sequence to produce mercapturic acids. Detecting cysteine conjugates and mercapturic acids in bile or urine provides evidence for previous GSH conjugation.

3 Drug Metabolism in Plants

Depending on their physicochemical properties and their interaction with soil, pharmaceuticals present in irrigation water can enter plants and undergo metabolic transformations as observed for other organic xenobiotics such as pesticides. Plants can detect, transport, and detoxify these molecules by setting a wide array of molecular mechanisms constituting the xenome [21]. Shimabukuro first described the detoxification cascade of organic xenobiotics in plants and divided it into three phases, in analogy to human hepatic drug metabolism [22]. Based on these observations, Sandermann established the green liver concept [23]. As far as we know, plants metabolize pharmaceuticals through a sequence of intermediates. Nowadays, the complete biotransformation pathway for the majority of pharmaceuticals in plants is not understood; however, the metabolic reactions described in other studies indicate similarity to those that pesticides undergo. Since plants are sedentary, they rely significantly on biochemical mechanisms of defense [24]. Plants further resemble the liver of higher vertebrates insofar as they are able to metabolize xenobiotics with great specificity [25].

In phase I reactions, the parent compound is chemically modified by introducing a functional group to the molecule typically in one or more enzymatic reactions. This phase is called the activation phase and in most instances renders more hydrophilic metabolites. Increased solubility avoids the partition of the compound in biological membranes and shortens their half-life. The primary metabolites formed in phase I reactions are often identical to those in animals. Although most of the time metabolites display reduced affinity towards the target organism than the parent compound, in some cases the phytotoxicity can increase after activation. The generated metabolites are chemically more reactive as they contain functional groups suitable for phase II metabolism, where the metabolite is deactivated by covalent binding to endogenous molecules to form water-soluble conjugates. Conjugates formed in plants differ from those found in humans and animal species by relying on glycosyl and malonyl transferase-mediated metabolism to produce glycosyl and malonyl conjugates. These metabolic pathways are unknown in humans although glycosylation is mechanistically analogous to glucuronidation with the only difference residing in the structure of the saccharide moiety. The formation of GSH conjugates in plants, involving GSTs, may be more prominent than in humans where their detection in in vitro test systems such as hepatocytes is generally regarded a warning sign because it indicates the formation of reactive electrophilic metabolites. In fact, detection of GSH adducts during compound screening in pharmaceutical research settings usually leads to the rejection of GSH conjugate-forming entities. Further conjugates reported to be generated in plants are the products of amino acid conjugation, sulfation, and O-methylation. These inactive conjugates are considered as non-toxic or less toxic than the parent compound. In phase III reactions (compartmentation), conjugates are extracted from the site of formation and transferred into different compartments. For example, glycosyl, malonyl, and GSH conjugates are sequestered in the vacuole with the aid of specific ATP-binding cassette

transporters. In other cases, conjugates are transported to the apoplast where they can bind or not to the cell wall.

Here we describe the basic reactions governing the formation of pharmaceutical metabolites in plants. This will help researchers when attempting to identify novel metabolites by suspect screening techniques.

3.1 Phase I Metabolism

In this phase, a variety of chemical reactions are involved in the xenobiotic metabolism in plants such as oxidation (CYP, peroxidases, dehydrogenases, and laccases), reduction (aldo-keto reductases), and hydrolysis (esterases, carboxylesterases, amidases, and epoxide hydrolases). Like in humans, the CYP family is the single most important enzyme class which commonly catalyze monooxygenation of substrates (including hydroxylations, epoxidations, dealkylations, decarboxylations, and isomerizations), but they are also able to act as peroxidases or reductases.

3.1.1 Oxidation

Oxidation is an important reaction for the detoxification of xenobiotics in plants and comprises carbon hydroxylation, N-dealkylation, O-dealkylation, epoxidation, desulfuration, sulfoxidation, and nitrogen oxidation. The most common CYP-mediated carbon oxidation reactions are, like in humans, hydroxylation of aromatic rings and alkyl side chains of the substrates (see Fig. 1). For instance, the metabolism of the nonsteroidal anti-inflammatory drug IBU in plants is initiated by hydroxylation of the isobutyl group (see Table 1) yielding hydroxy-IBU (the exact position of the hydroxyl group remains to be identified) which is further oxidized to the dihydroxy metabolite and carboxy-IBU as reported for *Phragmites australis*, *Arabidopsis thaliana* (cells), *Typha angustifolia*, and *Lemna gibba*. These two metabolic pathways have also been documented to occur in mammals and microorganisms [41–47]. In plants the anticonvulsant CBZ is transformed to trans-10,11-dihydroxy-CBZ catalyzed sequentially by CYP and epoxide hydrolases [28–31]. As secondary pathways, monohydroxylation (2- and 3-hydroxy-CBZ and, to much lower extent, 4-hydroxy-CBZ) and dihydroxylation were found to take place in tomato plants. A single phase I metabolite of DCF (4′-hydroxy diclofenac) was reported to be formed in *Hordeum vulgare* and hairy root (HR) cell cultures of *Armoracia rusticana* [37–40].

An example of O-demethylation in plants is the O-desmethyl-metabolite of naproxen (NPX) [41]. Therefore, NPX follows the same metabolic pattern in plants and humans, the only phase I metabolite being O-desmethyl-NPX which is extensively metabolized to phase II conjugates [41]. Caffeine in radish also undergoes demethylations and suffers stepwise N-demethylation yielding paraxanthine, theobromine, and theophylline. Successive demethylations then generate 7- and

Table 1 Comparison of the metabolism of drugs in humans and plants

Compound	Human metabolites	Plant metabolites	Plant species	Ref.
Acetaminophen (AAP)	Phase I: N-acetyl-p-benzoquinone imine (NAPQI) Phase II: AAP-Gluc, AAP-Sulf, **AAP-Cys**	Phase II: AAP-Hex, **AAP-Cys**; AAP-GSH	*Armoracia rusticana* (cells)	[26]
Caffeine	Phase I: **Paraxanthine, theobromine, theophylline**, **3-methylxanthine, 7-methylxanthine**, **1,3,7-trimethyluric acid**, 1-methylxanthine, 1-methyluric acid, 1,7-dimethyluric acid Phase II: 5-acetylamino-6-formylamino-3-methyluracil	Phase I: **Paraxanthine**, **theobromine, theophylline**, **3-methylxanthine**, **7-methylxanthine**, **1,3,7-trimethyluric acid**, 8-OH-1,3,7-trimethyl-3,7,8,9-tetrahydro-1H-purine-2,6-dione, 7-OH-1,3-dimethyl-3,7-dihydro-1H-purine-2,6-dione, xanthine	*Lactuca sativa*, radish	[27]
Carbamazepine (CBZ)	Phase I: **2-OH-CBZ, 3-OH-CBZ**, **2,3-diOH-CBZ**, **10,11-dihydro-10-OH-CBZ, 10,11-epoxy-CBZ**, **10,11-diOH-CBZ**, **2,3-dihydro-OH-CBZ**, CBZ-o-quinone, 2, 3-epoxide-CBZ, **acridine, acridine-carboxaldehyde, acridine-carboxylic acid, 9-OH-acridine, acridone** Phase II 10,11-dihydro-10-OH-CBZ-Gluc	Phase I: **2-OH-CBZ, 3-OH-CBZ**, **2,3-diOH-CBZ**, **10,11-epoxy-CBZ**, **trans-10,11-diOH-CBZ**, **cis-diOH-CBZ**, **10,11-dihydro-10-OH-CBZ**, **2,3-dihydro-OH-CBZ**, 10,11-dihydro-CBZ, 2,3-dihydro-2,3-OH-CBZ, CBZ-2,3-quinone, 10,11-dihydro-10,11-epoxy-CBZ, **acridine, acridine-carboxaldehyde, acridine-carboxylic acid, 9-OH-acridine, acridone** Phase II: 10,11-dihydro-10-OH-11-GSH-CBZ, CBZ-Hex, CBZ-Cys, 10,11-dihydro-10-OH-CBZ-Cys, CBZ-Cys-Gly	*Phragmites australis* (culture), *Solanum lycopersicum*, *Cucumis Sativus*, *Carrot culture*	[24, 28–32]
Ciprofloxacin (CIP)	Formyl-CIP, oxo-CIP, desethylene-CIP, Sulfo-CIP	Phase I: OH-CIP, N-desalkyl-CIP, desethylene-CIP	*Brassica parachinensis*	[33]

Clomipramine (CMP)	Phase I: **N-desmethyl-CMP**, N, N-didesmethyl-CMP, CMP-N-oxide, 2-OH-CMP, 8-OH-CMP, 10-OH-CMP, 8-OH-N-desmethyl-CMP Phase II: 2-OH-CMP-Gluc, 8-OH-CMP-Gluc, 2-OH-N-desmethyl-CMP-Gluc	Phase I: OH-CLP (unidentified position of OH), **N-desmethyl-CMP**, OH-N-desmethyl-CLP (unidentified position of OH)	*Lepidium sativum*	[34]
Diazepam (DZP)	Phase I: **N-desmethyl-DZP** (nordazepam)**, OH-DZP** (temazepam), **N-desmethyl-OH-DZP** (oxazepam)	Phase I: **N-desmethyl-DZP** (nordazepam), **OH-DZP** (temazepam), **N-desmethyl-OH-DZP** (oxazepam)	*Raphanus sativus*, *Beta vulgaris*, *Cucumis sativus*	[35, 36]
Diclofenac (DCF)	Phase I: **4-OH-DCF**, 3′-OH-DCF, 4′,5-diOH-DCF Phase II: DCF-AGluc, 3-′-OH-4′methoxy-DCF, 3-′-OH-DCF-Sulf, 4-OH-DCF-Sulf, 3′-OH-DCF-Gluc, 4-OH-DCF-Gluc	Phase I: **4-OH-DCF** Phase II: 4-OH-DCF-Hex, DCF-Hex-Mal, OH-DCF-Hex-Mal, DCF-Gln, DCF-Glu, 4-OH-DCF-GSH	*Hordeum vulgare*, hairy root cell culture of *Armoracia rusticana*, Cucumber and radish (horse radish), *Typha latifolia*	[37–40]
Ibuprofen (IBU)	Phase I: 1-OH-IBU, 2-OH-IBU, 3-OH-IBU, **carboxy-IBU** Phase II: IBU-AGluc	Phase I: OH-IBU (unidentified position of OH), 1-OH-IBU, 2-OH-IBU, diOH-IBU, **carboxy-IBU** Phase II: IBU-Glu, OH-IBU-Gluc, IBU-Hex, IBU-Hex-Hex, OH-IBU-Hex, OH-IBU-Glu, IBU-Glu, OH-IBU-Glu, OH-IBU-Ser, acetyl-IBU-Hex, OH-acetyl-IBU-Hex, IBU-Hex-Mal, IBU-DeOHex-Hex, acetyl-IBU-DeOHex-Hex	*Phragmites australis*, *Vigna unguiculata L. Walp*, *Arabidopsis thaliana* (cells), *Typha angustifolia*, *Lemna gibba*	[41–47]
Iopromide (IOP)	Not metabolized	Phase I: Dehydro-IOP, di-dehydro-IOP, IOP-carboxylic acid, dehydro-IOP-carboxylic acid, IOP-dicarboxylic acid, dehydro-IOP-dicarboxylic acid, de-carboxy-didehydro-IOP-carboxylic acid, di-iodo-IOP	*Typha latifolia*	[48]

(continued)

Table 1 (continued)

Compound	Human metabolites	Plant metabolites	Plant species	Ref.
Ketoprofen (KPF)	Phase II: KPF-AGluc	Phase II: KPF-Hex, KPF-Hex-Mal, KPF-Gln, KPF-Glu	*Lepidium sativum*	[39, 40]
Mefenamic acid (MFA)	Phase I: 3-methylhydroxy MFA, 3-carboxy-MFA Phase II: FA-AGluc, MFA-Gly	Phase II: MFA-Hex-Mal, OH-MFA-Hex, OH-MFA-Hex-Mal	*Lepidium sativum*	[39, 40]
Metformin (MFM)	Not metabolized	Phase I: N-desmethyl-MFM	*Typha latifolia*	[49]
Naproxen (NPX)	Phase I: **O-desmethyl-NPX** Phase II: NPX-O-Gluc, O-desmethyl-NPX-Sulf, O-desmethyl-NPX-AGluc, O-desmethyl-NPX-O-Gluc	Phase I: **O-desmethyl-NPX** Phase II: NPX-Hex, NPX-Hex-Mal, NPX-Ala-Glu, NPX-Gln, NPX-Glu, NPX-Gln-Glu, NPX-Ile-Glu, NPX-Leu-Glu, NPX-Phe-Glu, , NPX-Thr-Ala, NPX-Tyr-Gly-Gln, NPX-Val-Glu, OH-NPX-Tyr-Gly-Gln	*Lepidium sativum*, *Arabidopsis thaliana* (cells)	[39–41]
Ofloxacin (OFL)	Desmethyl-OFL, OFL-N-oxide	Phase I: OH-OFL, O-ethyl-OFL, O-methyl-OFL, decarboxy-OFL, N-desmethyl-OFL, OH-N-desmethyl-OFL Phase II: OFL-Hex, OFL-Hex-Mal	Tomato and lettuce	[50, 51]
Sertraline (SRT)	Phase I: N-desmethyl-SRT, SRT ketone Phase II: SER N-carbamoyl-Gluc, α-OH-SER ketone-Gluc	Phase I: two OH-SRT (unidentified position of OH) Phase II: N-desmethyl-SRT-Phe, N-desmethyl-SRT-Tyr, OH-N-desmethyl-SRT-Phe (unidentified position of OH)	*Lepidium sativum*	[34]
Trazodone (TZD)	Phase I: 4-OH-TZD**, m-chlorophenylpiperazine**, triazolopyridinone dihydrodiol, triazolopyridinone epoxide	Phase I: OH-TZD (unidentified position of OH), **m-chlorophenylpiperazine**	*Lepidium sativum*	[34]

Triclosan (TCS)	Phase I: OH-TCS Phase II: TCS-Gluc, **TCS-Sulf**	Phase II: **TCS-Sulf,** TCS-Hex, TCS-Hex-Sulf, TCS-Hex-Hex, TCS-Hex-Mal, TCS-DeOHex-Mal-Hex-Sulf, TCS-DeOHex-Hex-Sulf, TSC-Hex-Mal-diSulf, TCS-DeOHex-Hex-Sulf, TCS-Hex-Mal-Sulf, TCS-DeOHex-Hex, TCS-acetyl-Hex; OH-TCS-Hex-Mal, OH-TCS-Hex-Mal-Sulf, and more	Radish in vitro	[52]
Trimethoprim (TMP)	Phase I: 3-NO-TMP, 1-NO-TMP, 3-Desmethyl-TMP, 4-Desmethyl-TMP, Cα-OH-TMP, Phase II: Cα-NAC-TMP	Phase I: (2,4-diaminopyrimidin-5- yl)-(3,4,5-trimethoxyphenyl)methanone (oxo-TMP)	Tomato and lettuce	[50]

Gluc glucuronide conjugate, *AGluc* acyl glucuronide conjugate, *Sulf* sulfate conjugate, *GSH* glutathione conjugate
Glucoside conjugates, *Hex* hexose; *DeOHex* desoxyhexose. Amino acid conjugates: *Ala* alanine; *Cys* cysteine; *Gln* glutamine; *Glu* glutamic acid; *Gly* glycine; *Ile* isoleucine; *Leu* leucine; *Phe* phenylalanine; *Ser* serine; *Thr* threonine; *Tyr* tyrosine; *Val* valine; *Mal* malonyl. Bold font indicate metabolites detected in both human and plants

3-methylxanthine and ultimately the end product xanthine [27]. In humans, in turn, caffeine is primarily metabolized in the liver where it undergoes also C8 oxidation apart from demethylation. The initial N-demethylation reactions are common in humans [53], but not up to the stage of xanthine. While paraxanthine represents the large fraction of the primary metabolites (up to 80%) during the first step of demethylation, it seems that theobromine is the most abundant intermediate in radish. A further example of N-demethylation is the antidiabetic agent metformin (MFM), a reaction that was shown to occur in *Typha latifolia* as well as in humans [49]. Hydroxylation of the methylene group attached to the piperazine ring in trazodone in *Lepidium sativum* (garden cress) results in the formation of m-chlorophenylpiperazine [34].

Other compounds undergo sequential demethylation and hydroxylation. For instance, diazepam is metabolized in plants (*Raphanus sativus*, *Beta vulgaris*, *Cucumis sativus*) to two primary metabolites, nordazepam as the N-demethylation product and temazepam as the result of hydroxylation of the methylene group adjacent to the carbonyl group in the seven-membered ring. Both metabolites can then converge into oxazepam, thus constituting the same metabolic pathway as identified in humans. The antidepressant sertraline (SRT), in turn, is extensively metabolized to N-desmethyl-SRT in humans, which is followed by oxidative deamination to the ketone. When *Lepidium sativum* (garden cress) was exposed to SRT, only two hydroxylated metabolites were detected [34], while no evidence for N-demethylation was presented. In the same study, a second antidepressant, clomipramine (CMP), formed an N-demethylated biotransformation product, previously reported to be formed in humans [34], which are also able to hydroxylate both CMP and its N-desmethyl-CMP.

A special type of carbon oxidation is epoxidation. This CYP-mediated reaction at aromatic rings or double bonds releases typically highly reactive species due to the ring tension and the polarized carbon-oxygen bond. One of the few stable examples is 10,11-epoxide of CBZ, which is a major transformation product in plants. In humans, the formation of this symmetrical epoxide is a rare example of a metabolite being sufficiently stable to be excreted into human urine (see Fig. 1).

While carbon oxidation of drugs is well documented in various plants species, there are no cases yet that have demonstrated the oxidation of heteroatoms. Such reactions, though, can be expected based on the observation of such pathways for pesticides. For instance, the herbicide prometryn, a methyl-aryl thioether, is subject to S-oxidation, while the pyridazine ring in the herbicide credazine undergoes N-oxidation in this heterocycle [54].

3.1.2 Hydrolysis

As of today, there are no studies that provide evidence for drug hydrolysis in plants. Nonetheless, the presence and function activity of hydrolytic enzymes in plants have been demonstrated for several pesticides; for instance, propanil undergoes cleavage

of the amide bond, whereas the nitrile group of bromoxynil is hydrolyzed to the carboxylic acid [54].

3.1.3 Reduction

In contrast to oxidation, enzymatic reduction of organic contaminants occurs less frequently in both plants and humans [55]. A plant-specific metabolite not occurring in humans is 10,11-dihydro-CBZ, whose formation can be explained by hydrogenation of the 10,11 double bond in the central ring of CBZ [28–30, 56]. Furthermore, CBZ was shown to yield two reductive metabolites in tomato plants originating from internal cyclization of the carbamoyl group with the carbon in position 6, followed by the conversion of two aromatic double bonds into saturated moieties [28].

3.2 Phase II Metabolism

The metabolic transformations of drugs identified in phase II reactions are very similar to those documented for humans. One of the major differences between human and plant metabolism with respect to phase II reactions is that the former catalyzes the transfer of glucuronic acid to substrates bearing carboxyl, hydroxyl, or amine group, whereas the latter makes use of glucose for the analogous reaction with the drug molecule. As mentioned above, in phase II metabolism, plants are able to transfer D-glucose to suitable nucleophilic substrates under the catalytic effect of glycosyltransferase, whereas the analogous reaction in the human body involves glucuronic acid (Fig. 9). Direct conjugation of drugs without previous functionalization by phase I reactions can occur in plants provided the substrate contains appropriate functional groups for conjugation. Apart from the important role of glycosyltransferases, further phase II enzymes are GST, malonyltransferases, SULT, and methyltransferases [57].

3.2.1 GSH Conjugates

An important role of GST in humans is the inactivation of reactive metabolites of electrophilic nature. As illustrated in Fig. 9, acetaminophen (AAP) is bioactivated in a CYP-mediated N-oxidation/dehydration to yield a species known as NAPQI. The detection of the GSH conjugate in AAP-treated *Armoracia rusticana* hairy roots (HR) strongly suggests that the bioactivation in this plant species is identical to hepatic bioactivation in mammals [26]. In general terms, GST enzymes are important in detoxifying xenobiotics across a broad variety of organisms, including mammals, bacteria, fungi, plants, and insects. GSH conjugates of xenobiotic compounds result in highly polar metabolites that can be more easily translocated [56]. The residual reactivity of the epoxide 10,11-epoxy-CBZ (see above) becomes

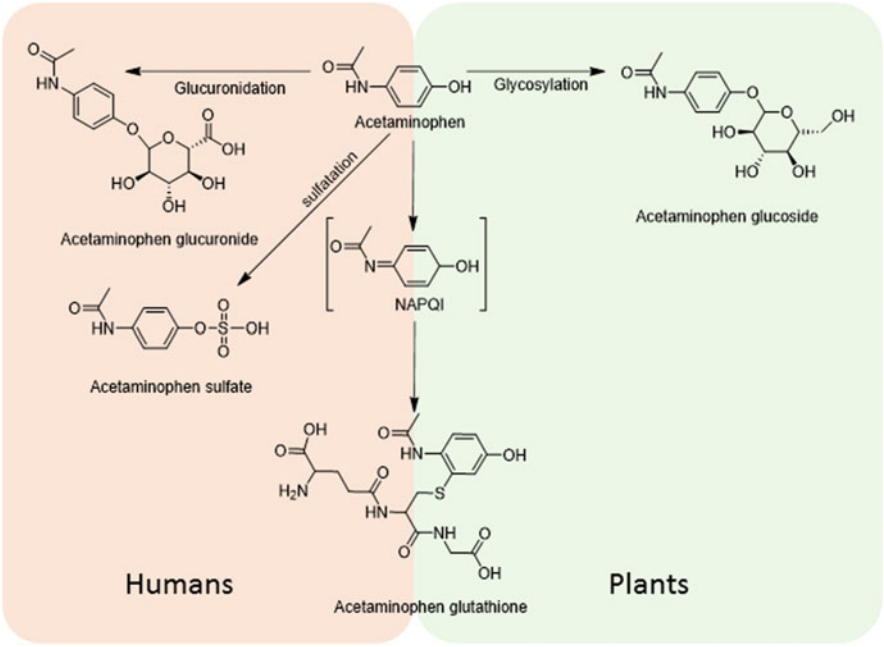

Fig. 9 Proposed metabolic pathways in humans and plants of acetaminophen. Adapted from [26]

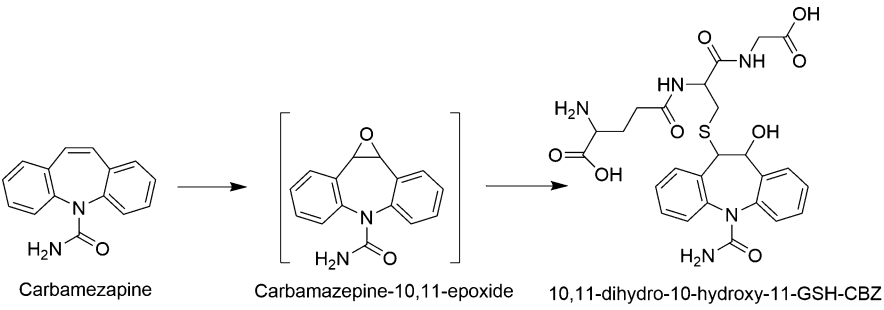

Fig. 10 Formation of 10,11-dihydro-10-hydroxy-11-GSH-CBZ in roots inoculated with *R. radiobacter* [29]

evident in the identification of 10,11-dihydro-10-hydroxy-11-GSH-CBZ in roots inoculated with *R. radiobacter* [29]. The formation of this metabolite can be rationalized by the ring opening of the epoxide through addition of GSH as depicted in Fig. 10. The detoxification capabilities of GST were also proven for *Typha latifolia* (bulrush) upon exposure to DCF. In root tissues, the cytosolic GST-CDNB (1-chloro-2,4-dinitrobenzene) activity was significantly increased forming 4-OH-DCF-GSH through reaction of GSH with the active metabolite 4-OH-DCF [38].

3.2.2 Carbohydrate Conjugates and Derivatives

The most frequently detected phase II metabolic transformation of xenobiotics in plants is the conjugation with sugars. This glycosylation facilitates the conjugation of chlorinated phenols, anilines, and thiophenols as well as of carboxylic acids [21]. When these functional groups (OH, NH, SH, COOH) are already present in the structure of the parent compound, it can be directly conjugated; otherwise it requires a preceding phase I reaction to unmask or introduce the respective functionality. For instance, AAP is transformed to AAP-Hex (Fig. 9) which is the dominant plant metabolite (64%) in analogy to the prominent glucuronidation in humans (40–67%) [26]. Plant glycosyltransferases also transform triclosan (TCS) into TCS hexoside (TCS-Hex) and subsequently to disaccharide conjugates such as TCS hexosyl hexoside (TCS-Hex-Hex) and TCS desoxyhexosyl hexoside (TCS-DeOHex-Hex) [58] by linking a second sugar unit (hexose and desoxyhexose, respectively) to the TCS-bound hexose [58].

Carbohydrate conjugates of xenobiotics can undergo further transformation with malonic acid which is an important transformation step in plant detoxification. The function of malonyltransferases resides in the transfer of malonyl from malonyl-CoA to the substrate which in case of glucosides is attached to the 6-hydroxyl group in the glucose ring. In addition, the amino groups of anilines and *S*-cysteinylated conjugates are also amenable to malonylation [21]. Malonylation has been hypothesized to protect the saccharide conjugates against enzyme cleavage and to render the products ready for storage in vacuole or cell walls in phase III [59]. In horseradish cultures, the UV filter oxybenzone (OBZ) undergoes initial conjugation with glucose to form OBZ-Hex followed by malonylation to yield OBZ-Hex-Mal (Fig. 11) [61]. Excretion of OBZ-Hex into the growth medium was observed, while the corresponding OBZ-Hex-Mal remained stored in root cells, a fact supporting the hypothesis of vacuolar compartmentation of glucoside-malonyl conjugates.

In horseradish culture TCS was transformed to a monohydroxylated metabolite, which alongside the intact parent compound underwent malonyl conjugation to yield the corresponding hexosyl malonyl conjugates (TCS-Hex-Mal and OH-TCS-Hex-Mal) [52]. Analogous reaction sequences were observed for the phenylacetic acids

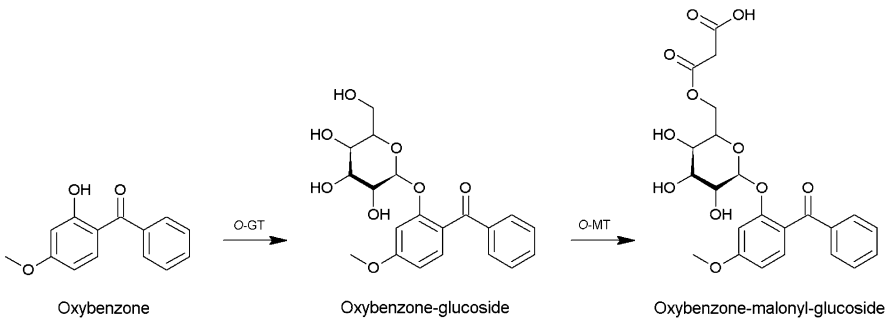

Fig. 11 Proposed metabolic pathway of oxybenzone in *A. rusticana*. Adapted from [60]

DCF and IBU in radish [27, 37]. Mefenamic acid (MFA) with its benzoic acid core was oxidatively metabolized in 3-methylhydroxy MFA [40] at the methylphenyl ring to the benzylic alcohol. Both compounds can then serve as substrates for glycosylation to generate MFA-Hex-Mal and OH-MFA-Hex-Mal [40]. By contrast, treatment of *Lepidium sativum* with ketoprofen allowed only to detect the hexosyl malonyl conjugate, while no evidence for any oxidative pathways could be provided [40].

An analogous reaction of the hydroxyl group with a carboxylic acid of the glycosylated phase II conjugates is performed by acetylation [52]. The acetylation of TCS and IBU hexosides in one OH-group of the glycone part gave rise to the acetylhexoside in radish [27, 46, 52]. Sulfation of glucosides in plants was also reported [57]. So far, TCS is the only glycosylated compound that was shown to generate sulfo- and disulfosaccharide conjugates in plants [52].

3.2.3 Amino Acid Conjugates

The reaction of drugs to form amino acid conjugates is an infrequent reaction in human drug metabolism observed mostly for carboxylic acids to yield taurine or glycine conjugates. By contrast, plants appear to be more susceptible to form amino acid conjugates. For instance, drugs, bearing a carboxyl group such as DCF [37], IBU [41], and NPX [41], form conjugates with glycine, glutamine, leucine, and phenylalanine. For example, IBU was conjugated directly with glutamine and glutamic acid to form IBU-Gln and IBU-Glu [41]. Hydroxy-IBU likewise formed their respective conjugates as well as the one with serine (Table 1). These results, together with findings for NPX, clearly show that conjugation with amino acids, especially Glu and Gln, was a major route of biotransformation of profens in *Arabidopsis* [41]. Amino acid conjugation though is not limited to substrates with carboxyl group to yield the amide, but it was also observed for the secondary amine in N-desmethyl SRT in garden grass treated with SRT whose carboxylic acid functionality reacted with the aromatic amino acids tyrosine and phenylalanine [34]. The formation of AAP-Cys did not originate from a direct reaction with cysteine but was formed by two consecutive hydrolytic steps of the two amide bonds which yielded glutamic acid from the phase II metabolite, AAP-Glu, already formed in *Armoracia rusticana* [26].

3.3 Phase III Plant Metabolism

To get rid of phase II products, plants transport them into the vacuole where they are stored or incorporate them to the cell wall. This phase III of compartmentation is unique to plants because plants cannot excrete xenobiotics as animals do. Phase III products are no longer toxic for plants but can be reconverted in the original phase II or even phase I metabolites after ingestion by herbivores or after reincorporation of

decaying biomass to the environment. Transport into the vacuole is driven by ATP-binding cassette (ABC) protein-mediated transporters. ABC transporters use MgATP to drive the transport of ligands, a process unaffected by transmembrane H+ electrochemical potential but strongly inhibited by vanadate ions [62]. The importance of these transporters in xenobiotic detoxification has been clearly demonstrated by showing that transgenic *Arabidopsis* overexpressing an ABC protein showed an enhanced tolerance to multiple herbicides [63].

Vacuolar storage of xenobiotic-malonyl conjugates has been observed in plants using the herbicide 2,4-D and the pesticide pentachlorophenol [59, 64]. Malonylation is characterized by enhanced chemical stability and improved solubility and deposition of target compounds in vacuoles [60, 65–67]. Transporters for pharmaceutical-malonyl conjugates have so far not been identified, but transporters for malonyl conjugates of physiological importance are described since long. The central molecule for ethylene biosynthesis, 1-aminocyclopropane-1-carboxylic (ACC), is present in plant cells in free and conjugated forms. One of these conjugates is N-malonyl-ACC (MACC) and can be translocated between the cytosol and the vacuole by ATP-dependent tonoplast carriers [68, 69] suggesting that MACC formation and storage in the vacuoles might be important to control the pool of available ACC.

GSH conjugates are by far much well studied being glutathionylated pesticides the best studied routes of xenobiotic conjugate metabolism [67, 70]. The ABC subfamily C is responsible for the transport of glutathionylated xenobiotics into vacuoles [62]. Beyond their contribution to vacuolar sequestration of model GSH conjugated xenobiotics, they have evolved to fulfil other physiological transport roles [71]. Pharmaceutical metabolism in plants through conjugation to GSH was observed for several compounds like AAP, CBZ and DCF (Table 2). Their transporters have not yet been identified. More research is needed in this area specially to investigate possible recycling and further processing of GSH conjugates, a fact that has been observed previously in vacuoles and cytoplasm [75].

Another compartmentation route for xenobiotic detoxification is the incorporation of degradation intermediates into bound residues, typically polysaccharide or polyphenolic biomolecules located in the cell wall or more occasionally proteins or lipids [76]. A study using ^{14}C-labelled IBU and NPX allowed the quantification of non-extractable phase III metabolites integrated in cell walls [41]. Bound residues are insoluble and are not detectable using conventional solvent extraction techniques. However, studies have shown that between 1 and 70% of the herbicide metabolite can be incorporated into structural components of the plant [76, 77].

4 Plant Models for the Study of Pharmaceutical Metabolism

Pharmaceutical metabolism in plants has been studied by using different approaches. Uptake, translocation, metabolism, and compartmentation of transformation products are a complex process depending on many variables from soil structure,

Table 2 Studies involving hairy roots for the identification of pharmaceutical transformation products in plants

Compound	Concentration	Identified metabolites	Plant species	Reference
Acetaminophen (AAP)	151 ppm	Phase II: AAP-Hex, AAP-GSH, AAP-Cys	*Armoracia rusticana*	[26]
Diclofenac (DCF)	2.96 ppm 29.6 ppm	Phase I: 4-OH-DCF, Phase II: 4-OH-DCF-glycopyranoside	*Armoracia rusticana*	[37]
Diclofenac (DCF)	59.4 ppm	Phase I: DCF-2,5-iminoquinone	*Armoracia rusticana*	[72]
Oxybenzone (OBZ)	22.8 ppm	Phase II: OBZ-Hex, OBZ-Hex-Mal	*Armoracia rusticana*	[61]
Carbamazepine (CBZ)	59 ppm	Phase I: 10,11-epoxy-CBZ, 10,11-diOH-CBZ, 10-OH-CBZ, 2,3-dihydro-2,3-diOH-CBZ, 2,3-diOH-CBZ, CBZ-2,3-quinone, acridine, acridine-carboxaldehyde, 9-OH-acridine, acridone Phase II: CBZ-GSH, CBZ-Cys, CBZ-Cys-Gly	*Armoracia rusticana*	[29]
Phenol, 2,4-dichlorophenol	100 ppm	Not identified	*Brassica napus*	[73]
Tetracycline, oxytetracycline	1 to 10 ppm	Phase I: oxidation at the BCD chromophore	*Helianthus annuus*	[74]

composition, moisture, and pH to the physiology of each plant species or the associated plant microbiota. This complexity can be simplified by using models allowing to answer basic mechanistic questions.

4.1 Whole Plants

Metabolism of xenobiotics can be studied by using whole plants. This approach involves long growth periods and complex matrices to analyze, being necessary the development of specific extraction and cleanup methods adapted to each situation in particular [78]. Moreover, the formation and distribution of metabolites in different parts of the plant are the result of complex interactions between the physicochemical properties of the molecule, the soil characteristics, the rhizosphere microbiome, the plant species, the plant physiological and health status, and the endophytic microbiota. Hydroponically grown plants are a good compromise between field conditions and the use of a real model. Hydroponics have the advantage of working in controlled conditions, limiting the interferences with the pharmaceutical applied and the soil matrix and its microbiota and the possibility to clean the roots prior to extraction. Additionally, the remaining growth solution used to provide water and

nutrients for the plants can be easily screened for root exudates and metabolites produced in the rhizoplane. A wide array of studies identifying uptake of pharmaceuticals in plants using both approaches can be found in the literature [79]. These approaches can be used to study the transfer of pharmaceuticals and their metabolites to edible plants and transformed products when using reclaimed wastewater in agriculture. Additionally, they may provide useful information about translocation and distribution of metabolites in plant tissues [38, 43, 48].

4.2 In Vitro Models

Researchers have made use of models to avoid the complexity of soil-grown plant systems and to simplify their studies. Even though mechanisms like transpiration-driven uptake and translocation or interactions with the soil and plant microbiota are absent or disturbed in these model systems, they are a viable approach when it comes to identifying new metabolites and describe metabolic pathways exclusive of plant cells. Most known models involve the use of cell suspensions of different plant species [41, 58, 80–83].

In vitro studies can be rapidly set up in order to identify metabolites in plant tissues by incubating cut stems devoid of their root system or excised leaves or roots in a solution containing pharmaceuticals and/or metabolites. Pioneering studies who served to establish the general model of xenobiotic detoxification in plants were conducted using excised leaves and roots exposed to different herbicides [84, 85]. Furthermore, by using cut stems or detached leaves, it is possible to quantify phytotoxicity and plant short-term response towards pharmaceuticals [86].

Pharmaceutical metabolism can be studied also in enzymatic extracts obtained from plant tissues. Some in vitro studies using enzyme extracts incubated with pharmaceuticals have found similar metabolic patterns both in radish tissue enzyme extracts and in the intact plants [27]. Plant enzyme extracts have been used to investigate metabolism of MFM in specific plant compartments [49] and synthetic estrogens at specific plant development stages [87].

4.3 Examples of Method Applications

The particularities and complementarities of these different approaches can be illustrated using CBZ as an example of a recalcitrant pharmaceutical that can be taken up and metabolized in plants. Wu and coworkers studied CBZ metabolism using carrot cell suspensions [80]. They could determine that only about 5% of the initial CBZ amount was metabolized in plant cells and transformed into CBZ-epoxide and CBZ-10,11-diol (Fig. 12). Both metabolites were also identified as main metabolites of CBZ in leaves of tomato, cucumber, sweet potato, and carrot and fruits of cucumber and tomato [31].

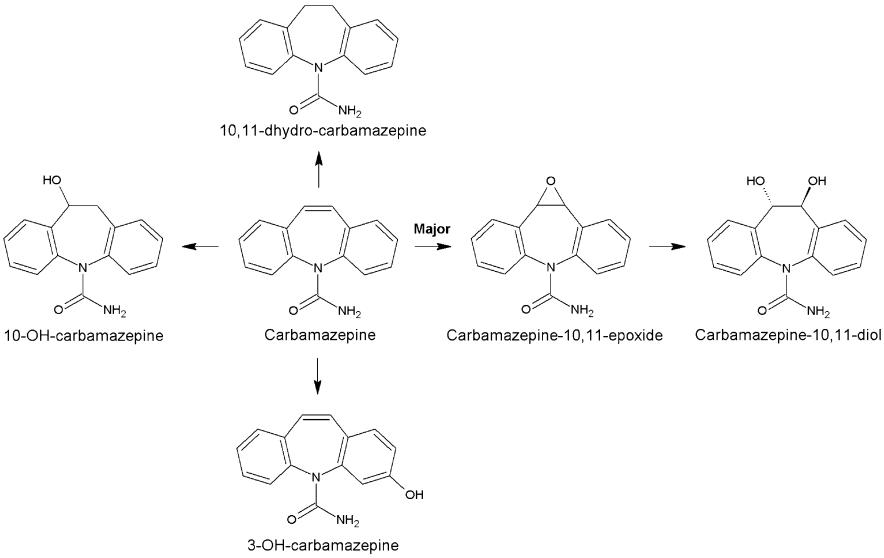

Fig. 12 Metabolism of carbamazepine in carrot cell suspension. Adapted from [80]

In a field experiment with vegetables grown with reclaimed wastewater, the distribution of CBZ and nine related metabolites, also found in wastewater, was studied in different crops and plant tissues [88]. Using this approach, researchers could establish an accumulation pattern leaves>roots>shoots>fruits for these compounds and could identify tissues where specific metabolites accumulated (e.g., acridone in carrot leaves). In another study, a total of 11 metabolites (mainly phase-I) were quantified in tomato plant tissues grown hydroponically [28]. This approach concluded that an intensive transformation of CBZ occurred in tomato fruits in addition to metabolism in roots and leaves and completed the metabolic pathway for CBZ in plants (Fig. 13).

These findings show that major metabolic pathways of CBZ in plant cell cultures and in whole plants are similar proving that plant cell cultures can serve as a simple, rapid tool to study the metabolism of pharmaceuticals in plants. However, studies with whole plants and soil are needed to identify accumulation and compartmentation of specific metabolites in different plant tissues.

4.4 Hairy Roots as Model for the Study of Root Metabolism

In 1930 a disease called “hairy root” (HR) disease was identified in apple trees. The causative agent identified by Riker and coworkers was *Phytomonas rhizogenes* (later on reclassified as *Agrobacterium rhizogenes*), a bacterium inducing abnormal growth of adventitious hairy roots in affected plants [89]. The molecular

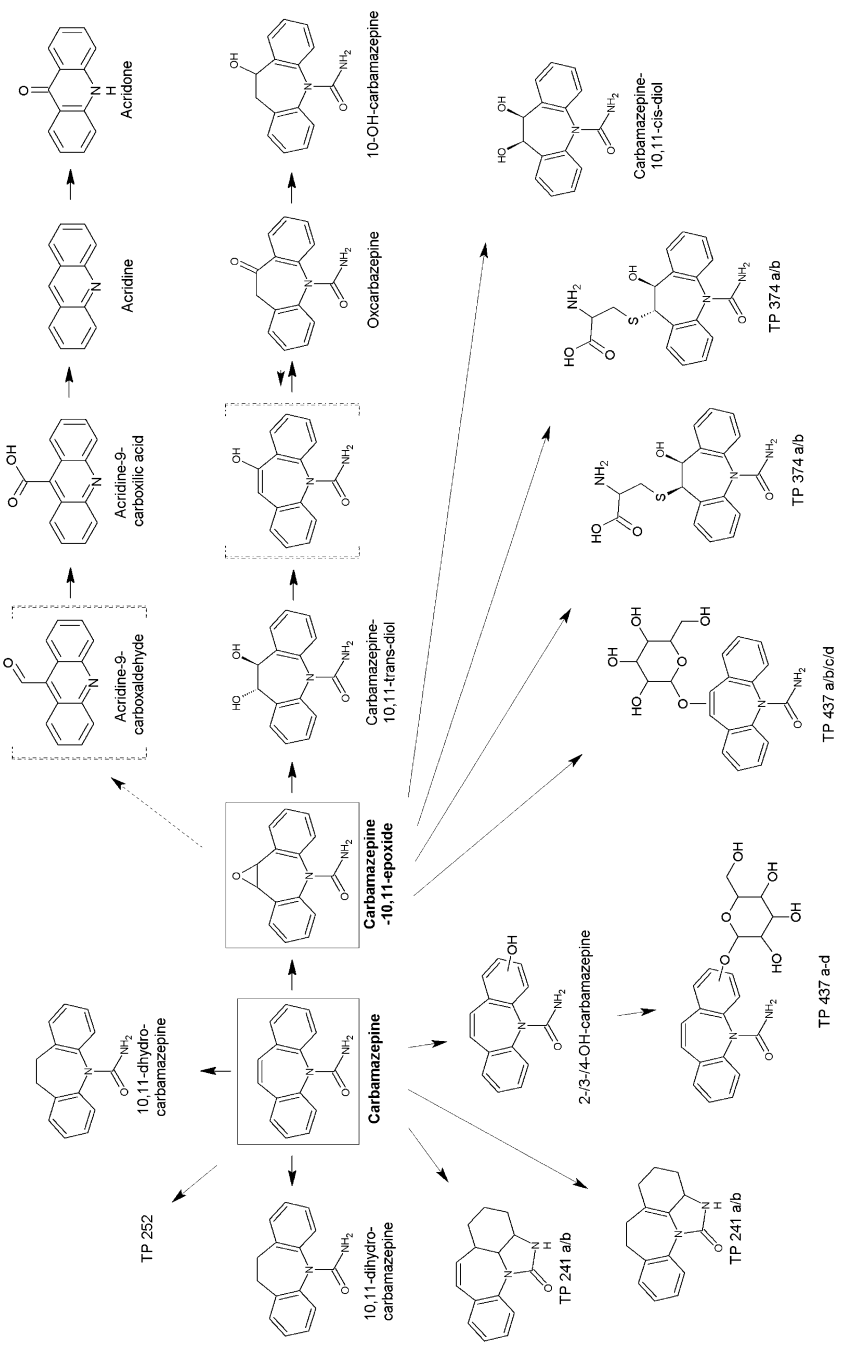

Fig. 13 Metabolism of carbamazepine in tomato plants. Adapted from [28]

mechanisms that *A. rhizogenes* uses to induce HR formation are similar to those used by *A. tumefaciens* to induce gall formation and consist in the transfer of a DNA segment (T-DNA) to plant cells. The T-DNA of the Ri (root inducing) plasmid contains several virulent genes causing rhizogenic growth to the transformed cells [90, 91]. Both bacteria transform plant tissues introducing genes through their T-DNA to produce opines, which serve as specific nutrients for the bacteria [92]. However, the physiologic basis of the tumorigenesis disease is different. Alteration of auxin metabolism has been proposed to contribute significantly to the expression of the HR phenotype [93, 94]. These findings have established the foundations for the development of HR cultures with different valuable biotechnological applications such as secondary metabolites production, biotransformation processes, or phytoremediation studies [73, 95].

In the last two decades, the use of HRs has been consolidated as study model for the degradation and metabolism of organic pollutants, heavy metals, radionuclides, and more recently of pharmaceuticals [96]. Additionally, HRs can be used to study plant detoxification mechanisms and activity of detoxifying enzymes such as peroxidases and laccases [97, 98]. Considering (as mentioned previously) that metabolic pathways are conserved in plant cell cultures, HRs have emerged as a model for the description of pharmaceutical metabolism in plants. HRs have some advantages over whole plants or other models like cell suspensions. They have a stable genotype and phenotype, a fast in vitro growth with no requirement of additional phytohormones, and a high production of secondary metabolites, a reason for which they are often called "phytochemical factories" [99]. Additionally, they are easy to maintain, by subculturing in sterile media, avoiding interactions with rhizosphere microbial populations present in whole plants. For this reason, they can be used also to study interactions with single microorganisms, especially rhizospheric and endophytic bacteria or fungi [29, 73, 100, 101]. To date, HR cultures have been obtained from a large number of plant species, predominantly dicotyledonous. Monocotyledonous species (like many macrophytes used for phytoremediation) have remained recalcitrant to the transformation by *A. rhizogenes*. Nonetheless, in the last years, advances in transformation techniques have allowed to obtain HR cultures from several monocotyledonous species [102].

HRs have been used for their enzyme activity in pharmaceutical research. *Brugmansia candida* HRs were shown to possess a glycosyl transferase very effective in biotransforming the toxic depigmenting agent hydroquinone into a less toxic alternative as arbutin [103]. For a long time, this model has been used for the biotransformation of natural compounds (e.g., thymol, geraniol, coumarin, or flavone derivatives) into molecules of improved pharmaceutical properties [104]. These biotransformations rely on HRs inherent enzymes and are governed by hydroxylation, glycosylation, oxidoreduction, and hydrolysis reactions. As these enzymes are normally present in roots of the selected plant species, this system is now used for the identification of xenobiotic metabolites, using plant species known for their production of enzymes involved in phase I metabolism such as peroxidases and laccases [97, 98].

Several metabolites have been identified in HRs exposed to pharmaceuticals (Table 2). With the development of new analytical methods, HRs are helping researchers to identify novel metabolites and describe metabolic pathways in plants. The first study of uptake and metabolism of pharmaceuticals was done using sunflower HRs to biotransform tetracycline and oxytetracycline [74]. Even if the transformation products were not fully identified, authors could evidence oxidation reactions affecting the UV absorption spectra of the parent compounds. Interestingly, it was observed that these modifications originated also in the liquid media, suggesting an active role of root exudates. It is known that roots can exudate organic acids, sugars, or amino acids but also enzymes such laccases or peroxidases. Mechanisms of DCF oxidation by peroxidases were recently studied using crude enzyme extracts from HRs [72]. Using stopped flow spectroscopy in combination with liquid chromatography-mass spectrometric analysis, authors could identify the formation of the highly reactive diclofenac-2,5-iminoquinone, which may be the precursor of several biological conjugates and breakdown products in plants.

Dismissed of the aerial part, HRs are deprived of transpiration, the main driving force of water uptake in whole plants. As a consequence, pharmaceutical uptake is the result of passive diffusion through the membrane, direct entrance into wounds, or active transport in case of molecules requiring transporters (e.g., MFM). For the same reason, phase I metabolites that are normally free in the cytosol can cross membranes by diffusion, being therefore detectable in the culture media [29]. Phase II metabolites are normally immobilized in the vacuole or in the cell wall and require a tissue extraction for their identification. Following this procedure, glucoside and GSH conjugates were identified in *Armoracia rusticana* HRs after exposure to acetaminophen [26], DCF [37], and CBZ [29]. In some cases, glucoside conjugates are exudated into the medium where the parent compound can be released after cleavage of the glucose. Malonylation has been hypothesized to protect the saccharide conjugates against enzyme cleavage and to render the products ready for storage in vacuole or cell walls [86]. A mass balance can be calculated by combining the analysis of both intra- and extracellular matrices. Using this approach, it was observed that *A. rusticana* could metabolize up to 82% of the initial acetaminophen amount after 6 hours of incubation with a distribution in the cells of 18% acetaminophen, 64% acetaminophen-glucoside, 17% acetaminophen GSH conjugate, and 1% of the corresponding cysteine conjugate [26].

5 Role of Microbiome in Pharmaceutical Metabolism and Plant-Microbe Interactions

With the recent developments on the omics technologies, the study of the microbiome has gained attention. Plants are no longer considered as standalone organisms but as holobionts in which many different microbiomes interact in a specific ecological context, contributing to major functions such as plant nutrition

and plant resistance to biotic and abiotic stresses [105]. Researchers are addressing the impact of pharmaceuticals on plant microbiome in wetlands [106, 107] and crops [108], contributing to elucidate the role of the plant microbiome in xenobiotic metabolism as well as its contribution to plant fitness in polluted environments.

5.1 The Rhizosphere Is a Hot Spot for Pharmaceutical Metabolism and Metabolite Exchange Between Plant and Microorganisms

The contaminant concentration in soil is a major factor determining rhizosphere and root endosphere microbiome structure and function [109]. Bacteria abundance and diversity are high in the rhizosphere and decrease from the outer region to the inner compartments. Bacterial abundance has been estimated to be 10^7–10^9 cfu per g of fresh soil in the rhizosphere, 10^5–10^7 in the rhizoplane, and 10^3–10^4 cfu/gfw in the aerial endosphere [110]. The rhizosphere is a zone of exchange between the plant and the soil, where soil nutrients and root exudates allow the growth of a complex microbial community. Rhizospheric bacteria are key players in the metabolism of xenobiotics present in the soil, being the first to enter in contact with foreign molecules. It has been observed that degradative genes are enriched [111] and expressed [112] in the rhizosphere microbiome of plants growing in contaminated sites, revealing a selective control of the plant over rhizospheric microbial communities, favoring microbes with effective degradative traits. Plant selection of microbes can be exploited for cultivation and isolation of plant-associated bacteria with degradation and beneficial properties. Several studies have addressed microbiome composition and role in plants exposed to pharmaceuticals, some of them using culture-dependent methods (Table 3). Isolated microorganisms with in vitro degradation capabilities can be used for bioaugmentation in nature-based solutions for wastewater treatment.

Several studies have revealed how plant-associated microbial communities respond to the presence of pharmaceuticals in constructed wetlands (CW) for wastewater treatment highlighting the importance of plant-bacteria interplay for the remediation of pharmaceuticals [119]. In general, the concentration of pharmaceuticals affects bacterial community richness and diversity. It was observed that TCS affects the development of certain bacteria and, eventually, the bacterial community structures in CWs [117]. Moreover, the plant species selected for the CWs affected the selection of microbial strains involved in TCS degradation. Whereas in *Typha angustifolia* and *Hydrilla verticillata* CWs, beta-Proteobacteria were enriched after exposure to TCS, in *Salvinia natans* CWs, delta- and gamma-Proteobacteria and Sphingobacteria populations were significantly increased, and could relate to TCS biodegradation [117]. Similar results were obtained by Liu and coworkers in CWs planted with emergent cattail, submerged hornwort, and floating duckweed [118]. Accumulation of TCS in sediment and plants was high in hornwort and

Table 3 Studies involving plants and their associated bacteria for the removal and metabolism of pharmaceuticals

Plant species	Compound	Type of bacteria	Bacteria	Comments	Reference
Coffea Arabica, *Coffea robusta*	Caffeine	Endophytic	*Bacillus lentimorbus*, *Bacillus megaterium*, *Bacillus subtilis*, *Bacillus cereus*, ***Pseudomonas putida***, *Pseudomonas chlororaphis*, *Pantoea ananatis*, *Pantoea agglomérons*, *Stenotrophomonas maltophilia*, *Kluyvera cryocrescens*, *Kocuria kristinae*	Isolation In vitro degradation	[113]
Phalaris arundinacea	Diclofenac, sulfamethoxazole	Endophytic	*Variovorax boronicumulans*, *Bacillus wiedmannii*, ***Microbacterium flavescens***, *Agrobacterium tumefaciens*	Isolation	[114]
Juncus acutus	Bisphenol-A, sulfamethoxazole, ciprofloxacine	Endophytic, roots and leaves	*Sphingomonas* sp. U33 (leaf), *Bacillus* sp. R12 (root), *Ochrobactrum* sp. R24 (root)	Bioaugmentation study using *Juncus acutus*	[115]
Phragmites australis	Carbamazepine	Endophytic	***Chryseobacterium taeanense***, *Pseudomonas moorei*, ***Diaphorobacter nitroreducens***, ***Achromobacter mucicolens***, *Rhizobium radiobacter*, ***Rhizobium daejeonense***	Isolation, removal in liquid cultures	[116]
Phragmites australis	Carbamazepine	Endophytic	***Diaphorobacter nitroreducens***, ***Rhizobium radiobacter***	Inoculation of *Armoracia rusticana* hairy roots and degradation in vitro metabolites identified	[29]

(continued)

Table 3 (continued)

Plant species	Compound	Type of bacteria	Bacteria	Comments	Reference
Miscanthus giganteus	Diclofenac, sulfamethoxazole	Endophytic	*Streptomyces curacoi*, *Streptomyces neopeptinius*, *Streptomyces griseorubiginosus*, *Streptomyces curacoi*, *Microbacterium natoriense*, *Glycomyces lechevalierae*, *Glycomyces lechevalierae*, *Pedobacter panaciterrae Gsoil*, *Staphylococcus succinus* subsp. *casei*, *Streptomyces griseorubiginosus*, *Streptomyces lincolnensis*, *Microbacterium saccharophilum*, *Neorhizobium alkalisoli*	Isolation and sequencing	[106]
Typha angustifolia, *Hydrilla verticillata*, *Salvinia natans*	Triclosan	Sediment bacteria in CWs	*Betaproteobacteria*, *Deltaproteobacteria*, and *Gammaproteobacteria*, *Sphingobacteria*	Sequencing, removal in CWs	[117]
Typha latifolia, *Anthocerotae*, *Lemna minor*	Triclosan	Sediment bacteria in CWs	*Betaproteobacteria*, *Gammaproteobacteria*, and *Bacteroidetes*	Sequencing, removal in CWs	[118]
Typha angustifolia	Ibuprofen	Rhizospheric bacteria	*Dechloromonas* sp., *Clostridium* sp., *Clostridium saccharobutylicum*, *Sphingobacteriales*, *Cytophaga* sp.	Sequencing, removal in CWs	[42]
Cyperus alternifolius, *Cyperus papyrus*, *Juncus effusus*	Sulfonamides (sulfadiazine, sulfamerazine, sulfamethazine, sulfamethoxazole, sulfapyridine)	Rhizospheric bacteria	*Methylosinus*, *Methylotenera*, *Methylocaldum*, and *Methylomonas*	Sequencing, removal in CWs	[107]

Bold font indicates bacteria with confirmed degradation properties

duckweed, while in cattail, biodegradation likely played an important role. *Betaproteobacteria*, *Gammaproteobacteria*, and *Bacteroidetes* were detected and might have significant correlations with TCS degradation [118]. Even if these studies did not document TCS metabolites, they identified bacterial genera with the potential to contribute to TCS metabolism in CWs. Rhizospheric bacteria *Dechloromonas* sp., *Clostridium sp*., the order *Sphingobacteriales*, and the *Cytophaga* sp. identified in roots from *Typha angustifolia* were most probably responsible for the rhizodegradation of IBU [42]. IBU accumulated in leaves and was partially transformed to IBU carboxylic acid, 2-hydroxy IBU, and 1-hydroxy IBU. Bacteria participating in the degradation of antibiotics were also identified. Man and coworkers observed how sulfonamides inhibited some functional microorganisms related to the sulfur and nitrogen cycles in the rhizosphere of wetland plants [107]. On the other hand, sulfonamides significantly enriched methylotrophs with potential to degrade the antibiotics such as *Methylosinus*, *Methylotenera*, *Methylocaldum*, and *Methylomonas* [107].

5.2 *Endophytic Bacteria Can Enhance Degradation of Pharmaceuticals in Plants*

Endophytic bacteria have been found in intercellular space in root tissues but also in the xylem and in the cytosol of some cells. Since much of pharmaceutical metabolism in plants takes place in the cytosol and xenobiotic's ultimate fate is often conjugation and storage of the conjugates in the vacuole, endophytic bacteria can influence metabolic pathways with their enzymes involved in phase I reactions, introducing new metabolites in the plant xenome. Although the endosphere is a habitat with lower bacterial diversity than the rhizosphere, the presence and concentration of pharmaceuticals have shown effects also on the endophytic bacterial diversity in legume species irrigated with reclaimed wastewater [108] and in CW species exposed to pharmaceuticals [115, 117].

Specific endophytic bacterial groups are enriched after plant treatment with pharmaceuticals. In *Miscanthus* x *giganteus*, 16S rRNA amplicon sequencing of the endophytic bacterial community showed an enrichment of *Actinobacteria* after treatment with sulfamethoxazole and DCF [106]. Cultivation-dependent techniques revealed similar results, and some isolated strains (e.g., *Microbacterium aoyamense* and *Streptomyces curacoi* with additional plant-growth promoting traits) were able to degrade DCF and sulfamethoxazole in vitro [106]. *Alphaproteobacteria* (*Novosphingobium* and *Oligotropha*) and *Betaproteobacteria* (*Herminiimonas*, *Methylophilus*, *Cupriavidus*) were enriched in roots of *Juncus acutus* exposed to a high concentration of bisphenol-A, metals (Zn, Ni, Cd), and pharmaceuticals (sulfamethoxazole and ciprofloxacin) pollution [115]. The work of Syranidou et al. is one of the few studies using previously isolated endophytic strains for bioaugmentation of CWs and shows how bacterial strains may improve the

phytoextraction potential of a wetland plant like *Juncus acutus* [115]. Another study focused on the isolation and identification of endophytic bacteria from *Phragmites australis* exposed to the antiepileptic CBZ [116]. The authors could identify several strains with abilities to degrade CBZ and with potential plant-growth properties. CBZ metabolism has been studied in rats, humans, fungi, bacteria, and plants, being probably one of the most studied pharmaceuticals in plants. Following uptake, transformation products have been identified in a wide array of plant species including macrophytes like *Typha* spp. [30], vegetables like tomato [28], carrot [80, 88], lettuce [88, 120], potato, and zucchini [88]. Most of these metabolites are found also in human urine, rat liver, fungi, or bacterial [121–123]. Only a few metabolites seem to be exclusive to one organism. This specialization depends on the detoxifying enzymes that have evolved in different organisms. While plants do not rely on organic xenobiotics to obtain energy for growth and development, endophytic bacteria may find in these compounds an available source of carbon. Equipped with enzymes for the complete degradation of organic compounds, they contribute to the metabolism of pharmaceuticals in the plant endosphere.

Attempts to identify the contribution of endophytic bacteria to the metabolism of pharmaceuticals in the plant holobiont are scarce. Root cultures have been used to study the interaction between plants and endophytic bacteria during CBZ metabolism [29]. Two strains isolated from *Phragmites australis* plants exposed to CBZ were shown to use specific metabolic pathways in synergy with the plant (Fig. 14). Metabolites accumulated in *Armoracia rusticana* roots belonged to phase I transformations and to a GSH conjugation (phase II) both occurring after 10,11-epoxidation (Fig. 14). Enzymes involved in these steps were likely CYP or peroxidases (epoxidation), epoxide hydrolase (cleavage and hydroxylation), and GST (GSH conjugation). When HRs were inoculated with endophytic bacteria, two other pathways were favored. Application of *Diaphorobacter nitroreducens* resulted in the formation of a group of metabolites with an acridine-related structure. This pathway involves the cleavage of the carbamoyl group and rearrangement of the central ring of CBZ and had been previously described in fungal cultures growing in anoxic conditions [122] or as result of photo-oxidation [124]. The enzymatic machinery behind such transformations is still unclear. *Rhizobium radiobacter* activated a pathway involving successive oxidation reactions at the carbons of the side aromatic benzene and leading to the formation of 2,3 dihydrodiols and subsequent 2,3 diol compounds. This is a conserved mechanism in bacterial degradation of polycyclic aromatic hydrocarbons (PAHs) such as phenanthrene, naphthalene, fluoranthene, pyrene, and benzopyrene and is generally catalyzed by dehydrogenases.

6 Conclusion and Perspectives

Wastewater reuse for agricultural irrigation has been increasing over the years. In the last 20 years, there has been an explosion of studies concerning the occurrence and fate of pharmaceuticals and other emerging contaminants in crops and their possible

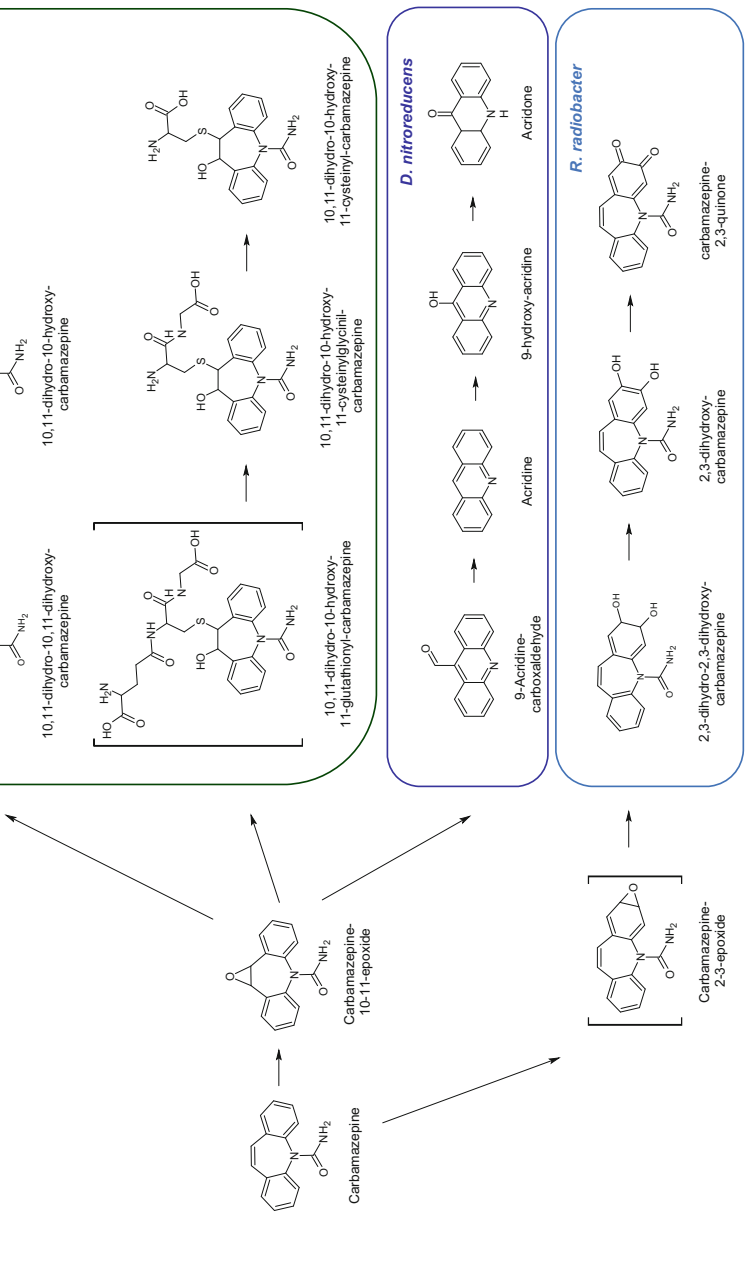

Fig. 14 Metabolic pathways of carbamazepine in *Armoracia rusticana* hairy roots inoculated with the endophytic bacteria *Diaphorobacter nitroreducens* and *Rhizobium radiobacter* isolated from *Phragmites australis* plants exposed to carbamazepine. Adapted from [29]

impact on the food chain and human health. New regulations will have to include many of these compounds to produce water of sufficient quality to satisfy the public opinion towards the use of reclaimed wastewater for agriculture.

With the recent advances on analytical methods, scientists are able to quantify more and more compounds and follow their metabolites using non-target or suspect screenings. Pharmaceuticals are subjected to a wide array of chemical modifications during their passage through our wastewater treatment facilities. And beyond this, their fate is subjected to plant metabolism once applied to the field. As some studies show, the concentration of metabolites can exceed largely that of the parent compound, emphasizing the importance of metabolite analysis in monitoring studies [50].

The analysis of metabolites and understanding of plant metabolism is crucial to set good agricultural practices. Plant metabolism of xenobiotics has been historically studied on the basis of herbicides and agrochemicals application [70]. In the last years, these studies have been extended to contaminants of emerging concern. Even if common mechanisms have been unraveled, a huge number of pharmaceuticals and their metabolites remain to be studied. Further studies are needed in order to answer questions related to plant species and agricultural specificities and metabolite distribution in different organs of the plant (edible or not).

Furthermore, this information provides the scientific community with applications for the improvement of remediation techniques based on phytomanagement. Constructed wetlands and algal pond treatment systems sit at the forefront of innovations in contemporary wastewater treatment aimed at the food-water-energy nexus, and biotechnological advances in this field will still be necessary in the coming years [125]. Moreover, studies using plants as monitoring devices for assessing the fate and environmental presence of pharmaceuticals will be helpful to protect our agroecosystems [126].

With recent advances in the field of plant microbiome, it has been shown that plant microbiota can be used to enhance degradation of pharmaceuticals in CWs. Some beneficial strains with potential degradative abilities have been identified. However, recent studies use integrative approaches to prioritize improvement of microbial networking rather than inoculation with single strains [127, 128]. A deeper understanding on plant-microbial functions for pharmaceutical degradation will lead to the development of minimal rhizosphere or plant microbiome for phytoremediation [129].

Thus, monitoring plants for pharmaceutical exposure and identifying their metabolites and their spatiotemporal distribution will be crucial for the innovation in treatment techniques and for the safety of reutilization of reclaimed wastewater for agricultural irrigation.

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Part III
Remediation and Impacts

Impact of PhACs on Soil Microorganisms

Sara Gallego and Fabrice Martin-Laurent

Contents

Abstract The use of reclaimed water in crop irrigation helps to mitigate water shortage. The fertilization of arable soils with sewage sludge, biosolids, or livestock manure reduces extensive application of synthetic fertilizers. However, both practices lead to the introduction of pharmaceutical active compounds (PhACs) in arable soil, known to host a wide range of living organisms, including microorganisms which are supporting numerous ecosystem services. In soils, the fate of PhACs is governed by different abiotic and biotic processes. Among them, soil sorption and microbial transformation are the most important ones and determine the fate, occurrence, and dispersion of PhACs into the different compartments of the environment. The presence of PhACs in soils can compromise the abundance, diversity, and activity of the soil microbial community which is one of the key players in a range of soil ecosystem services. This chapter reviews the current knowledge of the effects

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of PhACs, commonly found in wastewater effluents and derived organic fertilizers, on the soil microbial community.

Keywords Ecosystem services, Microbial activities, Microbial ecotoxicology, Microbial function, Pharmaceuticals

1 Ways of Entrance of PhACs in Arable Soils

Every year, million tons of pharmaceutical active compounds (PhACs) are consumed worldwide for prophylaxis and curative treatments in human and veterinary medicines [1, 2]. Following their ingestion, formulated PhACs enter the body where they are partially assimilated by the organism and, thereafter, largely excreted through feces and urine [3, 4]. On the one hand, excreted residues of PhACs used in human medicine are collected in domestic and hospital sewage disposal systems to reach wastewater treatment plants [5, 6]. Direct dumping of unused or expired medication [7, 8] and illegal drugs [9] can also contribute to wastewater contamination. Since PhACs are relatively stable, conventional wastewater treatment plans have proven to be moderately effective at removing them [10]. As a result, complex mixtures of PhACs and their main metabolites are frequently found in treated wastewater effluents discharged directly in the river and/or in sewage sludge applied to arable soil as organic fertilizers [11, 12]. On the other hand, excreted veterinary PhACs accumulate in livestock manure [13–16] in concentrations that can be severalfold greater than in sewage sludge [17].

In arid or semiarid regions, such as the Mediterranean rim, where rainfalls are uneven and water resources limited, the use of treated wastewater in crop irrigation and groundwater recharge constitutes a promising alternative to release green water pressure on water cycle. Irrigation of crop with wastewater provides not only water but also nutrients to plant [18–20]. This agricultural practice may thereby reduce the application of agrochemical fertilizers, improve plant growth, and limit the wastewater discharged in rivers, thereby decreasing the PhACs pressure on surface water resources especially during the low-water period. Similarly, organic amendment of arable soils with livestock manure and/or sewage sludge/biosolid is also known to be beneficial for mineral fertilization of soil (especially nitrogen) and plant nutrition: it contributes to the maximization of crop yields [21, 22]. However, both practices lead to the release of numerous micro-pollutants including PhACs into arable soils with unknown consequences on both their abiotic and biotic components [23–28]. Although introduced PhACs concentrations are quite low, their repeated input in soil may lead to their accumulation, cause toxic effects to in soil living organisms, and transfer to surrounding aquatic compartments [29, 30].

In addition to diffuse contamination sources in arable soils, improper disposal of drugs or pharmaceutical waste products and accidental spills from pharmaceutical manufacturing plants and hospitals constitute important point sources of

contamination. PhACs residues from these polluted sites [31–34] can contaminate water resources (runoff, surface water; leaching, groundwater), which can be used for crop irrigation, and indirectly contribute to both soil pollution and crop contamination.

2 Processes Involved in the Fate of PhACs in Arable Soils

As described above PhACs reach the environment via different entry routes. They reach soil via organic amendment (sewage sludge and farmyard manure) and crop irrigation (wastewater) and water resources via discharge of treated wastewater from wastewater plants in rivers and runoff and leaching from amended arable field. Once they enter the environment, the principal processes governing their fate are found at different degrees in both terrestrial and aquatic compartments. PhACs present in solid and liquid phases interact with both abiotic and biotic compartments of the environment.

In soils, PhACs are subject to several abiotic (sorption, photolysis, chemical transformation) and biotic (bioaccumulation and biotransformation) processes, which determine their ultimate distribution into the different environmental compartments [30, 35]. The rate and degree of each of those processes are determined by PhACs physicochemical characteristics as well as pedoclimatic conditions including temperature, humidity, and soil physiochemical characteristics [36–38].

Among the different mechanisms involved in the environmental fate of PhACs, sorption to soil components is by far one of the most important. It implies their close interactions with organic matter and mineral constituents of soils, involving ion exchange, surface adsorption to mineral constituents, hydrogen bonding, and formation of complexes with ions such as Ca^{2+}, Mg^{2+}, Fe^{+3}, or Al^{3+} [30]. Examples of PhACs with a strong tendency to bind to soil particles are found among those that are poorly soluble such as the analgesic paracetamol, [39], the biocides triclosan and triclocarban, and some antibiotics such as tetracyclines, macrolides, sulfamethazine [40, 41], and quinolones, which form stable complexes through cation bridging to clay minerals. As a result, PhACs remain adsorbed in soils for a long period of time although lowly bioavailable to in soil living organisms [41–51].

On the contrary, the analgesics and anti-inflammatory compounds diclofenac, ibuprofen, and naproxen, the β-blocker propranolol, and some antibiotics such as sulfamethoxazole are less adsorbed to soils [38, 52–54] from where they can runoff to surface waters or leach to groundwater after a heavy rainfall event [25, 54–58]. This was also observed for carbamazepine, meprobamate, trimethoprim, and primidone applied to soil via crop irrigation with spiked wastewater, thereby confirming their low sorption to soil components and their relatively high mobility in soil [56, 59–64]. In addition, PhACs present in the soluble fraction are not only ready to leach to groundwater but also available for plant uptake [24, 65–70], macro- and mesofauna bioaccumulation [71–73], and/or microbiota uptake and further transformation [74].

Additionally, PhACs in soil can be transformed by biotic or abiotic reactions, leading to transformation products that can be more stable, more toxic, and persistent than their parent compounds [75, 76]. Among abiotic processes, photodegradation [77] and hydrolysis [78] are known to transform PhACs in aquatic media. The anti-inflammatory drugs diclofenac, naproxen, ibuprofen, and the diuretic agent amiloride were found to be transformed to hydroxyl metabolites, presenting higher toxicity, after a photocatalytic treatment [79–84]. Additionally, studies from Yamamoto et al. [85] reported a slow rate in sunlight photodegradation of acetaminophen, mefenamic acid, as well as ibuprofen and carbamazepine. In soils, photodegradation was observed for sulfonamides and tetracycline antibiotics which spread on the soil surface and pig slurry following first and biphasic kinetics, respectively [86].

Biotic transformation of PhACs is mainly achieved by microorganisms, which have developed during their long-lasting evolution an impressive enzymatic array able not only to detoxify their environment but also to get access to nutrients for their growth. PhACs biodegradation is achieved by two types of microbial guilds catalyzing two types of transformation: one the one hand, co-metabolic transformation is catalyzed by non-specific enzymes (such as P450 monooxygenase also involved in the biodegradation of other xenobiotics such as pesticides) [74, 87–96]. On the other hand, metabolic transformation is catalyzed by specific enzymes leading to partial or full mineralization of PhACs that are used as nutrients and energy sources for the growth of the degrading microbial guild [87, 90, 97–112]. From this point of view, transformation of PhACs by fungi and bacteria is a key process for their dissipation in the environment [113–116]. Since PhACs are designed to remain active after ingestion, most of them are relatively recalcitrant to biodegradation. However, it was shown that chronic or punctual exposure of soil microbial communities to PhACs can enhance their degrading capacities toward them [109, 117]. Biodegradation of PhACs in soils has been reported for naproxen [38, 74, 118]; ibuprofen [38, 114, 119, 120]; diclofenac [74, 114, 121–123]; paracetamol [39]; carbamazepine [62]; antibiotics such as sulfamethazine [109] and sulfadiazine [124]; triclosan [51, 125–133]; antifungals such as fluconazole, clotrimazole, and miconazole [25, 131, 134–136]; and caffeine [113].

3 Impact of PhACs on in Soil Living Microorganisms

Residues of human and veterinary PhACs enter terrestrial environments as complex liquid or solid biomixtures applied to crop as organic fertilizer or for watering. Like other active ingredients used for plant protection (pesticides), PhACs are relatively recalcitrant to biodegradation, active at rather low concentrations, and target key enzymes involved in essential biological functions that are widespread in the tree of life. During the last decades, the presence of pharmaceutical residues in the aquatic environment has raised special attention, and numerous studies have reported their effects on the aquatic living organisms and supported ecosystem services [137–140]. However, little is known regarding the effect of antibiotics and other PhACs on

soil ecosystem services supported by microbial guilds. Soil microorganisms play a pivotal role in multiple ecosystem services. They contribute to soil health, mediate in biogeochemical cycles, and regulate climate change among other processes. Thus, the exposure of soil microorganisms to PhACs can influence their functioning with direct consequences on soil ecosystems. On the one hand, PhACs such as antibiotics and antifungals can inhibit specific microbial guilds and supported functions and thereby compromise the survival and growth of certain microbial guilds. On the other hand, some microorganisms can either develop mechanisms of defense against toxic PhACs (development of antimicrobial resistance, for instance) or use them as nutrient source (biodegradation) for their growth leading to the emergence of specific bacteria. It is noteworthy that some of the PhACs, such as the antibiotics, are particularly of concern because, when they are released in the environment, they exert a selection pressure favorable to the development and dissemination of antimicrobial resistance that can impair human and animal health [141].

Here we report some studies regarding the characterization of the ecotoxicological effects of some PhACs on soil microbial communities. The compounds were selected based on their ubiquitous detection in different environmental matrices and relevance.

3.1 Non-steroidal Anti-inflammatory Drugs (NSAID): Naproxen, Ibuprofen, and Diclofenac

Non-steroidal anti-inflammatory drugs (NSAID) are medicines used to relieve pain, decrease fever, and reduce inflammation. These compounds inhibit the cyclooxygenase (COX) enzyme, required to convert arachidonic acid into thromboxanes, prostaglandins, and prostacyclins, preventing the platelet adhesion, vasodilation, and increasing body temperature [142]. Among the different types, naproxen, ibuprofen, and diclofenac are the most frequently detected NSAIDs in wastewater effluents [143–148].

3.1.1 Naproxen

Naproxen is an acidic compound frequently found in wastewater effluents and receiving waters [143, 147, 149, 150]. It was found to be rapidly biodegraded in liquid microcosms containing either natural microbial communities from river water [151, 152] or bacteria, fungi, and algae [90, 91, 153–157]. To date, only three studies have addressed the dissipation of naproxen on agricultural soils, and little information is available regarding its ecotoxicological effects on microorganisms [158]. On soil microcosms carried out with three different agricultural soils (sandy loam, loam and silt) never exposed to this NSAID, Topp et al. showed a rapid mineralization of naproxen after application of liquid municipal biosolids [118]. Naproxen was also

shown to be degraded in two soils collected from arid regions under aerobic conditions while it was more persistent under anaerobic conditions, suggesting that in terrestrial ecosystems its biodegradation is catalyzed by microorganisms under aerobic conditions. The differences in naproxen half-lives were attributed to specific soil types and microbial characteristics [38]. Studies from Grossberger et al. [74] on agricultural soils irrigated with reclaimed water showed a rapid dissipation of naproxen. Kinetics of dissipation were not enhanced in soils previously exposed to this NSAID, suggesting that in this experiment the naproxen was co-metabolically degraded.

Based on these studies, naproxen seems to be rapidly dissipated in soils where under aerobic conditions it does not remain for long period of time. However, as recurrent contaminant of reclaimed water that is repetitively applied in large volumes to irrigate various crops, it may persist long enough to impact in soil living microorganisms. Indeed, naproxen was found to irreversibly inhibit nitrite production in the ammonia oxidizing bacterium *Nitrosomonas europeae* following the loss of its membrane integrity, which can potentially compromise nitrogen removal in wastewater treatment plants [159]. Naproxen was also shown to change the abundance and the enzymatic activities of soil microorganisms inducing disturbances in soil functions [160].

3.1.2 Ibuprofen

Ibuprofen is a nonprescription drug widely used for the treatment of pain, fever, and rheumatic disorders. Ibuprofen is a chiral compound that contains two enantiomers, the S-enantiomer (pharmacologically active) and the R-enantiomer (inactive) [161–163]. During human metabolisms, R-ibuprofen undergoes chiral inversion, resulting in S-ibuprofen, which is excreted in urine [164, 165]. This pharmacokinetics transformation to S-enantiomer is consistent with the observation of a selective enrichment of S-ibuprofen not only in wastewater influents [166, 167] and effluents [168] but also in surface water [166, 169]. R-enantiomer biodegradation was reported in aquatic systems [169, 170]. However, the depletion of S-enantiomer was shown in wastewater effluents [167] and lake water microcosm spiked with ibuprofen [166] suggesting that ibuprofen enantiomerization may also happen after its release in in the environment.

The ability of both microbial communities [90] and pure microbial strain to degrade ibuprofen has been widely reported [171]. The bacterium *Nocardia.* sp. transforms ibuprofen to ibuprofenol and subsequently to the corresponding acetate derivative [172]. *Sphingomonas* sp. uses ibuprofen as a sole carbon and energy source via deoxygenation of the ring followed by meta-cleavage and catechol formation catalyzed by enzymes encoded by *ipfABDEF* genes [107, 108, 171]. *Bacillus thuringiensis* and *Serratia marcescens* degrade ibuprofen more efficiently in the presence of other carbons sources suggesting co-metabolic transformation [91, 92, 95]. Ibuprofen was also found to be degraded by white-rot fungi [153, 173] that yielded a number of transformation products more toxic than the parent compound.

Ibuprofen degradation was negligible in anaerobic and sterile soil [174] and water-saturated soil [119], further indicating that it is degraded by microorganisms and principally under aerobic conditions.

Ibuprofen has been found in different terrestrial ecosystems [175, 176] at different concentrations ranging between 0.2 and 610 μg/kg. In soils ibuprofen is rapidly degraded under aerobic conditions with half-lives values between 30 to 34.3 days, 10 to 15 days, and 1 to 6 days, respectively [38, 114, 119]. Similar maximum mineralizable amounts of ibuprofen were shown in both aqueous and soil microcosms but with about 3.5 times lower mineralization rate in soil systems [120].

To our best knowledge, the effect of ibuprofen on microorganisms has only been studied in liquid cultures and aquatic populations, and not yet on soil microorganisms. Ibuprofen has antifungal activity against dermatophytes [177] and inhibits the growth of some Gram-positive species [178, 179]. Ibuprofen caused the decrease in the biomass of riverine biofilms and inhibited the growth of *Cyanobacteria* and of alpha, beta-proteobacteria, cytophaga-flavobacteria, and SRB385 populations [180]. Additionally, ibuprofen was also shown to significantly modify the growth of the microbial community of a river sediment incubated at different temperatures and light exposure [181]. Pollution-induced community tolerance (PICT) analysis performed on fluvial biofilms exposed to wastewater effluents showed that at the highest concentrations of ibuprofen and diclofenac, they acquired a tolerance to these components accompanied by an alteration of the algal composition and metabolic profile of microbial organisms [182]. Recently, a mixture of ibuprofen, naproxen, and diclofenac was shown to change the composition of the microbial community (increase in *Actinobacteria* and *Bacteroidetes* and a decrease of *Micropruina* and *Nakamurella*) but not the total nitrogen removal in batch reactors [183]. Although the environmental risk assessment concluded that ibuprofen represents a risk for the aquatic environment [184], it was not included in the list of priority substances under the Water Frame Directive due to a lack of sufficient evidence for its environmental toxicity [185].

3.1.3 Diclofenac

Diclofenac, the most used NSAID in the world, is poorly removed in conventional sewage treatment plants [186–188]. Hence, diclofenac residues are frequently detected in the environment [53, 175, 189–192]. As a consequence, it is considered as a contaminant of emerging concern, and it was added to environmental quality standards (EQS) with a threshold value of 0.1 μg/L (European Community document (COM(2011)876)). More recently, diclofenac was included in the list of priority substances (PSs) of the Directive 2013/39/EU and Watch List of Decision 2015/495/EU [193–195].

Diclofenac is a polar pharmaceutical compound poorly adsorbed to soil components and therefore easily transferable to surrounding environmental compartments via leachates and runoff [38]. In agricultural soils, under aerobic conditions, diclofenac is readily biodegradable [74, 114, 121–123] within 10 days, whereas it

persists in sterile soils, indicating that soil microorganisms are responsible for its rapid dissipation. This was confirmed by the isolation and characterization of several fungal [156, 196–200] and bacterial strains able to degrade diclofenac as sole carbon source [87, 97, 201] or through cometabolism [87, 93, 94, 196, 202–205].

Ecotoxicity of diclofenac on Gram-positive [206, 207] and Gram-negative bacteria [208, 209] was reported because of the inhibition of DNA synthesis [210] or of the impairment of membrane activity [211, 212]. To date, only two studies have assessed the effects of diclofenac on soil microorganisms [123, 160]. Experiments performed by Cycon et al. [160] with different endpoints including substrate-induced respiration, soil enzyme activities, and enumeration of culturable bacteria and fungi showed that diclofenac exposure led to an increase in the number of culturable bacteria and fungi. At the highest dose (10 mg/kg), diclofenac increased soil respiration as well as the activity of some soil enzymes (acid and alkaline phosphatase, urease). On the contrary, it inhibited the activity of soil dehydrogenases, while it does not affect enzymatic activities (nitrification and ammonification) of N cycle. Experiments performed by Thelusmond et al. [213] by means of Illumina sequencing, STAMP and PiCRUST in agricultural soils observed an increase in *Proteobacteria*, *Gemmatimonadetes*, and *Actinobacteria* and identified four metabolic pathways positively impacted (propanoate, lysine, fatty acid, and benzoate metabolism) during diclofenac biodegradation.

3.2 Other Analgesics and Antipyretics: Paracetamol or Acetaminophen

Paracetamol or acetaminophen is one of the most widely used over-the-counter analgesic and antipyretic drug. The mechanism of action is complex and includes the inhibition of the cyclooxygenase isozyme COX-3 involved in the synthesis of prostaglandins and the activation of metabolites influencing cannabinoid receptors [214, 215]. As result of its popular use, paracetamol has been frequently found in wastewater treatment plants and in various environmental matrices all over the world [147, 175, 216–227].

Paracetamol is transformed by both fungal [228, 229] and bacterial cultures [96, 98, 99, 111, 230, 231]. In bacteria, two different biodegradation pathways via hydroquinone [101, 111] or pyrocatechol [232] have been characterized [233]. To date, only one study has addressed the fate of paracetamol in soil [39] showing that 17% of initial dose applied was mineralized in 120 days, while 73.4–93.3% was recovered as non-extractable residues. Additionally, eight different transformation products were identified, and new biodegradation pathways for paracetamol degradation in soil were proposed. In this study, paracetamol dissipation was mainly explained by the rapid formation of bound residues preventing the dispersion of paracetamol by leaching and/or runoff but accumulating in soil where it may represent a risk for in soil living organisms.

Although numerous studies have shown toxic effects of paracetamol on aquatic organisms [234–236], little information is available regarding its ecotoxicity toward microorganisms. Paracetamol has antibacterial properties on isolated Gram-positive strains [179]. In combination with doxycycline, it was found to inhibit the activity of nitrifying, denitrifying, and anaerobic ammonium oxidation (anammox) bacteria involved in N cycle from different batch reactors [237]. The microbial toxicity of paracetamol was assessed using the MARA (microbial assay for risk assessment), the Microtox, and the Ames microplate assay [96]. Gram-negative bacilli and Serratia were the most sensitive bacteria, while the most resistant were *Enterococcus* and yeast *Pichia anomala*. According to MARA performed with 11 different strains, the mean value of microbial toxic concentration (MTC equivalent of EC50) was 3,435.00 ± 129.90 mg/L, and the EC50 estimated values using Microtox with *Aliivibrio fischeri* were 7,923 mg/L and 9,487 mg/L after 5 and 15 min of paracetamol exposure, respectively. Ames assay concluded that paracetamol was non-mutagenic, according to the EPA standards [96].

3.3 Antidepressants: Fluoxetine (Prozac) and Citalopram Hydrobromide (Celexa)

Antidepressants are medications that can help ease symptoms of depression, anxiety, and affective disorders. Among them, selective serotonin reuptake inhibitors (SSRI) are the most commonly prescribed. They increase the levels of serotonin in the brain and block the reabsorption of serotonin into neurons. Examples of SSRI antidepressants are citalopram and fluoxetine, commonly marketed with diverse trade names such as Prozac and Celexa, respectively.

Citalopram is a chiral compound sold as a racemic mixture, but only the S-enantiomer (sold as Escitalopram) has the desired antidepressant effect. Similarly, fluoxetine is commercialized as a racemic mixture, with the S-enantiomer approximately 1.5 more potent than the R-enantiomer. In the human body, fluoxetine is metabolized to norfluoxetine. Several studies have found citalopram, fluoxetine, and its major metabolite norfluoxetine in different environmental matrices [222, 238–242]. Under laboratory conditions, citalopram and fluoxetine are relatively recalcitrant to hydrolysis, photolysis, and microbial degradation [243, 244]. Nonetheless, the biodegradation of fluoxetine by a single bacterium (preferably the R-enantiomer) [105] or microbial consortium has been reported [245, 246]. Fluoxetine biodegradation applied at 1 μg/L was reported in estuarine and coastal seawaters with half-lives ranging from 6 to 10 days [247]. Similarly, in activated sludge the biodegradation of citalopram was reported with 60% and 40% elimination rates under aerobic and anoxic conditions, respectively [248, 249]. In activated sludge [250], similar elimination rates (70%) of citalopram were observed under aerobic conditions, and this biotic transformation led to the formation of 14 different transformation products.

The ecotoxicity of fluoxetine and citalopram on aquatic organisms has been widely documented [251–253]. They affect the behavior, reproduction, development, and survival of aquatic invertebrates and vertebrates [254, 255]. On microbes, psychotropic drugs such as fluoxetine have been found to inhibit microbial activity [256]. In this regard, fluoxetine has significant antibacterial effect and potential antibiotic modulating activity against multiresistant bacteria [257]. Fluoxetine reduced the richness and increased the beta diversity of gut microbiota [258].

3.4 Antiepileptics: Carbamazepine

Carbamazepine is a relatively lipophilic antiepileptic drug used to control and prevent seizures [259, 260]. Due to its scarce removal in wastewater treatment plants [186, 188, 261–263], carbamazepine is frequently found in municipal effluents [63, 188, 260]. For this reason, it has been proposed as an anthropogenic marker of sewage contamination in aquatic environments [264–266]. Carbamazepine is also frequently detected in arable soils irrigated with wastewater, amended with biosolids or in soils where reclaimed water is used to recharge groundwater [239, 240, 267].

In soils carbamazepine was barely degraded (1.2% of mineralization after 120 days of incubation) and transformed to a range of transformation products not adsorbed to soil components (4.2% recoveries as non-extractable residues of initially applied carbamazepine) [62]. The persistence and accumulation of carbamazepine in soils have been reported by many authors [123, 268]. However, some fungi [153, 269–273], bacteria [102, 274, 275], or the combination of both [276] is able to degrade carbamazepine [277]. In this context, a recent study performed in four agricultural soils identified by means of shotgun sequencing the most abundant phytolypes (*Rhodococcus*, *Streptomyces*, and *Pseudomonas*) and associated functional genes [130]. The uptake and metabolism of carbamazepine by endophytic bacteria were studied by Sauvêtre et al. who reported a number of degrading endophytic isolates and identified several degradation products [278, 279].

The ecotoxicological effect of carbamazepine was studied on riverine biofilm communities where it was found to reduce the bacterial biomass and the abundance of gamma-proteobacteria, suppress the *Cyanobacteria*, and increase in algal biomass and abundance of beta-proteobacteria [180]. In soils, the ecotoxicological effects of carbamazepine on soil microorganism have been recently reported indicating an enrichment of *Sphingomonadaceae*, *Xanthomonadaceae*, and *Rhodobacteraceae* [213] and an increase in *Proteobacteria* and *Verrucomicrobia* possibly due to the emergence of carbamazepine degraders [123, 213]. In addition, the abundance of *Flavobacterium*, three genus incertae sedis and *Bacteroidetes* decreased [213] revealing the toxicity of carbamazepine toward these microorganisms.

It is noteworthy that carbamazepine applied at environmental concentrations can induce horizontal transfer of plasmids carrying antibiotic resistance among the bacteria community [280]. Given the co-occurrence of PhACs in environments,

these findings pointed out the potential threat of carbamazepine in the environmental spread of antimicrobial resistance.

3.5 Antibiotics

Antibiotics are natural or synthetic substances that kill (bactericidal) or inhibit the growth (bacteriostatic) of bacteria [281]. They are commonly used in human and veterinary medicines [282] as well as in agriculture [283–285] and aquaculture [286, 287] to prevent or treat infections, as growth promoters [288, 289] and sometimes as food preservatives [290]. There are about 250 different antibiotics which can be classified on the basis of their mechanisms in four different groups [281] such as those that inhibit the:

- Synthesis of the cell wall (beta-lactam and glycopeptides)
- Biosynthesis of proteins (aminoglycosides, tetracyclines, chloramphenicol, macrolides, oxazolidinones)
- DNA replication (quinolones)
- Metabolism of folic acid (sulfonamides and trimethoprim)

As a result of their extensive use and their recalcitrance to degradation, antibiotics are frequently found in various matrices such as wastewater [291–297], biosolids [240, 298–301], sewage sludge [302–308], and farmyard manure [309–320]. Applications of these matrices to arable soils to water crop or as organic amendment can lead to the dispersion of antibiotic residues in both terrestrial and aquatic ecosystems [321, 322]. Indeed, antibiotics can runoff or leach from the soil polluting surface water and groundwater, respectively [25, 323, 324]. The ubiquitous detection of antibiotic residues in environmental matrices is cause for a great concern since even at rather low concentration they exert a selection pressure favorable to the emergence and further dispersion of antimicrobial resistances among environmental microbial communities [325–330].

In addition, antibiotic residues may also inhibit specific microbial guilds or functions and therefore disrupt critical processes for ecosystem functioning. Indeed, they have been shown to affect degrading microorganisms, thereby impairing the removal of organic matter and chemicals in sewage treatment plants [331–334]. In addition, antibiotic residues contaminating wastewater or biosolids/manure that are applied on arable soils can inhibit microbial populations involved in carbon and nitrogen geochemical cycling [335, 336], climate regulation [337], and degradation of xenobiotics and therefore may alter soil fertility and ecosystem health [338–343] .

In soils, antibiotics are subjected to microbial transformation with variable degrading rates depending on their molecular structure and physicochemical properties [48, 344]. Amoxicillin (beta-lactam) and chlortetracycline are easily degradable [345, 346], while ciprofloxacine, norfloxacine (fluoroquinolones), azithromycin (macrolides), and doxycycline (tetracyclines) are more recalcitrant to biodegradation remaining for a long period of time in soils [131]. Interestingly, in several studies

performed on a long-term field experiment where various antibiotics were repeatedly applied, evidenced for enhanced dissipation of an impressive range of antibiotics (sulfamethazine, tylosin, chlortetracyclin, erythromycin, clarithromycin, and azithromycin) in exposed field plots as compared to control field plots [109, 117]. The number of studies reporting the degradation of different antibiotics in soils is important [124, 340]. Differences observed between studies for a given antibiotic are most likely due to variations in soil type, antibiotic concentrations, and environmental conditions.

Numerous bacterial strains able to degrade antibiotics have been isolated from various matrices including patient, animal, sediment, sludge, manure, and soil. For soils it includes strains belonging to the genera *Microbacterium* sp. (sulfamethazine, sulfadiazine, and sulfamethoxazole) [109, 347, 348], *Bacillus* sp. (penicillin) [110], *Escherichia* sp. (sulfonamides including sulfamethazine and sulfamethoxazole) [349], *Stenotrophomonas* sp., (tetracycline) [350], *Ochrobactrum* sp. (sulfamethoxazole and erythromycin) [351, 352], *Labrys* sp. (fluoroquinolones and sulfamethoxazole) [88, 351], and *Gordonia* sp. (sulfamethoxazole) [351]; the orders *Burkholderiales*, *Caulobacterales*, *Xanthomonadales*, *Pseudomonadales*, *Enterobacteriales*, and *Rhizobiales*; and the phyla *Bacteroidetes* (penicillin and neomycin) [112]. In this regard, bioaugmentation of sulfonamide-spiked soil microcosms with *Microbacterium* sp.C448 [109] was shown to reduce the persistence of antibiotic residues in soils and all associated side effects [353, 354].

3.6 Antiseptics and Disinfectants

Antiseptics and disinfectants, sometimes called biocides, are chemicals commonly used in a variety of medical and domestic settings to prevent or kill the growth of microorganisms. In general, biocides are less specific than antibiotics as their action mode has a broad spectrum of activity, generally not fully understood [355]. Among widely used biocides, triclosan has raised special concern due to its weak demonstrated benefit [356] and potential toxic effects on human health [357, 358]. At low concentrations, triclosan is a bacteriostatic, while at high concentrations, it is bactericidal agent effective against many types of Gram-positive and negative non-sporulating bacteria, some fungi, and certain parasites [359–363]. Although the use of triclosan was restricted in certain types of products [364–366], it is still found in many care products such as toothpaste, mouthwash, hand sanitizer, and surgical soaps. Due to its widespread use and incomplete removal from wastewater treatment plants [367–369], triclosan is frequently detected in several environmental matrices such as soil and surface waters [222, 370–373]. Triclosan was found to bioaccumulate in aquatic species, algae, snails, and earthworms [71, 373–375] in which it caused toxic effects [376–383]. Similarly, plants such as pumpkin, zucchini, onion, and tomato have been shown to bioaccumulate triclosan in the edible parts, thereby leading to the contamination of the food chain [384–386].

Although triclosan is an antimicrobial agent, some fungi [387, 388] and bacteria are able to degrade it co-metabolically or metabolically using it as sole carbon source for their growth [89, 100, 104, 106, 389–393]. In addition, repeated exposure to sublethal concentrations of triclosan may result in the development of resistant colonies [394, 395]. The mechanisms of triclosan microbial resistance share some similarities with those involved in antibiotic resistance [396, 397]. Several studies have demonstrated the development of cross-resistance between triclosan and antibiotics [398–400]. Therefore, triclosan like other biocides is suspected to take part to the selection pressure favorable to the emergence, spread, and maintenance of antibiotic resistances among environmental microbial communities [395, 401–404].

In soils, triclosan was reported to degrade to variable extent, with various half-lives depending on soil properties and conditions of incubation [51, 115, 125–132]. Regarding its ecotoxicological impact on soil microorganisms, triclosan was found to transiently inhibit microbial respiration, reduce microbial biomass [126], and sulfatase activity [405]. These effects were positively related to the dose of triclosan applied to the soil and inversely correlated with soil organic matter and clay content, suggesting that soil characteristics control its bioavailability and induced toxicity. Triclosan was also found to reduce the relative abundance of both Gram-positive and negative bacteria and fungi [406]. Recently, studies performed in four agricultural soils using shotgun sequencing observed an increase in *Pseudomonas*, *Sphingomonas*, *Methylobacillus*, and *Stenotrophomonas* and identified the most abundant functional genes associated with triclosan biodegradation [130].

3.7 Antifungals

Antifungals comprise a large and diverse group of drugs used to treat fungal diseases in humans, animals and plants. Based on their action mode, antifungals can be divided in three different classes: azoles, which inhibit the synthesis of ergosterol; polyenes, which physicochemically interact with fungal membrane sterols; and 5-fluorocytosine, which inhibits macromolecular synthesis [407]. Among the different azoles, of particular interest is the case of the triazoles, which constitute a synthetic group of heterocyclic compounds containing a five-membered ring of two carbon atoms and three nitrogen atoms commonly used for the control of fungal diseases in humans, animals, and plants. They include drugs such as fluconazole, clotrimazole, and miconazole and plant protection products such as tebuconazole and epoxiconazole. By inhibiting the activity of lanosterol 14α-demethylase (DMI), a member of the cytochrome P450 catalytic activity, triazoles alter the bioconversion of lanosterol to ergosterol, a fundamental component of the fungal cytoplasmic membrane, preventing fungal growth [407, 408]. Therefore, triazoles are fungistatic and not fungicidal, but although misleading, the term fungicide is commonly used in agriculture for this type of pesticide.

Due to their efficacy and broad spectrum of activity, triazoles are among the most common systemic fungicides used in the control of plant diseases [409]. Contrary to

other available antimycotics, they are applied not only to prevent but also to treat plant fungal diseases. Triazoles have also been shown to promote the growth of plant leading to increase in the crop yield [410, 411].

In the medical field, synthetic antifungal agents are widely used for the treatment and prophylaxis of many mycoses [412]. As a consequence of their common use, substantial amounts of azoles reach the wastewater treatment plants [413–416]. There, as observed for many other PhACs, due to their intrinsic stability, triazoles can remain stable and active with only slight changes in their chemical structure. Studies investigating the occurrence of azole fungicides in wastewater are limited [413, 414, 417–419]. However, a number of studies have identified wastewater effluents as triazole pollution point source of surface waters and agricultural soil [134, 420–425].

The dissipation of triazole plant protection fungicides in soils has been widely documented. Pesticides such as tebuconazole [426–433], epoxiconazole [434, 435], propiconazole [436–438] and cyproconazole [439] have been shown to be relatively persistent in soil. In soil tebuconazole was shown to be transformed in 34 different transformation products [440]. To date *Burkholderia* sp. and *Pseudomonas aeruginosa* are the only two soil bacterial isolates known to degrade the fungicide propiconazole [441, 442].

Similarly, antifungal medicines are highly resistant to microbial degradation. Experiments performed in soil microcosms showed that fluconazole and clotrimazole were scarcely degraded, with half-lives in the range of 73 to 85 days for fluconazole and of 29 to 126 days or of 36.2 to 130.8 days for clotrimazole [135, 136]. In field conditions, a higher persistence was found in biosolid amended soils for the azole biocides climbazole, clotrimazole, and miconazole [25, 131, 134], with differences in dissipation half-lives attributed to soil types and biosolid application rates. To date, only one study has reported the ability of one edible fungal specie to degrade bifonazole and clotrimazole [443].

As observed with antibiotics, the intensive and repeated use of triazoles has led to the emergence of fungal resistances. Among the different mechanisms of resistance involved, the overexpression of the CYP51 gene that codes for the lanosterol 14α-demethylase, due to mutations (insertions or duplications) in the promoter region, and the increase in molecular efflux by ABC (ATP-binding cassette) transporters caused by the overexpression of genes coding for membrane transport have been mainly observed [407, 444–446]. Clinical isolates with observed resistance to triazoles include the species of *Aspergillus*, *Candida*, *Fusarium*, *Zygomycetes*, *Trichosporon*, *Penicillium*, *Bipolaris*, and *Scedosporium*, among others [447–452]. The majority of cases of azole-resistant diseases are due to resistant *Aspergillus fumigatus* which causes a variety of diseases in humans and animals ranging from allergic, chronic, and acute invasive diseases, the latter posing a significant threat to immunocompromised patients [453]. The surge of resistant fungi of human pathogens in the medical field has been related to the exposure to fungicides used in agroecosystems [454–456]. The important use of triazoles in agriculture may indeed exert a selective pressure favoring the survival of certain human pathogenic fungi, increasing the risks and chances for humans to encounter such resisting microbes.

Pathogenic fungi that have their natural habitat in the environment are the fungi *Coccidioides*, *Histoplasma*, *Aspergillus*, *Colletotrichum*, and *Cryptococcus* [457–461].

While a number of studies have evaluated the ecotoxicological impact of triazole fungicides [462] (propiconazole [463, 464], tetraconazole [465], tebuconazole [429, 466–473]) on soil microorganisms, the effects of antifungal medicines on soil microorganisms have been scarcely documented [474]. Climbazole, an antidandruff and antimycotic agent, was shown to be toxic to algae, aquatic lentils (Lemna), and terrestrial plants and exhibited low toxicity toward the soil bacterium *Arthrobacter globiformis* with an EC_{50} of 456 mg/kg soil for inhibition of dehydrogenase activity [474].

4 Perspectives

Although PhACs are found as contaminants in almost all environmental matrices, including soils, their environmental fate and ecotoxicological impact on in soil living organisms and supported ecosystem services remain poorly described and scarcely understood. This evident lack of information is most likely due to the absence of regulatory requirements to monitor soil quality in the absence of a soil protection directive that was proposed almost 20 years ago to the European Commission, but that is still not adopted [475]. In addition, the current regulation to release on the market PhACs does not consider enough their possible effect on the environmental compartment, in particular on soil.

Most of the studies are laboratory experiments that consider contaminant one by one spiked at high concentration in microcosms. Only a few of them are done at field or environmental scale with complex mixture of contaminants but with the problem of the reference (normal operating range) to interpret the variations observed. Although it is the rule at the environmental scale, no studies consider the effect of complex mixtures of PhACs to soil [476]. Until now, there are no consensus to assess the fate and the ecotoxicological effects of PhACs on soil microorganisms and supported ecosystem services.

Given the fact that human and animal health are unambiguously link to environmental health under the concept of "One health," it could be concluded that there is an urgent need to unify current regulations on the release on the market of PhACs, biocides, and plant protection products in close connection with the regulations to protect the environment such as the water framework directive, air quality framework directive, and national directives on soil protection (pending the publication of the soil protection directive). This unification has to be done under a holistic policy embracing both a priori and a posteriori environmental risk evaluation assessment by targeting specific protection goals, including microbial communities that support soil ecosystem services.

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Biomarkers in Earthworms

Montserrat Solé

Contents

Abstract Soil-dwelling naturally occurring earthworms (e.g. *Lumbricus terrestris*) are valuable sentinels in soil pollution monitoring for their ecological role but also because they have shown to be sensitive to environmental contaminants. However, most laboratory studies have adopted epigeic earthworms as models (*Eisenia* spp.) in acute toxicity testing. In soil chronic toxicity assessment, it is essential to include sublethal responses that can have direct implications on species performance, reproduction and behaviour and thus be of ecological significance. In this sense, some biochemical biomarkers are regarded as early warning signals of further ecological consequences. Amongst those most frequently considered are specific responses to certain chemicals (e.g. metallothionein induction to metal exposure) but also those related to oxidative homeostasis of the organisms because prolonged stress may lead to adverse effects at the individual level (disruption of immune system, altered growth and reproduction). Biomarker measures can be applied in specific tissues, but, for methodological constraints, the consideration of the whole animal simplifies protocols and, once validated, they are informative and integrative. The use of

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non-destructive tissues (e.g. coelomocytes) that do not require sacrifice, the incorporation of "omic" disciplines and recent technical advances in metabolite identification are all encouraged to be incorporated into toxicity evaluation.

Keywords Biochemical biomarkers, Endogeic earthworms, Soil toxicity assessment

1 Introduction

This chapter focusses on the use of earthworms from different ecological categories, epigeic, endogeic and anecic (see Chap. 11), as sentinels in toxicity monitoring in relation to soil assessment [1]. It will address the works published after some comprehensive reviews [2] on the use of biomarkers in earthworms or particular review issues addressing pesticide pollution [3, 4]. The use of earthworms as sentinels and sublethal biomarkers in soil pollution assessment is well recognised in terrestrial ecotoxicology, mostly in relation to metal and pesticide pollution [5–8]. This chapter will focus on more recent work (from 2015 onwards) dealing with the use of biomarkers for assessing the toxicity associated with legacy chemicals (e.g. heavy metals, pesticides) and will also include emerging contaminants or contaminants of increasing environmental concern such as nanomaterials, pharmaceutical drugs, plastics, plastic additives and e-waste-related products as well as environmentally realistic mixtures of chemicals. Another chapter in this book by Sanchez-Hernandez (Chap. 11) addresses the role of earthworms as vermiremediators of chemical pollution in soils, and thus, this aspect will not be considered here. Only if the vermiremediation study includes earthworm biomarkers will it be mentioned in this chapter. In Fig. 1, a scheme of the topics described throughout the text is presented.

The first ecotoxicology tests adopted by the international Organisation for Economic Co-operation and Development (OECD) directives [9] were based on mortality and aimed at obtaining LC50 values for toxic chemicals. Subsequently, effects on sublethal responses, such as reproductive performance, were included [10]. More recently, the incorporation of enzymatic, molecular (genetic and metabolomics) and even behaviour biomarkers in the ecotoxicity tests has gained relevance as indicators of sublethal toxicity. The importance of using infra-individual responses (e.g. enzymatic activities and molecular alterations) rests mostly on being quick signs of potential toxic events and on the possibility of extrapolating to further individually and ecologically relevant consequences [11]. In relation to the selection of adequate bioindicator species, the epigeic *Eisenia fetida* and *Eisenia andrei* are those adopted in soil toxicity tests following OECD protocols, and therefore a larger body of research is conducted with them. However, this chapter will purposely give support to the studies conducted with the naturally occurring species in agricultural

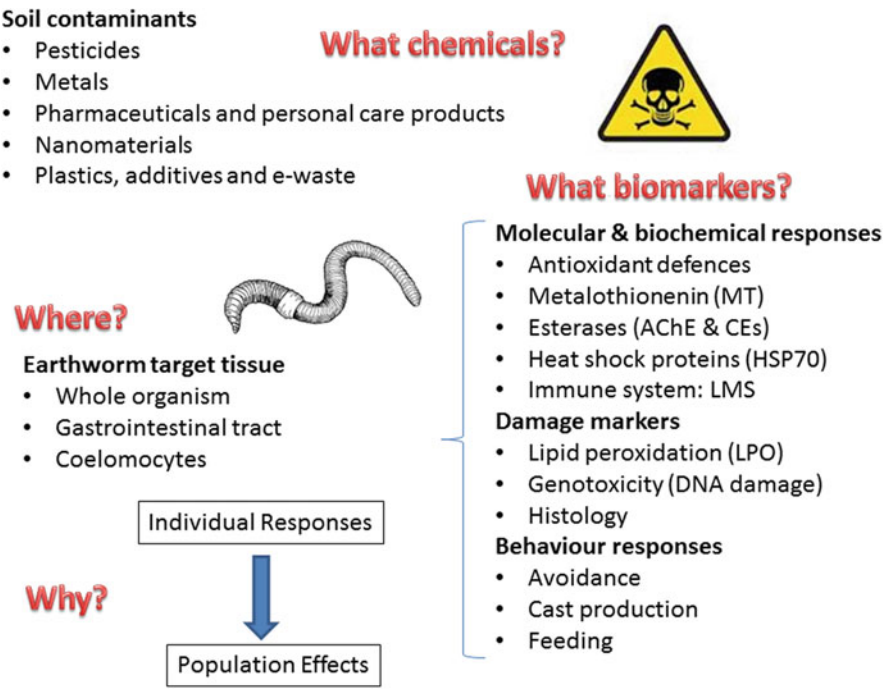

Fig. 1 Scheme on the chemicals, tissues and biomarkers more frequently used in soil toxicity assessment using earthworms

soils. Amongst these, soil-dwelling and deep-burrowing worms, such as the anecic *Lumbricus terrestris* and endogeic *Aporrectodea caliginosa* and *Allolobophora chlorotica*, are of relevance for their beneficial contribution to improving soil physico-chemical properties in agroecosystems. Nonetheless, when assessing emerging contaminant exposures, most literature available so far is almost exclusively centred on *Eisenia* spp.

In the review by Pelosi et al. [3], in search of the most sensitive earthworm species for pesticide homologation tests in Europe, the authors resolved that *L. terrestris* and *A. caliginosa* were amongst the most sensitive species. Moreover, since they contribute a high percentage to soil organism's biomass and are ecologically important in terrestrial ecosystems of many temperate regions, including cultivated soils, they are more suitable sentinels of soil pollution. In this chapter, considerations extracted from the review by Velki and Ecimovik [11] on the use of earthworms on ecotoxicology testing in terrestrial systems have been embraced, for instance, (1) the importance of using biomarkers that can be derived to higher hierarchy consequences, (2) the need to assess toxicity of naturally occurring chemical mixtures, (3) the use of microcosm models as a proxy of more realistic field conditions and (4) the need to consider additional environmental stressing factors such as those related to forecast climate change. Other considerations such

as implementing equally informative conservative techniques that do not require sacrifice or preliminary screening in vitro tools are encouraged. A detailed description on the most commonly used biomarkers in earthworm studies addressing soil pollution monitoring is given in a former and fairly recent book chapter [12]. However, in this issue, the most recent genomic and metabolomics applications in earthworm studies as promising biomarker molecular tools will be included.

The biomarkers considered here will refer mostly to sub-individual responses adopted in earthworm studies for the last 5 years on chemical toxicity assessment in laboratory exposures either using the filter paper contact tests or using spiked artificial or natural soils or even those conducted under realistic field conditions (mostly at a mesoscale). These studies embrace biomarkers informing on changes on enzyme activities and/or gene expression and endogenous metabolite composition, but also on the occurrence of damage to biomolecules such as DNA, proteins and lipids and alterations in lysosomal membrane stability, in immunology defences and in histological features caused by a range of anthropogenic chemicals acting either alone or in combination. A comprehensive table gathering many of these studies was already presented in the Pelosi et al. [4] review mostly in relation to pesticide exposures. Here a selection of more recent work (>2014) using earthworm biomarkers in relation to metal and pesticide exposures in agricultural soil relevant species will be considered, and emphasis will be placed on the toxicity studies addressing chemicals of more recent environmental concern known as emerging contaminants. As anticipated, studies screening for the toxicity associated with novel contaminants have mostly been based on the model earthworms (*Eisenia* spp.) adopted in soil ecotoxicology testing. Even though a larger body of literature correspond to this group, it is important to highlight the fact that a few research studies include more ecologically relevant species such as *L. terrestris*.

2 Ecotoxicological Biomarkers: An Overview

The biomarkers most frequently applied in earthworm studies are either those responsive to a particular type of chemical or those informative of a general stress status. For instance, metallothionein content specifically responds to metal exposures, but it has also been seen to be affected by other chemical stressors and given a protection role in invertebrates. Cholinesterases and in particular acetylcholinesterase (AChE) and carboxylesterase (CE) activities specifically respond to pesticides. While inhibition of AChE activity is considered a sign of neurotoxicity, the inhibition of CEs by pesticides (through stoichiometric binding) is considered a protective mechanism towards preventing neurotoxicity (AChE inhibition) under pesticide exposures. However, CEs also play a key role in endogenous as well as xenobiotic metabolism (phase I hydrolysis); thus, their modulation could compromise physiological and detoxication processes [13]. Other phase I oxidoreduction reactions such as those involving cytochrome P450 and its associated enzymatic activities (e.g. ethoxyresorufin-O-deethylase (EROD)) are present but less represented and

considered in earthworm studies. Markers indicative of an oxidative stress condition, potentially enhanced as a consequence of xenobiotic P450 metabolism, include the production of reactive oxygen species (ROS), the total antioxidant capacity (TAC) as well as the responses of antioxidant defences such as the activity of the so-called antioxidant enzymes such as catalase (CAT; transform H_2O_2 in H_2O and O_2), superoxide dismutase (SOD) involved in scavenging superoxide ($O^{2-\cdot}$) radicals, glutathione peroxidases (GPX) and guaiacol peroxidase (POD) involved in the reduction of a broad range of organic peroxides to the corresponding alcohols. Other enzymes with a dual role (antioxidant and phase II conjugation reaction) are the activities of glutathione *S*-transferase (GST) mainly involved in detoxification reactions and glutathione reductase (GR) engaged in maintaining the levels of reduced glutathione (GSH) from its oxidised disulphide form (GSSG) with a relevant antioxidant role. Endogenous molecules such as GSH and other molecular scavengers such as β-carotene and vitamins C and E also have a described antioxidant role.

Constitutive stress proteins, and in particular the heat shock proteins (e.g. HSP70), are also inducible and involved in the protection and repair of these cell biomolecules' integrity under multiple stress conditions, including heat. When the cell's natural defence mechanisms are overwhelmed, oxidation of biological molecules such as lipids occurs. This is known as lipid peroxidation (LPO), and the analytical assay (thiobarbituric acid reactive species or TBARS) to measure LPO quantifies the malondialdehyde (MDA) levels. In the case that oxidised molecules are proteins, its oxidation is measured as protein carbonyl (PC) formation. If the genetic material is that affected, then DNA damage occurs and can be measured in several ways, by the electrophoretic comet assay or single gel electrophoresis (SGE), by the formation of the oxidised nucleoside 8-hydroxy-2-deoxyguanosine (8-OHdG) using ELISA techniques and by measuring DNA degradation in the late stages of apoptosis by the TUNEL assay, amongst others. In earthworms, this effect biomarker (DNA damage) is especially relevant since it can be easily measured in coelomocytes as a validated non-destructive technique. Another effect marker widely used to measure damage occurrence in biological membranes is the lysosomal membrane stability (LMS) and the neutral red retention (NRR) assay a frequent method of evaluation. Immunology assessment is considered to be a general stress marker and has the advantage of being easily measured in biological fluids such as coelomocytes and can thus also be classed as a conservative technique. Histological alterations are morphometric measures considered signs of unequivocal damage consequences to higher hierarchy levels of biological organisation. Although damage evaluation is the most common measure included in many ecotoxicity studies with earthworms, this chapter places more emphasis on early warning biomarker responses such as those experienced by biochemical and molecular parameters. Some reviews have comprehensively addressed each particular biomarker in earthworms: ecogenotoxicity [14], genotoxicity, immune system, histology and antioxidant protective defences were addressed by Roubalova et al. [15] and esterases by Sanchez-Hernandez [6]. Other novel biomarkers applied to

toxicogenomics and toxicotranscriptomics in earthworms will not be discussed here as they have been accurately addressed elsewhere [16].

In addition to traditional biochemical markers, gene expression and metabolomics are new molecular tools gaining importance in biomonitoring although genetic changes and endogenous metabolite alterations should be translated into protein and physiological consequences, respectively, to gain ecological relevance. In fact, to relate sub-individual molecular and biochemical modifications to higher ecological consequences is one of the main objectives on the use of biomarkers for toxicity assessment. Therefore, studies aimed at linking responses at different hierarchy biological levels are of the utmost relevance.

In earthworms, the organs/tissues selected for biomarker determinations mostly refer to whole tissue homogenates due to constraints on the size of those most frequently used as sentinels. Moreover, to consider the whole tissue is relevant as it informs on the integrative response of the entire organism. In the case of larger earthworms, such as *L. terrestris*, and for research purposes, the use of particular organs/tissues has been encouraged as it discriminates the most sensitive ones to particular exposures [7]. Nonetheless, for monitoring purposes which frequently require sufficient biological tissue to perform a comprehensive set of complementary measures, and in which the speed for processing large quantities of samples is important, the use of earthworm whole tissue homogenates greatly simplifies the protocol. In this sense, some studies have been centred on validating the whole tissue approach in respect of more time-consuming accurate dissection techniques that require more expertise. It is also worth stressing the interest in validating biomarkers measured in whole tissue using traditional destructive tools with these same markers measured using alternative conservative techniques in biological fluids (e.g. coelomocytes). Some studies in earthworms are designed to achieve this goal since, in addition to having an ethical value, they allow follow-up of exposures in the same individuals and thus, reduce biological variability and the number of individuals required for experimentation. In this sense, the use of the coelomic fluid and the well-developed nervous system of larger earthworms is the targeted non-destructive matrix and will be discussed further.

In the next section, earthworm studies conducted using mostly biochemical biomarkers since 2014 will be described in relation to specific types of pollutants as listed in Table 1. Only a few studies have addressed particular exposures to pharmaceuticals; thus, a consideration to other chemicals has been given. However, general biomarker responses in terms of immune responses, oxidative stress and neurotoxicity are expected due to exposure to pharmaceuticals as has been observed in other invertebrate groups. Given the promising evidences with the incorporation of biomarkers in the field of gene expression and metabolomics, some recent studies including this approach will also be considered. An additional section will address the importance of metabolism and metabolite identification in earthworms for two main reasons: (1) it evidences the pathway of xenobiotic metabolism taking place within the organism and (2) it identifies by-products with even greater potential toxicity after their metabolism (metabolites) and therefore of the utmost importance not only for the individual but also in terms of food chain transfer.

Table 1 Biomarker studies using earthworms as sentinels in the assessment of environmentally relevant soil contaminants

	Earthworm species	Type of exposure	Tested biomarkers	Ref.
Pesticides				
Several agriculture pesticides	*Lumbricus terrestris* (A)	Field study. Conventional vs organic orchard	CEs in 3 tissues of the gastrointestinal tract	[18]
Carbamate (Pirimor®) and OP (Lorsban®) insecticides	*Aporrectodea caliginosa* (En)	Spiked soil for 1, 2, 3, 4 and 7 days	AChE in whole tissue and nerve conduction velocity (NCV) assay in medial giant fibres	[19]
Ethyl-parathion	*Allolobophora chlorotica* (En) and *A. caliginosa* (En)	Up to 7-day soil spiked (0.1, 1 and 10 mg kg^{-1}) under lab exposure	AChE, CE activities, behaviour responses (burrowing, casting, feeding)	[20]
OP pesticides: dimethoate and pirimiphos-methyl	*E. andrei* (Ep)	Filter paper contact test	AChE, CE activities in whole tissue vs coelomocyte extracts	[21]
Commercial formulations of glyphosate (GLF), tembotrione (TBT) and nicosulfuron (NCS)	*Dendrobaena veneta* (Ep)	Three concentrations of each formulation using filter paper contact test. Measures at 7 and 28 days	AChE, CAT and GST activities and LPO (malondialdehyde (MDA)) levels	[22]
Bifenthrin (pyrethroid)	*E. fetida* (Ep)	Soil-spiked test. After 3, 7, 14, 21 and 28 days	ROS formation, guaiacol peroxidase (POD), SOD, CAT, GST and EROD activities	[23]
Roundup® Alphée (glyphosate-based herbicide)	*Alma millsoni* (Ep), *Eudrilus eugeniae* (Ep) and *Libyodrilus violaceus* (Ep)	Soil exposure after 1-, 2-, 4-, 6- and 8-week herbicide application	Activities of GST, MT, AChE, LDH, SOD and GPX. Levels of GSH and LPO	[24]
Tebuconazole (fungicide)	*E. fetida* (Ep)	Soil spiked at 0.5, 5 and 50 mg kg^{-1} for 7 days	Biochemical (SOD, CAT, GSH and LPO), mRNA (SOD, CAT, TCPP and ANN-related genes) and untargeted metabolomics (NMR)	[25]
Dinotefuran (neonicotinoid insecticide) and two metabolites: UF and DN	*E. fetida* (Ep)	Filter paper contact test for acute toxicity (mortality) and soil exposure (0.1, 0.5, 1 and 2 mg kg^{-1}) for 2, 7, 14 and 28 days	Enzyme determinations (SOD, CAT), LPO 8-OHdG levels, gene expression Hsp70 and annetocin (ANN)	[26]

(continued)

Table 1 (continued)

	Earthworm species	Type of exposure	Tested biomarkers	Ref.
Metals				
Mixed metal pollution (Cd, Cr, Pb and Hg) due to chronic industrial and agricultural activities	*Metaphire posthuma* (En)	Field study in 7 polluted sites	Immune response (coelomocyte aggregation), ROS formation and antioxidant defences (phenoloxidase, SOD, CAT and GST) in coelomocytes	[29]
Mixed metal exposures (As, Cd, Pb, Cr, Hg, Mn, Fe, Co, Ni, Cu, Zn) of soils	*A. caliginosa* (En)	Field study	GSH content, GST, GPX and GR activities	[30]
Metal-contaminated soil (Cd, Pb and Zn)	*A. caliginosa* (En)	21-day microcosm study with natural field soil from 31 sites	Metallothionein gene (*MT*), CAT and GST activities, and protein, lipid and glycogen reserves and the integrated biomarker index (IBR)	[31]
Cd and Pb nitrates	*Aporrectodea rosea* (En), *A. trapezoids* (En) and *E. fetida* (Ep)	Artificial polluted soil after 7, 14 and 28 days	Genotoxicity (TUNEL assay), LPO (MDA) levels and total antioxidant capacity (TAC)	[32]
Mixed exposures				
Metals and polyhalogenated compounds	*Dichogaster curgensis* (Ep)	Lab study considering 6 polluted sites and a ref. Measures at 1, 7 and 14 days	CAT, SOD, GR, GPX, GST activities, LPO levels, histology in whole tissue and in coelomocytes: LMS (NRR) and DNA damage (comet assay)	[33]
Industrial waste water (IWW)	*E. fetida* (Ep)	Lab exposure with soil contaminated with IWW	MT content in whole tissue and LMS (NRR) in coelomocytes	[34]
Treated waste water (TWW)	*E. andrei* (Ep)	Lab artificial soil exposure after irrigation with TWW (10%, 50% and 100%) after 7 and 14 days	Enzymatic activities of CAT, GST and AChE, LPO levels and gene expression of *cat*- and *gst*-related genes	[35]

(continued)

Table 1 (continued)

	Earthworm species	Type of exposure	Tested biomarkers	Ref.
Treated waste water (TWW)	*E. andrei* (Ep)	Lab exposure, after 7 and 14 days, with field soils contaminated for 1, 8 and 20 years and 2 clean sites	CAT, GST and AChE activities, LPO levels, genotoxicity (micronuclei test) and gene expression of *cat*- and *gst*-related genes	[36]
Fertilisers and agrochemicals: urea, phosphogypsum, paper mill sludge, monocrotophos, glyphosate	*Eudrilus eugeniae* (Ep)	24 h lab soil exposure at 3 concentrations each chemical	LPO levels, LDH, AChE, CAT activities, histology	[37]
Textile mill sludge (TMS) with cow dung	*E. eugeniae* (Ep) and *Perionyx excavatus* (Ep)	Mixed ratio of TMS and cow dung soil 60-day exposures. Metals (Cd, Cu, Cr, and Zn) in tissue	MT content and histology analysis	[38]
Pharmaceuticals and personal care products				
Valsartan	*L. terrestris* (A)	7-, 14- and 21-day lab soil exposure at 5 mg kg^{-1} w.w.	AChE, GST and CE activities	[41]
Triclocarban	*E. andrei* (Ep)	Paper contact test (0.016, 0.13, 0.16, 0.33 and 0.5 µg cm^{-2}) at 24, 48 and 72 h	CAT and GST activities	[42]
Triclosan and methyl-triclosan	*E. andrei* (Ep)	Soil exposure to 35.0 ± 2.5 ng g^{-1}) for 28 d	DNA damage (comet assay) in coelomocytes	[43]
Cosmetic sludge (CS) and foundry sands (FS)	*E. fetida* (Ep)	CS and FS at different percentage. 1 h (ex situ) and 7 and 14 days (in vivo)	DNA damage (as single cell electrophoresis): ex situ and in vivo and coelomic cell composition	[44]
Livestock sludge (metals, veterinary drugs and pesticides)	*Amynthas gracilis* (Ep-En)	Soil exposure to sludge for 1, 7 and 14 days	AChE, SOD activities whole body tissue and in coelomocytes, LMS (NRR) and DNA damage (comet assay)	[45]
Nanomaterials				
Multiwalled carbon nanotubes (MWCNTs)	*E. fetida* (Ep)	0.03 and 0.3 mg g^{-1} d.w. for 3, 7 and 14 days	LMS, granulocyte morphometric analysis, MT content and AChE activity	[46]

(continued)

Table 1 (continued)

Zinc oxide nanoparticles (ZnO-NP)	*E. fetida* (Ep)	10, 50, 250 mg kg^{-1} after 7, 14, 21 and 28 days (in vivo)	In vivo ROS formation, LPO levels and SOD activity and in vitro ROS formation and LDH in coelomocytes	[47]
ZnO-NP and chlorpyrifos (pesticide) independently and as co-exposure	*E. andrei* (Ep)	ZnO-NP (up to 1,000 mg kg^{-1}), CPF (0–320 mg kg^{-1}) and ZnO-NP/CPF at 3 different ratios for 28 days	CAT, GST and AChE activities and LPO levels	[48]
Several copper oxide (CuO)-engineered nanomaterial variants	*E. fetida* (Ep)	200 and 1,000 mg kg^{-1} d.w. soil spiked with Cu-NP coatings for 14 days	GSH content, SOD and Na+/K+-ATPase activities and histology	[49]
Nickel oxide nanoparticles (NiO-NP)	*E. fetida* (Ep)	5, 50, 200, 500 and 1,000 mg kg^{-1} soil after 28-day exposure	Activity of SOD, POD, CAT and LPO (MDA) levels, DNA damage (as 8-OHdg) and histology examination	[50]
Plastics, plasticisers and e-waste				
Polyester-derived microfibres	*L. terrestris* (A)	0.1 and 1.0%w/w MF for 35 days in soil	Gene expression related to metal, oxidative stress Mt-2/hsp70/Sod-1. Behaviour (avoidance test and cast production)	[51]
Di-n-Butyl phthalates (DBP)	*E. fetida* (Ep)	At 5, 10, 50 and 100 mg kg^{-1} in soil. Samplings: 7, 14, 21 and 28 days	SOD, CAT, GST and peroxidase (POD) activities, GSH and LPO content	[53, 54]
Di-(2-Ethylhexyl) phthalate (DEHP)	*E. fetida* (Ep)	Spiked natural soil at 1, 3, 9 and 27 mg kg^{-1}. Samplings: 7, 14, 21 and 28 days	SOD, LPO, MT, HSP70, genotoxicity (comet), POD, LMS (NRR), mitochondrial membrane potential differential in coelomocytes	[55]
Polyethylene pellets (size 250 and 1,000 µm)	*E. andrei* (Ep)	62.5, 125, 250, 500 and 1,000 mg kg^{-1} d.w. in artificial soil for 28 days	Histology and immune-related damage assessment	[56]
Low-density polyethylene microplastics (MP) (size 250 and 1,000 µm)	*E. fetida* (Ep)	62, 125, 250, 500 and 1,000 mg MPs kg^{-1} d.w. in artificial soil. During 28 days	CAT, GST, LDH and LPO	[57]

(continued)

Table 1 (continued)

Bisphenol A	*E. fetida* (Ep) and *D. veneta* (Ep)	Artificial soil (1–2000 mg/kg) for 56 days Contact test (0.02, 0.2 and 2 mg ml^{-1}) for 48 h	Immune system (coelomocyte survival)	[58]
Bisphenol A	*E. fetida* (Ep)	Artificial soil (1–2000 mg/kg) for 56 days Contact test (0.02, 0.2 and 2 mg ml^{-1}) for 48 h	Gene expression: endocrine related (*EcR, MAPR, AdipoR)*, methyltransferases *(DNMTs)*, stress related *(HSC70 4)*, *metallothionein. Epigenetic mechanisms (Piwi2) and genotoxicity (PARP1)*	[59]
Tetrabromobisphenol A (TBBPA) and Cd alone and combined	*E. fetida* (Ep) and *Metaphire guillelmi* (En)	Cd (1 mg kg^{-1}) and TBBA (10, 50, 100, and 500 mg kg^{-1}) in artificial soil for 14 days	ROS formation, LPO and GSH levels, SOD, GPX and GST activities, histology examination	[61]
TBBPA	*M. guillelmi* (En)	^{14}C-TBBPA radiolabelled at 5 μg mL^{-1} chemical concentration measures after 5-, 10-, 15-, 20-, 25- and 30-day incubation	Biomarkers: SOD, CAT, GST and AChE activities, GSH and MDA (LPO) content and metabolite identification	[62]
Decabromodiphenyl ether (BDE209) + Pb alone and combined	*E. fetida* (Ep)	BDE209 (1, 10 mg kg^{-1}) and Pb (50, 250, 500 mg kg^{-1}) in soil (28 days) and contact tests (48 h)	DNA damage (comet assay) and avoidance behaviour	[63]
BDE209 (+ Pb) alone and combined	*E. fetida* (Ep)	BDE209 (100 mg kg^{-1}) and Pb (50, 250, 500 mg kg^{-1}) in soil (up to 28 days) after repeated exposures	SOD and CAT activities, LPO (MDA) contents and transcriptional levels of (SOD, CAT and Hsp70) genes	[64]
TBBPA, hexabromocyclododecane (HBCD) and BDE 209	*E. fetida* (Ep)	1, 10, 50, 100, 200, 400 mg kg^{-1} for 14 days	SOD, CAT and HSH70 gene transcription	[65]
BDE 47	*E. fetida* (Ep)	10, 50, 100, 200, 400 mg kg^{-1} for 14 days	SOD, CAT, GST and Hsp70 gene transcription	[66]
BDE 47 and BDE 209	*E. fetida* (Ep)	10, 50, 100, 200 mg kg^{-1} for 14 days	Metabolomics	[67]

(continued)

Table 1 (continued)

HBCD	*E. fetida* (Ep)	50, 100, 200, 400, 600 mg kg^{-1} for 14 days	SOD and GST gene expression and metabolomics	[68]
N-Ethyl perfluorooctane sulfonamidoethanol (EtFOSE)	*E. fetida* (Ep)	Quartz sand exposure to 0.960 nmol g^{-1} d.w. for 10 days	Activities of AChE, POD, SOD, CAT, GST and levels of ROS, LPO and 8-OH dG. Metabolite identification	[69]
Triphenyl phosphate (TPhP)	*P. excavatus* (Ep)	Acute filter paper contact test (1–2 days) and chronic soil 28-day test at 10 and 50 mg kg^{-1}	Metabolomics and metabolite identification	[70]
1-Butyl-3-methyl imidazolium tetrafluoroborate [Bmim]BF4 (ionic liquid)	*E. fetida* (Ep)	[Bmim]BF4 (5, 10, 20 and 40 mg kg^{-1}) in artificial and natural soil exposures after 7, 14, 21 and 28 days	ROS formation, guaiacol peroxidase (POD) and GST activities, DNA damage	[72]
1-Octyl-3-methylimidazolium hexafluorophosphate ([omim]PF6)	*E. fetida* (Ep)	At 5, 10, 20 and 40 mg kg^{-1} in artificial soil. Sampled on 7, 14, 21 and 28 days	ROS formation, antioxidant enzymes: SOD, CAT, POD and GST, LPO, DNA damage (SCGE) in coelomocytes	[73]
[Omim]BF4- and [Omim]Br-independent exposures	*E. fetida* (Ep)	Both at 5, 10, 20, and 40 mg kg^{-1}. Sampled on days 7, 14, 21 and 28	ROS formation, antioxidant enzymes: SOD, CAT, POD and GST, LPO, DNA damage (SCGE) in coelomocytes	[74]
1-Alkyl-3-methyl imidazole bromide ionic [$C_n$mim]Br ($n = 2, 4, 6, 10, 12$)	*E. fetida* (Ep)	Each at 5, 10, 20, and 40 mg kg^{-1}. Sampled on days 14 and 28	ROS formation, antioxidant enzymes: SOD, CAT, POD and GST, LPO, DNA damage (SCGE) in coelomocytes	[75]
1-Octyl-3-methylimidazolium chloride ([C8mim]Cl)	*E. fetida* (Ep)	At 5, 10, 20 and 40 mg kg^{-1} in artificial soil. Sampled on 7, 14, 21 and 28 days	ROS formation, antioxidant enzymes: SOD, CAT, POD and GST, LPO, DNA damage (SCGE) in coelomocytes	[76]

In brackets the ecological classification of earthworms as anecic (A), endogeic (En) and epibenthic (Ep) is indicated as reported in [1]. Abbreviation for biomarker acronyms is provided in the text

3 Biomarkers of Pesticide Exposure

The use of biomarkers in earthworms to assess soil contamination by pesticides has received the largest body of attention and has already been accurately reviewed [3, 5, 6, 8, 17]. Studies by Sanchez-Hernandez and co-workers [17] have described the tissue-specific differences in the CE sensitivity to organophosphorus pesticides. Their studies provided evidence on the role of *L. terrestris* in improving soil quality and increasing extracellular enzyme activities, including CEs, as well as soil microbial activity thanks to their casts' contribution. As mentioned before, and for biomonitoring of polluted soils, more recent studies using biomarkers measured in whole tissue homogenates or coelomocytes will be given priority.

A Chilean field study assessing pesticide exposures in sites following conventional versus organic agricultural management practices selected *L. terrestris* as sentinel. The study took place over the four seasons and in three tissues of the earthworms' gastrointestinal tract and demonstrated that CE measurements in this species were adequate biomarkers of pesticide exposure [18]. Also the soil-dwelling species *A. caliginosa* was selected in an experiment with soil spiked with two insecticides: a commercial brand of a carbamate (Pirimor®) and Lorsban® as organophosphate (OP). The aim of the study was to relate the neurotoxicity marker AChE activity in whole tissue homogenate with an electrophysiological technique named nerve conduction velocity (NCV) in medial giant fibres and therefore, to validate the use of a conservative technique (NCV) to assess neurotoxicity [19]. In the attempt to link biochemical (AChE and CE activities) to behaviour responses (burrowing, casting and feeding) of ecological significance, the study of Jouni et al. [20] exposed two endogeic earthworms, *A. chlorotica* and *A. caliginosa*, in soil contaminated with the pesticide ethyl-parathion for 7 days. The in vivo and in vitro exposures to this pesticide suggested that *A. caliginosa* was the most sensitive species [20]. The measurement of activities in non-destructive coelomocytes of *E. andrei* in response to two OP pesticide exposures (dimethoate and pirimiphos-methyl) was recently validated [21]. In this study, responses of AChE and CE enzymes in whole tissue homogenate were contrasted with those in this bio-fluid using the filter paper contact assay [21]. *Dendrobaena veneta*, also an epigeic species, was selected for testing the toxicity of three herbicides at three environmental concentrations in Petri dish exposures using the filter paper contact test. Two trials of 7 and 28 days were performed in which the biomarkers AChE, CAT, GST and LPO (measured as MDA) were considered in addition to reproductive endpoints [22]. The associated biomarker and toxicity responses were seen as herbicide- and dose-related. Toxicity due to pyrethroid and bifenthrin was assessed in *E. fetida* for up to 28 days following soil gradient exposures. Biomarkers dealing with oxidative stress and metabolism (ROS formation, POD, SOD, CAT, GST and EROD activities) were measured and their responses seen as concentration- and time-dependent [23]. Indigenous earthworm species from Nigerian soils, namely, *Alma millsoni*, *Eudrilus eugeniae* and *Libyodrilus violaceus*, were the sentinels of a bioremediation study after the application of Roundup® Alphée, a glyphosate-based herbicide (GBH) in potted soils,

and several biochemical endpoints suggestive of GBH toxicity were assessed for up to 8 weeks [24]. Their results revealed the usefulness of the targeted enzymatic endpoints and antioxidant defences in earthworms for monitoring GBH-contaminated soil and identified *E. eugeniae* and *L. violaceus* as the ones with higher vermiremediation potential. Another recent study that encompasses biochemical, mRNA and metabolomics complementary approaches by Zhang et al. [25] evaluated the effects on *E. fetida* due to exposure to the fungicide tebuconazole under artificial soil conditions for 7 days. Amongst the biomarkers assessed, a confirmation of an oxidative stress condition seen by SOD activity decreases translationally controlled tumour protein (TCTP) and annetocin (ANN)-related genes' downregulation as well as metabolic affectance of the AMP pathway (seen as the alteration of 12 endogenous metabolites) using untargeted nuclear magnetic resonance (NMR) techniques, all pointing to consequences on the reproductive outcome of the exposed earthworms. In the same species, neonicotinoid dinotefuran was tested, using the filter paper contact test and soil exposures, for enzymatic and effect oxidative responses including gene expression of Hsp70 and ANN genes [26] but also the toxicity associated with its metabolites as indicated in Sect. 9.

4 Biomarkers of Metal Exposure

The field study [27] considered a comprehensive set of biomarkers, in addition to MT as a specific marker, and pointed out *L. terrestris* as adequate sentinels of metal pollution. Furthermore, the same authors using the same species related the response of MT in the coelomic fluid to that in the whole tissue homogenate in order to validate the use of an alternative non-destructive marker for metal monitoring [28]. The naturally occurring species *Metaphire posthuma* was the species of choice in a study aimed at assessing toxicity in several metal-contaminated sites from India [29]. In this case, they also used coelomocytes from the local species to associate metal exposures to the response observed in biomarkers dealing with the immune response, ROS formation and antioxidant defences. The naturally occurring soil-dwelling earthworm *A. caliginosa* was the sentinel in a field survey conducted in Slovakia also following a metal-polluted gradient. In this case, the biomarkers selected and measured in whole tissue homogenates refer to GSH-dependent enzymes such as GSH content, GST, GPX and GR activities, considered as antioxidant defences [30]. A multidimensional analysis (PCA) of the selected biomarkers revealed site-dependent depletion on GSH content while showing an increase on the antioxidant enzyme activities in the local earthworms due to metal exposures. The same species *A. caliginosa* was used in a microcosm study with natural soils, and the consequences of the exposures were evaluated after 21 days in terms of *mt* gene expression, enzymatic activities and energy reserves [31]. The closely related anecic species, *Aporrectodea rosea* and *A. trapezoids*, but also the epigeic *E. fetida* were contrasted in relation to their sensibility to metal-spiked soils (Pb and Cd nitrates) at

different times of exposure (7, 14 and 28 days) [32]. In that study, non-conservative measures in whole tissue such as LPO (MDA content) and total antioxidant capacity (TAC), as well as measures in coelomocyte/genotoxicity (TUNEL assay), were related to metal partitioning in several subcellular fractions. Their conclusions confirmed the lower sensibility of *E. fetida* to metal exposures and therefore the need to include more ecologically relevant species in soil pollution assessment, as suggested previously in other earthworm studies.

5 Biomarkers for Mixed Chemical Exposure

Under a realistic field scenario, soil-dwelling earthworms will experience multi-xenobiotic exposures which are likely to require the consideration of a comprehensive set of biomarkers, including those dealing with general stress. This was the case of a field study in India using the earthworm *Dichogaster curgensis* as bioindicator in which the targeted chemicals were a consequence of fly ash pollution (mostly made of metals and polyhalogenated compounds). Amongst the biomarkers tested, there were antioxidant CAT, SOD, GR, GPX and GST activities and LPO levels, cytotoxicity, genotoxicity and histopathology as effect markers [33].

From a realistic perspective, and within the context of the usage of treated wastewaters (TWWs) in agriculture of arid and semi-arid climate areas, *E. fetida* was exposed to artificial soils irrigated with wastewater following different purification treatments [34]. The biomarkers of general stress, LMS (NRR assay) in coelomocytes, and the specific one, MT content in whole tissue homogenates, were measured after 28 days, and the authors claimed their approach proved suitable for assessing toxicological safety in reclaimed wastewaters' reuse [34]. Similarly, the species *E. andrei* was selected under laboratory soil exposure conditions for the potential use of TWW in agriculture, and a set of parameters involving enzymatic activities (CAT, GST and AChE) and gene expression (*cat* and *gst*) were considered as biomarkers in the exposed organisms [35]. The soil irrigated with an increasing percentage of TWW caused a reduction of CAT and AChE activities, while GST activity and LPO levels increased in the exposed earthworms, and the corresponding gene expression (*cat* and *gst*) was significantly downregulated with respect to controls. These researchers conducted the same type of study but considered natural soils that had been irrigated with TWW in a country with water scarcity problems (Tunisia) for an extended period of time: 1, 8 and 20 years [36]. The biomarkers considered in the long-term study were coincident with those considered before, but they also included genotoxicity (using micronuclei test). The longer exposures (20 years) impacted more negatively on the earthworm fauna, seen as enhanced genotoxicity, probably as a consequence of the higher metal and organic pollutant load detected in the longer-term irrigated soil experience. The vermicompost earthworm *E. eugeniae* was exposed for 24 h to contaminated soil containing three concentrations of several fertilisers and agrochemicals of concern in Indian agricultural soils, and the biomarkers measured comprised histology alterations, LPO

occurrence and the activities of LDH, CAT and AChE. Histological and enzymatic alterations were detected as soon as after 24-h exposures [37]. The suitability of the indigenous earthworm, *Perionyx excavates*, and the exotic one, *E. eugeniae*, was contrasted in a vermistabilisation study using combined mixtures of sludge from textile mill sludge (TMS) and cow dung at different ratios, and the biomarker MT content and histology damage were contrasted in the two epigeic species [38]. A higher MT content was revealed in the non-native *E. eugeniae*, and the mixture TMS/cow dung (1:1) resulted in a reduced histological damage in respect to when the exposure consisted exclusively of TMS.

Prior to the application of biomarkers to assess toxicity of cocktail contaminant mixtures, and in the context of wastewater recycling for agricultural practices, an adaptation of the OECD filter paper contact test was conducted with *L. terrestris.* The selected chemicals (lamotrigine and cocaine) had been previously detected in TWW from the urban catchment of a major NW Mediterranean city or are a cause of concern to wildlife species (fipronil). This approach considered independent exposures to non-realistic concentration of the chemicals in order to unequivocally identify metabolites with the current techniques available and to recommend adequate biomarkers. The biomarkers tested in the earthworm whole tissue homogenate were GST, AChE and CEs (using four different substrates as suggestive of several isozymes); a group of unexposed organism was considered as reference, and another one treated with the OP pesticide bis(4-nitrophenyl) phosphate (BNPP) was used as positive control. Confirmation of the exposures (although not environmentally relevant) and metabolism was complemented with the analysis of the parent compounds and the identification of the metabolites in the same whole tissue homogenates [39].

6 Biomarkers of Assessing Pharmaceuticals and Personal Care Product Exposure

Within the frame of water recycling for agricultural practices, the presence of urban pharmaceutical drugs and personal care products (PPCPs) in TWW has alerted for the potential transfer of these drugs to crops and the problem of antibiotic resistance in the exposed population. Concern on the negative impact of PPCPs also implies soil-dwelling organisms, considered valuable engineers for their beneficial role in improving soil biochemistry. The impacts on earthworms due to PPCPs have been studied under controlled laboratory conditions. The antihypertensive drug valsartan was selected due to its notable presence in TWW of a Mediterranean urban region [40], and *L. terrestris* was the sentinel exposed in soil contaminated with this drug. The dynamics of the drug (in the presence or absence of the earthworms) were followed up, and several biomarkers of exposure were determined over a 21-day period in the whole body homogenate of the earthworms. Earthworm enzymatic activities were related to soil biochemistry, microbial diversity and drug presence in

soil and whole tissue [41]. Microbial diversity increased over time with the presence of earthworms in soil as well as the overall enzymatic load in soil. Enzymatic responses (AChE, GST and CEs) in valsartan-exposed *L. terrestris* were mostly affected after 7 days and stabilised thereafter. Toxicity due to personal care products (PCPs) was evaluated in *E. andrei* after a short term (24, 48 and 72 h) using the filter paper contact test to several concentrations of triclocarban as modifications on CAT and GST activities [42]. Exposure to triclosan (together with its metabolite methyl-triclosan) was evaluated in *E. andrei* under longer (28 days) soil exposures as DNA damage occurrence in coelomocytes [43]. To gain in environmental relevance, the toxicity associated with drug chemical mixtures was evaluated in *E. fetida* exposed ex situ to cosmetic sludge (CS) and foundry sands (FS) from industrial activities, using DNA damage and coelomic cell composition as endpoints, after 1 h and in vivo after 7- and 14-day exposure trials [44]. The suitability of using a non-invasive method for coelomocyte extrusion and genotoxicity evaluation to variable percentage of water leachates, 1.5, 3, 6, 12.5, 25, 50 and 100%, was demonstrated as ex situ but also in vivo in coelomocytes as soon as after 7-day FS and CS leachate exposures. Toxicity evaluation of soil affected by livestock farming activities (mostly made of trace metals, veterinary pharmaceuticals and pesticide residues) was validated in the epi-endogeic earthworm *Amynthas gracilis* using sublethal responses in AChE and SOD activities in whole body tissue and lysosomal integrity (LMS) and DNA damage in coelomocytes after 1-, 7- and 14-day exposures [45].

7 Biomarkers of Nanomaterial Exposure

As anticipated, studies screening for the toxicity associated with this class of emerging contaminants has been carried out mostly in the model earthworms adopted in soil ecotoxicology testing (*Eisenia* spp.). Toxicity associated with multiwalled carbon nanotubes (MWCNTs) was assessed in *E. fetida* exposed to contaminated soil for up to 14 days, and, in addition to traditional endpoints (survival and reproduction), a set of biomarkers including morphometric measures, LMS, MT and AChE activity were considered [46]. A large proportion of soil toxicity assessment studies with these nano-contaminants using earthworms refer to engineered nanomaterial such as zinc oxide (ZnO). To select a few, a recent comprehensive study with *E. fetida* considered an in vivo approach on the effects of several concentrations of ZnO for up to 28 days in the responses on oxidative stress-related biomarkers (ROS formation, LPO levels and SOD activity). This in vivo approach was complemented by means of in vitro exposures to this nanomaterial in the coelomic fluid as ROS formation and LDH activity measures [47]. Since nanomaterial-induced toxicity is usually ROS-related, the highest ROS content and LPO levels were found on day 28 at the highest dose nano-ZnO concentration (250 mg/kg). Similarly, ZnO nanoparticles alone and combined with the pesticide chlorpyrifos were assessed in *E. andrei* in a laboratory soil exposure for up to 28 days and included responses of oxidative stress biomarkers (CAT and GST),

AChE activity and LPO as endpoints [48]. The responses observed were variable and dependant on whether they had addressed single or co-exposures, with the authors stressing the importance of assessing the biochemical responses to realistic mixtures.

Several copper oxide (CuO)-engineered nanomaterial (ENM) variants were spiked in artificial soil (freshly prepared and after 1 year of ageing), and the toxicity displayed in *E. fetida* was evaluated after 14 days in terms of GSH content, SOD activity, the osmoregulatory capacity indicator Na^+/K^+-ATPase activity and histology [49]. Their results revealed a coating-dependent difference in ENM toxicity to earthworms which was also modified after a year of soil ageing, and, in both cases, osmotic disturbance was revealed as the most sensitive endpoint.

Nickel oxide nanoparticles (NiO-NP) toxicity was assessed in the same earthworm model at a range of concentrations, 5, 50, 200, 500 and 1,000 mg kg^{-1}, in soil after 28 days. Biomarkers related to oxidative defences (CAT, POD and SOD), damage (as oxidised DNA and lipids) as well as histological examination of the whole tissue were evaluated [50]. Their results were also seen as dose dependant, and while POD activity, MDA (LPO) and 8-OHdG (DNA damage) levels increased, CAT and SOD defences decreased. Histology analyses also confirmed alterations of the epithelium layer, microvilli and mitochondria as well as potential adverse effects on the gut barrier.

8 Biomarkers of Plastics, Plasticisers and E-Waste-Related Exposures

The presence of plastic in natural soil is an issue of increasing concern especially for earthworm populations from natural and agricultural soils such as *L. terrestris*. Thus, this species was the sentinel selected for assessing microplastic exposures in natural soils polluted with polyester microfibres (MF) derived from textile laundering. During the 35-day trial, some gene expression endpoints related to metal, oxidative stress and general stress markers were considered [51]. The highest percentage of MF in soil (1%) caused a significant reduction in cast production and *hsp70* gene expression and an increase in the expression of *mt-2*, although metal content was not seen as a relevant contributor in the most polluted soil.

Phthalates are important plastic additives that in some cases can represent >50% of the polymer mixture (e.g. PVC) from which they can be easily released and be toxic to organism inhabiting polluted soils [52]. Phthalate exposures have been studied in the model *E. fetida* in standardised 28-day exposures. Di-n-butyl phthalate (DBP) was evaluated over time under a gradient up to 100 mg kg^{-1} dry soil, and several biomarkers related to antioxidant defences (SOD, CAT GST, POD and GSH content) and the indicator of LPO damage (MDA formed) were considered [53]. In general, the activity of certain enzymes (SOD, CAT, POD) showed a downward trend while LPO increased. The same experiment also included the measure of

damage to DNA macromolecules using the comet assay, and it revealed increasing damage over time and dose [54]. Another particular phthalate, di-(2-ethylhexyl) phthalate (DEHP), was spiked in natural soil (1, 3, 9 and 27 mg kg^{-1}), and several biomarkers were evaluated in *E. fetida* coelomocytes. Some parameters increased, SOD, LPO, MT, HSP70 and genotoxicity (comet assay), while others decreased: POD, LMS (NRR) and mitochondrial membrane potential difference in relation to concentration and over time [55]. The authors proposed a precautionary level of 3 mg kg^{-1} for potential risk of phthalates in soils (based on DEHP results). Assessment of toxicity due to polyethylene (PE) pellet (250–1,000 μm) exposure in *E. andrei* was conducted at a wide range of PE concentrations (max 1 mg kg^{-1}) in soil for 28 days [56]. Damage in gut tissue was revealed at all concentrations in a dose-dependent manner as well as toxicity of the immune system. More recently, the same authors evaluated low-density polyethylene microplastics (MP) toxicity in the same model species, following the same standardised protocol but considering additional oxidative stress parameters (GST and LPO) as well as the anaerobic metabolism marker (LDH) as endpoints [57]. The authors concluded that MP exposure caused oxidative stress and changes in energy metabolism more significantly after soil exposures over 250 mg kg^{-1} d.w.

Other plastic additives, such as the monomer bisphenol A, have also been screened for in vivo toxicity in the epigeic species *E. fetida* and *D. veneta*, and life-history traits and the immune system (measured as coelomocyte viability) were the targeted endpoints using chronic artificial soil and acute contact test exposures [58]. Although some species-related differences in sensitivity were seen, the immune system was not affected, while the reproductive outcome was compromised. The same researchers [59] selected *E. fetida* as bioindicator to search further into molecular gene expression effects due to BPA exposures in biomarkers related to endocrine function (*EcR, MAPR, AdipoR*), epigenetic mechanisms (*DNMTs*), genotoxicity (*PARP1*), stress responses (*HSC70 4*) and metabolism (*metallothionein*). Although soil exposure tests did not reveal effects at the molecular level in whole tissue homogenates, the particular analysis of the male organs showed effects on the endocrine-related genes, epigenetic mechanisms (*DNMT1* and *DNMT3b*), the genotoxic-related *PARP1* and the stress-related *Hsc70 4* genes. The contact test for acute exposures also indicated effects on detoxification and stress pathways in whole tissue homogenates such as *HSC70 4* and *metallothionein*, epigenetic mechanisms (*Piwi2*) and genotoxicity (*PARP1*) in addition to disruptive effects on male organs.

Flame retardants are also plastic additives of environmental concern in contaminated soils. The flame retardant tetrabromobisphenol A (TBBPA) is frequently present in plastics and electronic equipment and displays endocrine-disrupting properties [60]. TBBPA alone and under co-exposure with a toxic metal (Cd) are present in e-waste-contaminated soils. The consequences of single and combined exposures were evaluated in earthworms using several biomarker endpoints together with histological damage examination. The species were the standard model *E. fetida* and the soil-inhabiting and more ecologically relevant anecic *Metaphire guillelmi*. All biomarkers considered confirmed a higher sensibility in the natural-

dwelling species under co-exposures with respect to *E. fetida* [61]. Radiolabelled ^{14}C-TBBPA was used to spike soil and follow up metabolism, biomarker responses and metabolite identification in *M. guillelmi* [62] that will be further addressed in the next section. Exposures to other brominated flame retardants such as the more persistent decabromodiphenyl ether congener (BDE209) and the metal Pb, in single and combined mixtures, as common chemicals found in e-waste recycling sites, were evaluated in *E. fetida* in a soil test. In this case, a behaviour endpoint (avoidance test after 48 h) and the measure of DNA damage in coelomocytes (after 28 days) were the biomarkers selected [63]. A follow-up of these e-waste components was carried out in a soil test that considered three repeated exposures to these two contaminants for 10 days. After this preparation period, biomarker measures were related to antioxidant responses and gene expression at times: 2, 7, 14 and 28 days [64]. Antioxidant responses and gene expression were correlated to the exposures, and SOD activity seemed to be more sensitive than CAT activity measures. Gene expression of genes related to antioxidant defences (SOD and CAT) and HSP70 was studied after exposures at several concentrations (1–400 mg kg^{-1}) of the brominated chemicals: TBBPA, hexabromocyclododecane (HBCD) and BDE 209 [65]. Gene upregulation was chemical- and concentration-dependent suggesting the toxicity order TBBPA>HBCD>BDE209 and the HSP70 gene expression being the most sensitive biomarker. A particular BDE 47-associated toxicity in soil-exposed *E. fetida* was considered after 14-day exposures at a wide range of concentrations (10–-400 mg kg^{-1}) and gene expression evaluated [66]. Out of the four genes considered, SOD upregulation and Hsp70 downregulation stand out as well as growth rate inhibition as the parameters more consistently affected. In addition to more traditional biomarkers, a metabolomics consideration was incorporated into some of the former studies evaluating exposure in *E. fetida* to relevant flame retardants under similar experimental dose and time conditions. That is, BDE 47 and BDE 209 altered metabolites involved in energy metabolism, Krebs cycle, amino acid metabolism, nerve activities and osmotic/compatible solute balance [67]. However, HBCD exposure induced oxidative stress (SOD and GST gene expression) and impaired metabolic homeostasis including anaerobic metabolism as indicated by seven metabolite modifications in those exposed [68]. All former metabolomics approaches confirmed the sensitive nature of the NMR techniques in relation to ecotoxicological assessment of flame retardants.

Other organohalogenated chemicals of environmental concern are polyfluoroalkyl substances with perfluorooctane sulfonic acid (PFOS) as the main metabolite detected in environmental matrices and being N-ethyl perfluorooctane sulfonamidoethanol (EtFOSE) its main precursor. A recent study by Zhao et al. [69] evaluated the degradation of EtFOSE (0.9 nmol g^{-1} d.w.) over a 10-day period in a quartz sand experiment and measured the responses in *E. fetida* in biomarkers encompassing oxidative stress and damage. In addition to metabolite identification, a time trend activation of POD, SOD, CAT and GST enzymatic activities and damage revealed as DNA (8-OH dG) and ROS formation was evidenced at the longest exposures. As substitute alternative of more toxic halogenated flame retardant, organophosphorus flame retardants also require investigation. Triphenyl

phosphate (TPhP) toxicity was addressed under acute filter paper contact test and chronic soil exposures in the epigeic earthworm *P. excavatus* using a metabolomics and metabolite identification approach [70]. About ten endogenous metabolites and seven phospholipids were modified as seen by GC-MS and LC-QTOF methodologies due to TPhP.

Ionic liquids (ILs) are chemicals largely used in electric battery applications due to their chemical and thermodynamic stability and have been proposed as "green alternatives" to traditional solvents. However, their increasing use in technological, industrial and more recently scientific and medical applications could constitute a threat to biota present in soils contaminated with e-waste residues [71]. So far, all the studies conducted on assessing their toxicity have been based on *E. fetida* as the model. The potential toxicity of the imidazole-based IL, 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim]BF4), was evaluated in earthworms inhabiting artificial and natural contaminated soils in experiments for up to 28 days with greater toxicity observed in the earthworms from fluvo-aquic natural soils [72]. Another similar study but with the imidazole-based IL, 1-octyl-3-methylimidazolium hexafluorophosphate ([omim]PF6), was conducted in the same species under laboratory soil conditions for the same time period and with coincident endpoints [73]. Maximal ROS production and LPO occurrence were observed at concentrations of 40 mg kg^{-1} after 28 days. Likewise, DNA damage (using comet assay) was revealed as the most sensitive endpoint which increased over time and dose. The toxicity associated with two ILs, [Omim]BF4 and [Omim]Br, was contrasted under similar soil conditions, and the targeted endpoints revealed a negative impact time- and dose-dependent on DNA damage (measured as olive tail moment) even though antioxidant defences were also enhanced [74]. The influence of the length chain of the IL, 1-alkyl-3-methyl imidazole bromide ionic [$C_n$mim]Br, from $n = 2$, 4, 6, 10 and 12 was also tested in a sub chronic toxicity text in *E. fetida* in the conditions described above, but in this case the endpoints were measured only after 14- and 28-day soil exposures [75]. Their conclusions revealed that toxicity increased with length chain but decreased after C_{10}. The same experimental design and endpoints were applied to a chloride-derived IL, 1-octyl-3-methylimidazolium chloride ([C8mim]Cl), in *E. fetida* [76]. In this case, biochemical modifications of antioxidant defences did not prevent occurrence of damage (increased LPO and DNA levels) in a dose- and time-dependent fashion.

9 Earthworm Metabolism and Metabolite Identification

Metabolism may play a significant role in toxicity by two mechanisms: (1) it affects the bioaccumulation of chemicals and (2) it can originate metabolites with enhanced toxicity in respect to the parent compound. A comprehensive review on the earthworm's metabolism was published in 2015 by Katagi and Ose [77], but it was centred mostly on pesticide exposures. Depending on the chemical's nature, metabolism can lead to the formation of more reactive metabolites such is the case of OP

pesticides (oxon forms) and most polycyclic aromatic hydrocarbons (PAHs) and phenoxy herbicides (phenol formation) by oxidative reactions mostly catalysed by P450 enzymes. The authors from this former review concluded that in earthworms the main metabolic processes are carried out by the cytochrome P450 family, carboxylesterases (phase I oxidation and hydrolysis reactions, respectively) and glutathione S-transferases (conjugation phase II metabolism) although in quantitative terms they are less represented than in fish or higher vertebrates. Moreover, CE- and GST-catalysed reactions are mainly detoxification pathways, whereas those by phase I cytochrome P450 enzymes can lead to more toxic metabolites.

The identification of metabolites can also shed light onto the metabolic pathways experienced by the parental chemicals in earthworms and the further assessment of their associated toxicities. The study by Qin et al. [78] on the toxicity of the two racemic forms of the insecticide fipronil identified by means of HPLC-MS/MS analyses several phase I metabolites resulting from oxidation (sulphide), reduction (sulphone) and hydrolysis (amide) and revealing the *S*-fipronil form was more toxic than the *R*-fipronil one. Other phase II conjugation mechanisms significant in mammalian systems (conjugation with glucose, glucuronic acid or sulphate) are not regarded as prevalent in earthworms. However, with more recent technological advances in analytical methodologies, metabolite identification has greatly improved. In soil-dwelling organisms, *O*-methylation seems to be the preferred detoxification strategy, at least for phenolic compounds such as TBBPA [62]. Exposure to the OP flame retardant TPhP in the 28-day soil microcosm experience with the earthworm *P. excavatus* formerly described [70] identified several phase I and phase II metabolites by untargeted LC-QTOF methods, with the glucoside conjugates more abundant than the thiol-derived ones. A suggestion of potential metabolite formation in earthworms could also be provided by the enzymatic responses altered after the exposures and its confirmation by the application of state-of-the-art analytical technologies. This is the case provided by the [69] study formerly described in relation to EtFOSE exposures in which an elevation of GST activities was indicative of this conjugation pathway taking place and its further confirmation with metabolite identification by GC-MS.

The importance of metabolite identification for further toxicity assessment was given by the Liu et al. [26] study on dinotefuran (a neonicotinoid insecticide) and two of their main metabolites 1-methyl-3-(tetrahydro-3-furylmethyl) urea (UF) and 1-methyl-3-(tetrahydro-3-furylmethyl) guanidium dihydrogen (DN) exposures in *E. fetida*. The toxic effects induced by UF and DN metabolites showed a significant dose-effect and time-effect correlation. By increasing the concentrations (0.1–-2 mg kg^{-1}) and time (up to 28 days), more UF and DN were accumulated in earthworms, and these identified metabolites were responsible for changes in SOD and CAT activities, damage in lipid and nucleic acid and abnormal expression of Hsp70 and ANN genes confirming their toxic properties. More recently, an environmentally relevant study applied suspect and non-target screening QTOF-MS technology to identify 60 parental pesticides and pharmaceuticals and at least 50 of its transformation products in wastewater, resulting from biotic and abiotic degradation processes, and its further toxicity assessment was applied using the ECOSAR model [79].

10 Conclusions

A larger body of evidence on biomarker responses in earthworms is provided by the use of epigeic *Eisenia* spp. as model species of soil toxicity assessment. However, due to the ecological role of anecic species, their inclusion as sentinels is highly recommended because of their impact on soil physico-chemical and biological properties and abundance in agroecosystems. Moreover, the deep-burrowing species have shown themselves to be more sensitive to chemicals than the epigeic ones. From a monitoring perspective and practicability, the use of whole tissue homogenates rather than particular tissues facilitates the protocols and has already been validated for many parameters. The inclusion of biomarker measures using non-destructive techniques (i.e. coelomocytes) also deserves consideration. Amongst chemicals, traditional monitoring had focussed on pesticides and metal-contaminated soils. However, in recent years, other chemicals of increasing presence in natural soils (e.g. pharmaceuticals, plastics, e-waste and associated chemicals) have been targeted. This is especially relevant in countries enduring water scarcity as they may use contaminated wastewater for crop irrigation. Outstanding from amongst the more frequently selected biomarkers are early warning responses that can lead to consequences at higher levels of biological organisation. Since from an environmental perspective, contaminated soils endure many types of chemicals, including pharmaceuticals to different extents, the use of general stress biomarkers is encouraged, that is, immunological, oxidative stress exposure and effect and neuro- and genotoxic biomarker responses as common effects revealed by those experimental exposures. The development of state-of-the-art analytical protocols will allow (1) the incorporation of metabolomics biomarkers to identify the endogenous metabolic processes likely to be affected by contaminants and (2) metabolite identification, as suggestive of the xenobiotic metabolism pathways affected and to favour further toxicity assessment of the identified by-products, as a more realistic approach.

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Vermiremediation of Pharmaceutical-Contaminated Soils and Organic Amendments

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Contents

Abstract Human and veterinary pharmaceuticals reach agricultural soils via crop irrigation with treated wastewater and via soil fertilising with biosolids or manure. Compelling evidences on the environmental fate of pharmaceuticals suggest that accumulation of these emerging pollutants in soil is currently a serious risk for soil quality and food security. Currently, engineered remediation methodologies to remove pharmaceuticals from soils as well as those (e.g. aerobic composting) to treat biosolids and manure are not sufficiently efficient to full removal of pharmaceuticals. Moreover, these techniques are often economically prohibitive and may cause adverse side-effects in the environment. Microbes, soil fauna (e.g. earthworms) and their interactions exert a strong control in the organic matter decomposition and nutrient cycling of soil. By taking advantage of these naturally occurring processes, we propose the use of earthworms to clean biosolids and manure (ex situ vermiremediation) and to reduce pharmaceutical bioavailability in soil (in situ vermiremediation). The impact of earthworms on soil physicochemical

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and biological properties together to the tolerance of these organisms to pharmaceuticals makes these bioremediation strategies viable in soils receiving pharmaceutical-contaminated amendments and water. Additionally, some studies have evidenced that earthworms (*Eisenia* spp.) accumulate pharmaceuticals in their tissues, thus being an advantageous biological process in the vermicomposting of biosolids and manure.

Keywords Biochar, Bioremediation, Earthworms, Toxicity, Vermicomposting

1 Introduction

According to the US Food and Drug Administration [1], the term active pharmaceutical ingredient (API) refers to "any substance or mixture of substances intended to be used in the manufacture of a drug (medicinal) product and that, when used in the production of a drug, becomes an active ingredient of the drug product. Such substances are intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease or to affect the structure and function of the body". Many APIs and their metabolites are currently detected in treated (or reclaimed) wastewater, biosolids (sediments obtained from wastewater treatment plants) and animal manure. In this chapter, we will use the term API to refer to both human and veterinary pharmaceuticals.

The incomplete removal of APIs during wastewater treatment and the high use of veterinary pharmaceuticals in concentrated animal feeding operations (CAFOs) are the main reasons for detecting APIs in treated wastewater, biosolids and manure [2, 3]. In addition, pharmaceuticals' consumption is significantly high in densely populated areas, particularly in Asian countries, thus leading to discharge API-contaminated wastewater [4]. Likewise, crop irrigation with treated wastewater and the application of biosolids and manure as soil amendments are common agricultural practices in arid and semiarid areas, where, in addition to water scarcity, soils are characterised by a low organic carbon content [5, 6]. Therefore, these agroecosystems have a high risk of contamination by APIs.

Irrigation of agricultural soils with treated wastewater is, therefore, a significant route of continual input of APIs. Indeed, some of them are named "pseudo-persistent" pollutants because of concentrations in soil keep constant via irrigation [7], despite displaying short half-life times [8]. Fertilisation with biosolids/manure is another important source of API contamination. Although APIs are generally detected in treated water [8–10], some studies have reported the occurrence of these pollutants in biosolids [11, 12] and manure [13]. Moreover, the application of biosolids to soil has been shown that increases the persistence of certain APIs (triclosan and triclocarban) probably because of organic matter of biosolids that decreases the API bioavailability for microbial degradation [14, 15]. Accordingly,

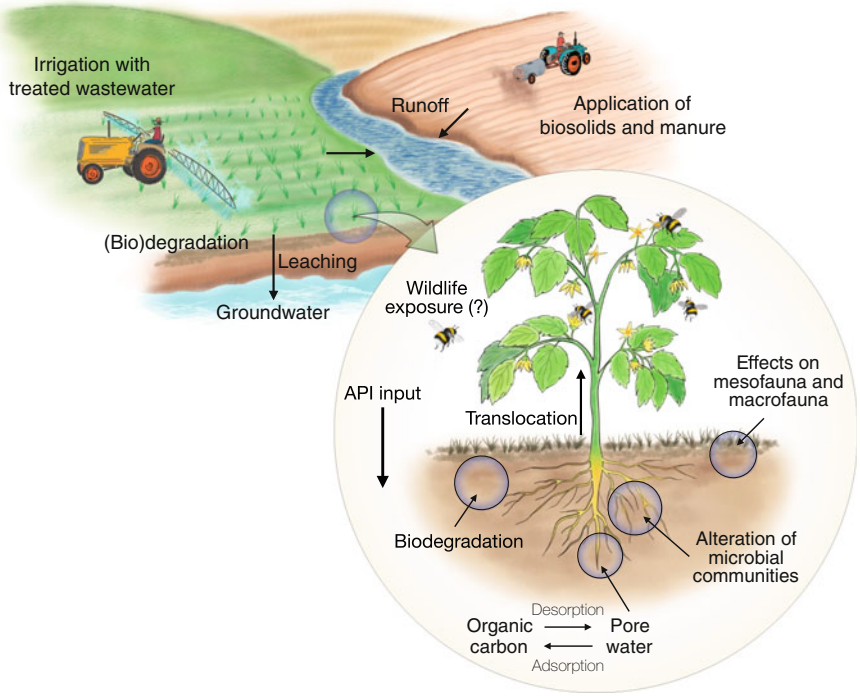

Fig. 1 An agroecosystem diagram illustrating the main routes of active pharmaceutical ingredient (API) input and dissipation in soil, with particular emphasis in the soil-plant system

API concentrations in the range of ng/g dry mass are detected in agricultural soils worldwide receiving this form of fertilisation [8, 9, 16], with high potential to be accumulated in edible crops [10, 13, 17].

Environmental fate of APIs largely depends on soil physicochemical and biological processes. A detailed description on physicochemical and transport processes governing API fate in soil is beyond of the scope of this chapter, but some generalisations are shown in Fig. 1. Environmental fate of APIs depends on intrinsic and extrinsic variables. The former are the physicochemical properties of the substance such as water solubility and dissociation of ionisable compound [18]. Some APIs are neutral (e.g. carbamazepine, diazepam, caffeine) and generally display a high capacity to bind to soil organic matter [7], whereas ionic pharmaceuticals (e.g. diclofenac, naproxen, ibuprofen, atorvastatin) tend to be less persistent in soil and their fate depends on soil pH. Among extrinsic variables, photodegradation, hydrolysis and biodegradation significantly contribute to API transformation and dissipation [11, 19]. Furthermore, soil properties also affect transformation and bioavailability of APIs. For example, the organic matter content of soil has a strong influence in the retention of hydrophobic APIs, therefore reducing their bioavailability and biodegradation [20].

It is now widely recognised that API accumulation in agricultural soils may be a serious threat to non-target organisms and natural resources. For example, ketoprofen, carbamazepine and caffeine were detected in groundwater samples from Europe at concentrations of 2.88, 3.60 and 4.50 μg/L, respectively [16], suggesting a high mobility of these chemicals in soil. Likewise, many studies have demonstrated that edible plants growing in API-contaminated soils accumulate and translocate APIs to aerial parts [11]. Furthermore, APIs may cause biochemical and physiological adverse effects in plants, negatively affecting their growth and development [7]. As a result, non-target organisms including human beings may be exposed to API through the consumption of contaminated edible plants. For example, bee exposure to pharmaceuticals accumulated in pollen and nectar from zucchini flowers was modelled for carbamazepine, and outcomes revealed that honeybee colonies as well as the bee behaviour could be seriously affected by moderate hydrophobic APIs [21]. Nevertheless, field surveys are still needed to draw solid conclusions about exposure levels of wildlife to API-contaminated plants.

Soil functioning is also altered by APIs. Although biodegradation is the major dissipation route [22, 23], these chemicals are able to alter soil microbial activity and community [19] and soil enzyme activities [24–26]. Because soil enzyme activities catalyse most chemical reactions involved in the transformation and decomposition of organic matter, and nutrient cycling [27], their alteration by APIs could lead to soil degradation. Therefore, affordable mitigating measures and remediation strategies should be taken into account to reduce the potential environmental risks of APIs. In this context, the use of earthworms emerges as a promising strategy for reducing API concentration and toxicity at the source (treatment of biosolids and manure) and in agricultural soils receiving continual input of APIs.

This chapter describes the mechanisms and technical aspects linked to earthworms' capacity to remediate API-contaminated soils and amendments. The first section makes a brief overview of the earthworm effects on soil functioning, therefore providing insights into the importance of these organisms in API degradation (Sect. 2). The third section provides data on toxic effects of APIs in earthworms: a knowledge needed to propose these organisms as biological vectors of API biodegradation. The fourth and fifth sections consider two options for using earthworms in managing API residues: vermicomposting of organic residues such as biosolids and manure (ex situ vermiremediation) and inoculation of soils with earthworms (in situ vermiremediation). The sixth section discuss how to improve API vermiremediation by using biochar. The last section will identify knowledge gaps that require further research to boost the use of earthworms for enhancing the natural attenuation of agricultural soil against APIs and other organic pollutants.

2 Impact of Earthworms on Soil Quality

The term *soil quality* defines the "capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health

and habitation" [28]. This capacity is achieved by an exquisite interplay between inherent physicochemical and biological properties and processes, which are originally defined during soil formation or pedogenesis [29]. Many exogenous factors such as land use, agrochemical inputs, global warming and introduction of exotic species, among others, alter soil quality with the risk of causing its degradation (i.e. the loss of actual or potential productivity or utility of soil as a result of natural and anthropogenic factors [30]). Current knowledge on soil biology indicates that biodiversity is a pivotal property in soil quality [31–33], and conventional agriculture (defined as the agricultural practices that use synthetic pesticides and fertilisers in short rotation crops [34]) seriously threats it [35]. Therefore, promotion and maintenance of soil biodiversity is determinant to boost sustainable agriculture that ensures reasonably high crop yields and food security.

Earthworms are annelids belonging to soil macrofauna (i.e. organisms of >2 mm in size [36]) and exert a profound impact on soil quality. In general, these organisms alter soil microbial and mesofauna (0.1–2 mm, body size [36]) communities with indirect effects on nutrient cycling and soil biodiversity [37]. The continuous burrowing and feeding activities of earthworms create a complex network of permanent (anecic species) and temporary (endogeic species) galleries [38], which have led these organisms to be considered as "soil engineers" [39]. In fact, earthworms have a significant contribution in soil bioturbation, i.e. "the biological reworking of soils and sediments by all kinds of organisms, including microbes, rooting plants, and burrowing animals" [40], whereby they largely affect microbial population dynamics [41] and facilitate microorganism dispersion in soil [42]. These functional capacities have led earthworms to have a particular interest in agronomy and environmental sciences because of their beneficial effects on plant growth and development [43, 44], control of soil-borne pathogens [45, 46], indirect degradation of organic pollutants [47] and buffering effect in polluted soils [48]. However, the agronomic and ecological benefits depend on feeding habits of earthworms. Soil ecologists classify earthworms into three categories according to preferred soil habitats, feeding habits and morphological traits [36, 49–51]: epigeic, anecic and endogeic (Table 1).

Epigeic earthworms are small-medium sized, inhabit soil surface and feeding on decomposing organic residues accumulated on the soil surface (Fig. 2). Epigeic earthworms rarely burrow into the soil and ingest it, so they are little or no exposed to organic pollutants occurring in the mineral soil. Some species of this ecological group such as *Eisenia fetida*, *E. andrei* or *Lumbricus rubellus* are used in the composting of municipal and industrial organic wastes (vermicomposting) [52]. Anecic earthworms are large sized and create long, permanent vertical burrows and feeding on decomposing litter that collect from the soil surface and drag into the burrow or accumulate at the entrance of the burrow, forming a deposit of litter mixed with cast named "midden" [53]. They also ingest mineral soil to obtain particulate organic matter [49]. The middens are considered hotspots of organic matter decomposition and faunal diversity [54, 55].

Endogeic species are medium sized soil-dwellers and ingest large amounts of soil to obtain nutrients. Earthworms of this ecological group intensively built temporary

Table 1 Ecological features of earthworms[a]

Functional classification	Feeding regime	Pigmentation and body size	Longevity	Burrowing capacity	Mobility and predation	Examples
Epigeic	Feed on decomposing organic residues (litter), and associated microflora, accumulated on soil surface (decomposers)	Heavily pigmented (reddish), usually ventral and dorsally Small-medium size	Short lived with short generation times They survive drought in the cocoon stage	No burrowing activity or limited to topsoil (A horizon)	Rapid movements in response to disturbance Predated by arthropods, birds and mammals	*Eisenia fetida* *Eisenia andrei* *Lumbricus rubellus* *Lumbricus castaneus* *Dendrodrillus rubidus* *Dendrobaena octaedra*
Anecic	Feed on litter from the soil surface that they collect on night, although they also ingest soil to obtain nutrients	Darkly dorsally pigmented Large size	Long-lives with long generation times They survive drought in a quiescent stage	They construct and live in permanent, long, vertical burrows. Some species form organic residue stackings at the burrow's entrance called middens	Rapid withdrawal into burrow when disturbed, although they are slow moving on the soil surface They are predated when are at the soil surface	*Lumbricus terrestris* *Lumbricus polyphemus* *Aporrectodea longa* *Aporrectodea nocturna* *Dendrobaena platyura* *Octodrilus mimus* *Eophila tellinii*

Endogeic	Feed on the organic matter in soil (geophagous)	Unpigmented or lightly pigmented Medium size	Intermediate longevity with short generation times They survive drought by entering in diapauses	They make temporary, sub-horizontal burrows in the mineral horizon of soil (uppermost 10–15 cm soil deep)	Generally sluggish They are predated by ground-dwelling animals (e.g. moles) Slow-moving earthworms	*Aporrectodea caliginosa* *Aporrectodea rosea* *Aporrectodea icterica* *Allolobophora chlorotica* *Octolasion lacteum* *Octolasion cyaneum* *Octolasion tyrtaeum* *Proctodrilus oculata*

[a]Römbke et al. [57], and Briones [36]

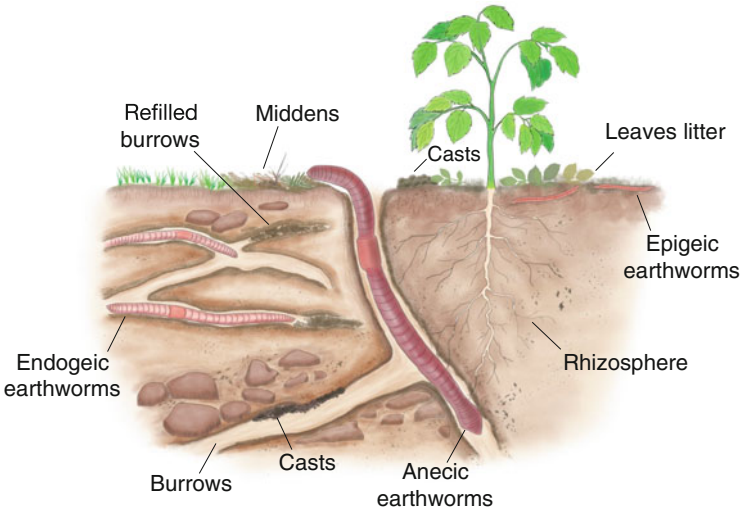

Fig. 2 Functional classification of earthworms. Epigeic earthworms are litter-dwelling which feed on organic matter accumulated on the soil surface. Endogeics are geophagous earthworms which construct subhorizontal, non-permanent burrows (they refilled the burrows with casts). Anecic earthworms built long, vertical, permanent burrows and feed on litter that collect from the soil surface and drag into the burrows. These earthworms also form an accumulation of litter mixed with casts around the burrow's entrance which is known as middens

horizontal burrows in the uppermost 10–15 cm of soil. This group is subdivided, in turn, into polyhumic, mesohumic and oligohumic endogeics, depending on the amount and quality of organic matter in soil [36]. Polyhumic endogeics are small filiform earthworms that live in the topsoil (A horizon) feeding on fine, organic matter-rich soil. Mesohumics are medium-sized endogeic earthworms that ingest soil with no selection (A and B horizon dwellers), whereas oligohumics are large-sized earthworms that live at higher depth soil (30–60 cm, B and C horizon dwellers) feeding on soil with a low amount and quality of organic matter [50, 56, 57].

3 Impact of Earthworms on Environmental Fate of Pharmaceuticals

The impact of earthworms on the environmental fate of APIs will depend on their ecological and biological traits. Many APIs are highly hydrophobic with K_{OW} values around 3.0 [58], so exposure of epigeic earthworms to APIs will be maximum as long as these chemicals are present in biosolids and manure applied to soil, or remain adsorbed to the organic matter-rich A horizon of soil where epigeic earthworms live. However, anecic and endogeic earthworms are suitable organisms to investigate the API transport in soil because of their constant burrowing activity. The feeding

behaviour of anecics means a vertical transport of APIs towards deeper soil layers, thus increasing the risk of plant exposure to these compounds via the root system. For example, some studies have reported that anecic (e.g. *L. terrestris*) and endogeic (e.g. *A. caliginosa*) earthworms facilitate translocation of water-soluble organic contaminants [59], metals [60], Ag nanoparticles [61] and microplastics [62, 63] from soil surface to deeper soil layers through the bioturbation process. Moreover, anecic earthworms could contribute to environmental fate of APIs via the following three processes: indirect microbial degradation occurring in the burrow walls and middens, vertical transport of APIs from the soil surface towards the deep soil via leaching through the burrows and via burying of API-contaminated litter, and trophic transfer of APIs to earthworm predators (e.g. birds). Past studies with pesticides [59], metals [64] and more recently with microplastics [65] also lead to hypothesise that APIs could be lixiviated by the action of earthworms. Likewise, the high microbial and mesofauna activity and diversity in burrow walls [66, 67] and middens [54, 68] make them hotspots for API biodegradation. In fact, a wide range of soil organisms such as springtails, enchytraeids, mites, nematodes and millipedes are generally found in earthworm casts and in the burrow linings [54, 66, 69].

All three ecological groups of earthworms will contribute to pollutant degradation in different ways, and the magnitude of this effect largely depends on feeding habits (litter feeders versus geophagous) and the impact on soil microorganisms, which are the major drivers of contaminant biodegradation. Furthermore, the burrow system holds a high microbial activity and biomass [67, 70], which is reflected in the higher enzyme activity of burrow walls respect to that in undisturbed soils [71–73]. Dissipation of APIs in earthworms' biostructures (burrow walls, casts and middens) needs to be further explored to know the impact of both anecic and endogeic earthworms in the environmental fate of APIs in agricultural soils. Recently, Briones and Álvarez-Otero [74] reported marked differences in the cuticle and epidermis thickness of the three ecological groups of earthworms. Anecic species have thickest cuticle (4.03 ± 1.6–5.72 ± 1.7 μm, range of mean ± SD) and epidermis (42.7 ± 16.7–46.3 ± 9.7 μm) than epigeic (cuticle = 1.51 ± 0.4–3.21 ± 1.5 μm, epidermis = 24.7 ± 5.2–39.4 ± 14.5 μm) and endogeic species (cuticle = 0.46 ± 0.15–1.22 ± 0.52 μm, epidermis = 31.1 ± 7.5–38.9 ± 10.5 μm). Beside the taxonomical and ecological meaning, these species-specific differences in the tegument thickness may be relevant in ecotoxicology. Past studies using *E. andrei* as model already demonstrated that the uptake of organochlorine pollutants takes place across the skin and the gastrointestinal epithelium [75]. Using a three-compartment model (soil-earthworm tissue-gut content), the researchers found that the uptake of organochlorine compounds via gastrointestinal tract was a significant bioaccumulation route for highly hydrophobic chemicals (log $K_{OW} > 6$) [75]. In addition, the transfer across the skin decreased as the K_{OW} value of the organochlorine compounds increased. Probably, the mucous secretion of skin and the cuticle layer contributed to reduce the uptake of highly hydrophobic pollutants through the skin. The role of the cuticle thickness in API bioaccumulation may be supported by the data in the study by Carter et al. [76]. These researchers compared the bioconcentration factors and uptake rates of four APIs (carbamazepine,

diclofenac, fluoxetine and orlistat), and found that *L. terrestris* had lower uptake rate constants through the skin (0.12–1.35 mL g^{-1} day^{-1}) than *E. fetida* (1.48–-4.46 mL g^{-1} day^{-1}). The variation in the cuticle thickness between both species could explain this marked difference in the API uptake rates [74], although contribution of other potential variables linked to experimental procedures (temperature of incubation, soil pH, feeding habit of earthworms) should not be excluded. Indeed, bioconcentration factors and uptake rate constants of APIs largely vary with the type of soil [77].

4 Pharmaceutical Toxicity in Earthworms

There is a huge body of literature dealing with the impact of APIs on soil microorganisms [19, 78]. Alterations in microbial community structure and microbial activity as well as emergence of antibiotic-resistant microorganisms are frequently detected in soil receiving APIs [79, 80]. However, toxicity of these substances on soil macrofauna is still scarce. Most data are obtained from laboratory incubation studies (standardised toxicity testing), which being important in a regulatory context for API marketing authorization [81], the outcomes provide limited information about the real impact on soil macrofauna in an ecological context. For example, the European Medicines Agency (EMA) guidelines recommend that assessment of API adverse effects on terrestrial ecosystems should follow the standardised acute toxicity tests issued by the Organization for Economic Co-operation and Development (OECD), such as OECD 207 [82] and OECD 222 [83], or the International Organization for Standardization (ISO), such as ISO 11268-1 [84], ISO 11268-2 [85] and ISO 17512-1 [86]. The recommended earthworm species in all these tests are *Eisenia fetida* and *E. andrei*. These two species display a set of advantages for running standardised toxicity testing such as the high reproduction rate, the ease of measuring the toxicity endpoints (e.g. mortality, body mass change, reproduction rate, behaviour), the low cost of maintenance in laboratory conditions and the availability of individuals from local suppliers (e.g. fishing stores, vermiculture centres).

Toxicity testing has revealed that *Eisenia* species tolerate API-contaminated soils compared to other soil organisms. For example, *E. fetida* was used in a standardised multi-test study to identify the ecological risk assessment of the antiparasitic ivermectin [87]. The earthworm was less sensitive to ivermectin with no mortality recorded after 28 days of exposure to soil spiked with 0.47–5.71 mg/kg dry soil respect to collembolan and predatory mites. Similarly, the acute toxicity of fluazuron (an insect growth regulator used to control ticks) was evaluated using *E. andrei* and *Folsomia candida*. The acaricide was lethal to earthworms at high concentrations (14d-LC_{50} = 111.3 mg/kg dry soil), reduced its reproduction rate (50% decrease respect to controls) at concentrations $\geq$20 mg/kg, and the animals avoided soils contaminated with $\geq$3.0 mg/kg fluazuron [88]. Likewise, the earthworms were also less sensitive to fluazuron than collembolans. *Eisenia andrei* and *F. candida* were also used for testing the acute toxicity of the veterinary pharmaceuticals nicarbazin

and monensin [89]. Nicarbazin was not toxic to both species at concentrations between 10 and 1,000 mg/kg dry soil, although monensin was lethal to earthworms (14d-LC_{50} = 31.6 ± 1.13 mg/kg, mean ± SD) and significantly decreased the reproduction rate of collembolans (28d-EC_{50} = 95.5 ± 28 mg/kg). The median lethal concentration of monensin for earthworms was similar to that reported in a previous study with *E. andrei* (28d-LC_{50} = 49.3 mg/kg dry soil) [90], although the incubation time was double than that of the study by Menezes-Oliveira et al. [89].

However, cautions must be taken when extrapolating outcomes from lab-scale toxicity testing to the field. First, the earthworm ecology and distribution should be considered in the environmental risk assessment of APIs. *Eisenia fetida* and *E. andrei* are epigeic earthworms, which mean that they live above the mineral soil surface and feed on plant litter [36]. These species rarely burrow into the soil as anecic and endogeic earthworms do, so exposure of epigeics to API-contaminated mineral soils should be lower than that for geophagous earthworm species [91]. Additionally, because agricultural soils are continually altered by tilling in successive crop seasons, *Eisenia* spp. are not abundant in these soils. Conversely, anecic and endogeic species are well represented in agroecosystems [92–94]. Second, the toxicity tests recommend the use of artificial soils (e.g. OECD soil or LUFA 2.2 soil), which obviously cannot be considered agricultural soils. A myriad of fluctuating variables of field soils may influence API degradation, bioavailability and mobility that are not considered in artificial soils, such as quantity and quality of organic matter content, microbial communities, aggregate distribution, etc. Third, the risk of species confusion in toxicity testing is another potential disturbing variable. In the case of *E. fetida* and *E. andrei*, both species can be easily confused with the risk of obtaining non accurate results. They are different species [95], with probably different responses (ecotoxicological biomarkers) to environmental pollutants [96]. Therefore, caution should be taken when using *Eisenia* spp. in the assessment of API toxicity. Finally, species-specific differences in earthworm sensitivity to environmental contaminants should be also considered when assessing API toxicity. For example, a meta-analysis study revealed that *L. terrestris* and *A. caliginosa* are more sensitive to pesticide toxicity than *E. fetida*, which questions the role of the latter for establishing environmental protection limits [97]. Indeed, earthworm species other than *Eisenia* spp. are now suggested as model organisms for standardised soil toxicity testing [91, 98, 99]. Therefore, despite the improvements made by EMA on the original guideline document for the environmental risk assessment of APIs [81] – discussed in Whomsley et al. [100] – the inclusion of other earthworm species highly representative of agroecosystems is not considered yet.

Earthworm biomarkers have been also included in toxicity testing as indicators of API bioavailability and to assess the potential adverse effects of APIs. For example, signs of oxidative stress (antioxidant enzyme activities and lipid peroxidation) and genotoxicity (DNA breaks) induced by chlortetracycline were observed in *E. fetida* incubated in antibiotic-spiked soils for 28 days, although such responses were not dose-dependent [101]. The researchers also found neither dead worms nor significant decrease in reproduction rate (number of juveniles and cocoons) at the highest antibiotic concentrations (100 and 300 mg/kg). Using the contact filter paper test

(OECD 1984), McKelvie et al. [102] investigated the nuclear magnetic resonance-based metabolomic profile of *E. fetida* exposed for 48 days to caffeine (19.3 μg/cm^2), carbamazepine (1,000 μg/cm^2) and estrone (1,000 μg/cm^2). These researchers found that carbamazepine and estrone caused a decrease in the concentration of certain metabolites in the whole earthworm body, although at a level of statistical significance of $\alpha = 0.1$. Despite the promising potential of metabolomics to elucidate the mode of action of APIs, several questions related to tissue-specific metabolic alterations or whether the natural environment (e.g. soil or organic matter-rich substrates) can modulate the earthworm metabolite profile remain unanswered at present. Genotoxic and oxidative stress have also been evaluated in *E. fetida* exposed to API-spiked soils by Dong et al. [103]. DNA damage assessed by the comet assay was the only biomarker that provided a consistent dose-dependent relationship with tetracycline, chlortetracycline, and the combination of both antibiotics. The antioxidant enzymes catalase and superoxide dismutase had erratic responses to the antibiotic exposure. The low number of replicates ($n = 3$ earthworm/treatment) in that study could be a limiting factor in concluding whether tetracycline, and chlortetracycline are oxidative stress inducers in earthworms.

Although the primary scope of ecotoxicological biomarkers is to predict adverse effects at individual and population levels, no study reports consistent data linking sub-individual level responses (e.g. DNA damage, antioxidant enzyme responses) with adverse effects at higher levels of biological organisation. Therefore, the impact of environmentally realistic concentrations of APIs on earthworms remains to be elucidated. Moreover, the functional association between biomarker responses and API toxicity is a challenge when the mechanism of toxic action in non-target organisms as earthworms is unknown. The reader can find a detailed analysis of earthworm biomarker applications in the Chap. 10 in this book.

The range of API concentrations in ecotoxicity testing normally are unrealistic, although they could represent a worst-case scenario defined by a continue input of APIs via biosolids application or irrigation with treated wastewater, low environmental degradation rate of APIs and soils with a high organic matter content. Nevertheless, the effective API concentrations estimated from laboratory toxicity testing are generally higher than those regularly detected in agricultural soils. For example, an acute toxicity testing with 18 pharmaceuticals using *E. fetida* and the standard OECD artificial soil revealed that only 8 drugs were lethal to earthworms after 14 days of exposure. The 14d-LC_{50} values were higher than API concentrations frequently found in soil, varying between 64.8 mg/kg (ibuprofen) and 3,298 mg/kg (propranolol) [104]. Therefore, data collected from standardised toxicity tests suggest that environmentally relevant pharmaceutical concentrations in soil, defined in the context of background concentrations reported in the literature, do not represent a serious risk to *Eisenia* species, at least at short term. However, because these epigeic earthworms are typically used in the aerobic decomposition of solid organic waste (particularly *E. andrei* [105, 106]), the question arises as: are API concentrations measured in cattle manure or biosolids high enough as to be toxic to composting earthworms, so compromising the vermicomposting process?

Soil-dwelling earthworms have also been used to test API toxicity and, like with epigeic earthworms, the results point out to a certain degree of tolerance. For example, toxic effects from the antibiotics tylosin and oxytetracycline were assessed using the endogeic earthworm *A. caliginosa* incubated in an agricultural sandy loam soil [107]. The researchers did not find significant effects after 21 days of exposure to the antibiotic-spiked soils (500–5,000 mg/kg dry soil). Therefore, assuming a certain degree of tolerance of soil-dwelling earthworm species to APIs, we propose that inoculation of agricultural soils with earthworms could be an eco-friendly strategy to alleviate potential toxic effects of these chemicals on soil microbial activity, and to reduce the uptake of APIs by plants. The next two sections provide an overview on how earthworms may function as "bioreactors" of API degradation in the feedstocks to be used as soil amendments as well as in agricultural soils.

5 Pharmaceutical-Contaminated Soil Amendments (Ex Situ Vermiremediation)

Fertilisation of agricultural soils with biosolids and treated (or untreated) manure is one of the main routes of soil contamination with APIs. Biosolids are stabilised organic materials resulting from treatment of municipal or industrial sewage that meet regulatory guidelines for its application as a soil amendment [108]. It is now recognised that biosolids application to agricultural lands increase the concentration of APIs in soil, the risk of surface water and groundwater contamination and the uptake of API (and metabolites) by plants [109]. Furthermore, biosolids application is between 5 and 50 times greater in forest and degraded sites than in agricultural soils [109], which represent a high ecological risk for soil biodiversity and soil biological processes.

One of the environmental risks of CAFOs is the occurrence of veterinary pharmaceuticals (e.g. antibiotics) in manure [110]. The high consumption of antibiotics in CAFOs together to the fact that antibiotics are not completely metabolised by animals [111], lead to their presence in urine and manure. The most frequently antibiotics found in animal manure belong to fluoroquinolones, sulfonamides and tetracyclines [110, 111]. Concentrations of these pharmaceuticals may be so high that direct application of untreated manure to soil is discouraged or forbidden. Accordingly, manure is aerobically or anaerobically treated to reduce the risk of soil contamination by APIs and other environmental contaminants and to obtain thereby value-added organic fertilisers. The most frequent treatments are composting, anaerobic digestion and accumulation in aerobic/anaerobic open-air ponds. Among them, composting provides the most technically easy and low-cost option, but there are still uncertainties about the extent of API biodegradation during composting. Although composting generally removes >90% of APIs [111], some studies show that this technique is not efficient for the full elimination of some types of APIS. For example, 17–31% of the initial concentration of ciprofloxacin

(fluoroquinolone) in swine manure was found in the resulting compost [112]. Similarly, composting of turkey litter spiked with some antibiotics led to the full removal of chlortetracycline, whereas reduction of monensin and tylosin varied between 54 and 76% of initial concentration and sulfamethazine was not removed at all [113]. It is postulated that sorption processes seem to be the most feasible elimination pathway for many APIs during composting [110, 111], thus hampering the mineralisation of these chemicals. However, most studies on composting-induced degradation of APIs do not consider the mass evolution of feedstock (e.g. formation of humic substances) during composting and the mechanisms underpinning the API degradation, so leading to inaccurate conclusions on the composting efficiency in the removal of APIs [114]. In addition, the impact of composting on API degradation has been a research topic mainly investigated at lab scale using API-spiked manures, so the aging effect has not been considered. Aging of hydrophobic organic pollutants in soil is a well-known phenomenon whereby pollutant availability and biodegradation decrease as the time that pollutants remain in soil increases [115]. A similar assumption has not been considered in composting studies of API-contaminated feedstocks where organic matter content is higher than that in agricultural soils. Likewise, complementary strategies such as vermicomposting (use of earthworms in composting of solid organic residues) have not been deeply investigated. Indeed, some benefits could be obtained with vermicomposting technology compared to aerobic composting. For example, the quality of compost, in terms of physicochemical properties, produced from green waste (trimmings and litter) was higher with vermicomposting than with composting [116]. Additionally, enzymes such as phosphatase and β-glucosidase showed a higher activity in the vermicompost than in compost, both produced from cattle manure [117]. The impact of vermicompost on soil physicochemical and biological properties was reviewed by Lim et al. [118], who concluded that vermicompost has a higher beneficial impact on plant growth and soil fertility than compost, because the former contains a larger amount of available nutrients and plant growth-stimulating substances (phytohormones), which probably degrade during the thermophilic phase of aerobic composting.

Vermicomposting is an oxidative process mainly driven by earthworms and microorganisms, whereby organic wastes are broken down and transformed into a fine and porous peat-like material named vermicompost [119]. This bio-oxidative process occurs in a mesophilic environment (<30°C) created by the continue activity of epigeic earthworms (e.g. *Eisenia* spp.), which aerate and facilitate heat dissipation during organic matter decomposition. Vermicomposting of organic waste has been described by Domínguez [106] in two actions: the earthworm gut-associated processes (GAPs) and the cast-associated processes (CAPs) (Fig. 3). The GAPs involve the physical break down (e.g. grinding in gizzard) and biochemical transformations of organic matter ingested by earthworms. Secretion of enzymes from the earthworm gut epithelium and exoenzymes secreted by gut symbionts provide a biochemical cocktail to decompose the organic matter [120, 121]. Nutrients are absorbed at the gut epithelium, and secretion of substances such as mucus, urea and ammonia will form the chemical composition of the egested material (casts). It is interesting to highlight that during GAPs, the initial microbial composition and activity of the

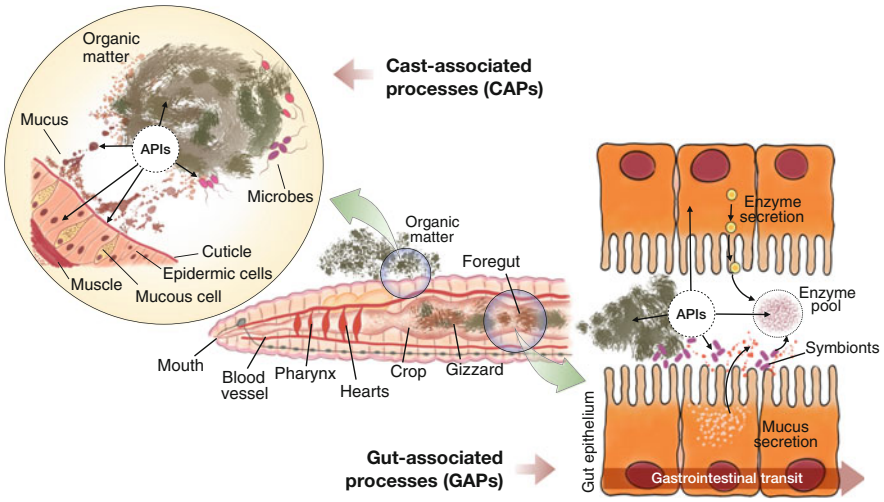

Fig. 3 Hypothesised model on vermicomposting of active pharmaceutical ingredient (API)-contaminated feedstocks (e.g. biosolids and manure). Fate of APIs during vermicomposting may follow multiple pathways (biodegradation or immobilisation) depending on the physicochemical properties of APIs and the biological cast-associated processes (CAPs) and gut-associated processes (GAPs) occurring during vermicomposting. In CAPs, pharmaceuticals may be bound to the cuticle, cross the earthworm tegument or bound to the organic matter of fresh feedstock, casts and mucus. Likewise, microorganisms of the feedstock and casts may degrade APIs. In GAPs, ingested pharmaceuticals may be breakdown by enzymes released from both symbionts and the earthworm gut epithelium. Additionally, APIs may be co-metabolised by symbionts or cross the gut epithelium. Adapted from Sanchez-Hernandez et al. [167] with permission from Elsevier

ingested material change during the gastrointestinal transit [121, 122]. Some studies have reported that pathogens generally occurring in cattle manure are significantly reduced in the earthworm cast probably as a consequence of the digestive processes occurring in the gastrointestinal tract of earthworms [123, 124]. The CAPs occur in the earthworm casts, and microorganisms and other decomposer fauna (e.g. collembolan) actively participate in the further decomposition of more recalcitrant organic wastes such as lignin, cellulose and hemicellulose (maturation stage). Therefore, CAPs prolong the decomposition of the feedstock although earthworms are no longer present. Indeed, changes in the enzymatic profile, microbial composition and nutrient concentration still happen in the maturation phase (earthworm free) of vermicomposting [125, 126].

In summary, it can be postulated that vermicomposting provides a source of microorganisms and extracellular enzymes with potential capacity for breaking down organic pollutants present in the feedstock (intrinsic remediation potential) and to remediate polluted soils when vermicompost is used as a soil amendment (extrinsic remediation potential).

Vermicomposting of biosolids and manure requires the assessment of three critical issues: (1) earthworm tolerance to APIs, (2) biodegradation of APIs and metabolites and (3) development of resistant microbial strains. For example,

vermicomposting of biosolids experimentally contaminated with tetracycline revealed that the concentration of 100 mg/kg had a stimulating effect on earthworm growth and organic matter decomposition, whereas that higher concentrations (500 and 1,000 mg/kg) led to a significant decrease of the decomposition process and to the emergence of antibiotic-resistant genes, thus compromising the quality and environmental safety of the final vermicompost [127]. Similarly, degradation of oxytetracycline and its main metabolites in chicken manure mixed with shredded paper waste was monitored in a co-composting system, which consisted in a first thermophilic composting phase followed by vermicomposting. Results from that study revealed that the additional phase of vermicomposting increased the degradation of oxytetracycline and its metabolite 4-epi-oxytetracycline in the feedstock containing a C:N ratio of 40 [128]. Despite these studies, there are still many unknowns on the efficiency of vermicomposting in reducing the concentration and toxicity of APIs and their metabolites. Furthermore there is no data available on the microorganisms and enzyme activities implied in API biodegradation, so that the vermicomposting process can be externally modified to facilitate removal of APIs.

Earthworms can accumulate biosolids-bound APIs. For example, *E. fetida* accumulated around 20% of ciprofloxacin and 40% of azithromycin present in soils amended with anaerobically digested biosolids which were contaminated with these antibiotics [26]. Although the study suggests ecological implications of the moderate bioaccumulation of APIs by earthworms, as these organisms may introduce APIs in food webs, their bioaccumulation capacity can be also regarded as an opportunity for removing APIs during biosolids vermicomposting.

6 Pharmaceutical-Contaminated Soils (In Situ Vermiremediation)

Soil bioturbation by earthworms has been exploited as a bioremediation strategy [129]. Earthworms are able to facilitate biodegradation of organic contaminants via three processes: (1) stimulating soil microorganisms, which may co-metabolise pollutants; (2) mobilising contaminants entrapped in soil organomineral complexes, thus rendering them bioaccessible to microbial biodegradation; and (3) altering the soil physicochemical properties (e.g. pH), which may contribute to contaminant degradation. Besides these external degrading processes, the gastrointestinal tract of earthworms contributes to contaminant degradation by the action of the gut symbionts and digestive enzyme secretion [47, 130]. Many studies have shown that earthworm activity in soils contaminated by environmental pollutants such as pesticides, polycyclic aromatic hydrocarbons (PAHs) or polychlorinated biphenyls (PCBs) reduces the initial concentration of these organic pollutants [47]. However, most of these studies have been performed under controlled conditions of laboratory (microcosm), and the real impact of earthworms in soil persistence of contaminants requires field validation [131]. Nevertheless, earthworm activity may also have no

effect on contaminant degradation rate. The most reliable explanation for this effect is the change in soil organic matter content and quality (humification) by earthworm activity. However, earthworms exert a positive effect on soil microbial activity and exoenzyme production even in the presence of environmental contaminants [48]. Taken together these studies suggest that inoculation of agricultural soils with earthworms could be a suitable strategy to remove or immobilise APIs, thus reducing the risk of being available to plants.

To date, remediation technology aimed to remove APIs is focused on the treatment of wastewater [2]. In soil remediation, only physical and chemical engineering systems have been tested in API-contaminated soils. For example, the electrokinetic technique, which consists of applying an electric field using two or more electrodes introduced in soil, has been used to remediate soils spiked with a mixture of sulfamethoxazole, ibuprofen, triclosan and caffeine [132]. The soil physicochemical alterations induced by the electric field, mainly on soil pH, caused a significant API degradation (13–85% of initial concentration) within 7 days of continual electrokinetic treatment (10 mA of current intensity). Among the chemical remediation methods, the use of the oxidant chemical persulfate alone or in combination with activating agents (iron), heat, alkaline chemicals or electrokinetic is widely used in the degradation of a variety of environmental contaminants such as PCBs, PAHs, pesticides, phthalates and APIs [133]. For example, ibuprofen (46–48 μM/kg soil) was fully removed from soils after a 60-min treatment with persulfate (20 mM/kg soil) activated by thermal treatment of soil (60°C) [134]. In a similar laboratory study, the antibiotic sulfamethoxazole was almost fully degraded (87.6% of initial concentration) in agricultural soils incubated for 4 h at 30°C with persulfate activated with nanoscale zero-valent iron (nZVI) nanoparticles [135]. However, persulfate-assisted remediation technologies have three main drawbacks: (1) alterations in the soil physicochemical and biological properties with potential adverse consequences to soil quality, (2) the need of external energy supply (e.g. electrokinetic technique and heating-activated persulfate treatment) and (3) the high costs associated with the application of these remediation techniques in real-field scenarios [132]. For example, remediation of ibuprofen-contaminated soils using both Fenton oxidation and nZVI nanoparticle methodologies led to toxic soils showing phytotoxicity [136].

Bioremediation of API-contaminated soils has not been extensively investigated. As with other organic pollutants, API dissipation is mainly due to microorganisms [23]. Additionally, aerobic conditions largely facilitate their degradation [137, 138]. Because anecic and endogeic earthworms continually aerate soil via the creation of burrows, they should be excellent “bioreactors” of API degradation. Table 2 summarises the main advantages and limitations of using soil-dwelling earthworms in the bioremediation of API-contaminated soils as well as some uncertainties that demand further research. The effect of earthworms on API degradation must be seen not only as a biodegradation process but also as a strategy of chemical immobilisation leading to reduce bioavailability and toxicity of these pollutants. Many studies have documented that the earthworm feeding activity and cast deposition on the soil surface and the burrow walls contribute to decrease the degradation

Table 2 Potential advantages and drawbacks of using earthworms in the bioremediation of pharmaceutical-contaminated soils and feedstock (biosolids and manure)[a]

Advantages
Increase of soil aeration via burrowing activity, so aerobic biodegradation of APIs may be facilitated (e.g. laccase activity)
Stimulation of microbial activity and exoenzyme production in burrow walls, casts and middens, these biostructures being hotspots for potential API biodegradation
Low or null API toxicity upon earthworms (epigeic and endogeic species), which means that vermicomposting and in situ vermiremediation could be viable strategies for removal APIs
Earthworms contribute to disperse soil microorganisms in soil and composting feedstock, so their use in these media should increase API biodegradation
Earthworm activity (feeding and burrowing) facilitates bioaccessibility of soil microorganisms (and earthworm gut symbionts) to APIs
Earthworm burrowing activity and dragging of organic residues into the burrows (anecic species) facilitate plant root development, thus being a complementary strategy for phytoremediation (non-food crops) of API-contaminated soils
Drawbacks
Viability of soil inoculation with earthworms largely depends on soil characteristics, climate conditions and crop management. Indeed, the system is only affordable in crops continually irrigated (e.g. drip irrigation) such as horticulture and fruit crops. Food supply is also required preferentially in the form of an organic mulching
Biodegradation of APIs could fail because of binding of the chemicals to organic matter (and humific organic matter), which is increased by earthworm activity. But such an effect can also be seen as an opportunity to reduce plant accumulation of APIs via root uptake
Introduction of exotic earthworm species in agroecosystem should be avoided or their introduction monitored to avoid dispersion and colonisation of non-agricultural soils
Uncertainties
Treated wastewater, biosolids and manure generally contain a mixture of different APIs, which occasionally coexist with other environmental contaminants (e.g. metals). Therefore, it is needed to know the potential mixture toxicity to earthworms and how earthworm-assisted biodegradation of APIs could be affected in the presence of other environmental pollutants
Metabolites of certain APIs are more toxic than the parent compounds, thus affecting the biodegradation process and increasing the toxicological risk for soil organisms and plants
Earthworms' interaction with plants (rhizosphere) and biochar could be a functional strategy for bioremediating API-contaminated soils while increases soil quality. However, further knowledge is still needed to recommend this combined system of bioremediation in the agroecosystem
It is well known that APIs alter soil microbial communities and may induce the emergence of antibiotic resistant microorganisms. Therefore, these chemicals could also induce earthworm gut dysbiosis (i.e. imbalance of gut microbial diversity). Knowledge on the impact of APIs (and metabolites) on earthworm gut microbial diversity is necessary to elucidate potential adverse effects on digestive processes, which could lead to vermicomposting failure (ex situ vermiremediation) or to a limited gastrointestinal decomposition of organic matter ingested with soil (in situ vermiremediation)
Pharmaceuticals are accumulated in earthworms, but detoxification (mainly performed in the chloragogen tissue) has not been investigated in detail. This topic requires further knowledge to propose vermicomposting earthworm species (*Eisenia* spp.) to clean biosolids and manure from APIs

[a]Elaborated from Sanchez-Hernandez et al. [162, 167], Morillo and Villaverde [131], Rodriguez-Campos et al. [47]

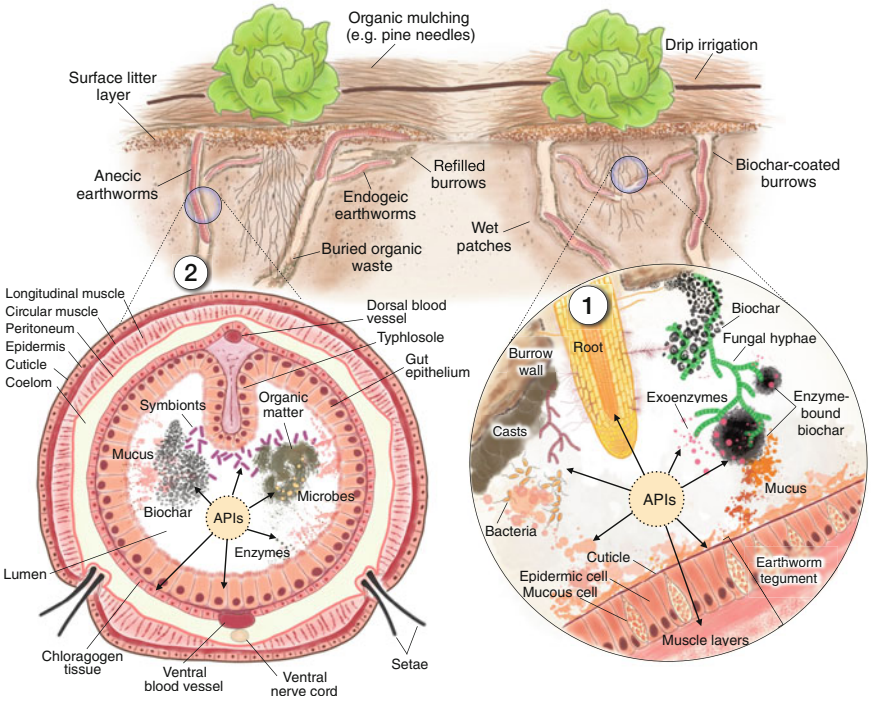

Fig. 4 Conceptual model of in situ vermiremediation of agricultural soils contaminated by active pharmaceutical ingredients (APIs). The system exploits the feeding behaviour of anecic and endogeic earthworms to improve soil quality and reduce API uptake by plants. In addition, biochar can be co-applied with earthworms to increase immobilisation of APIs. Fate of APIs is driven by the interplay between biological processes occurring in the earthworm biostructures (sphere 1: burrow walls, casts and middens), and those occurring in the gastrointestinal tract (sphere 2: cross-sectional view of the earthworm). Arrows denote the multiple pathways of API dissipation, which include microbial biodegradation, adsorption to organic matter and biochar, breakdown by exoenzymes, bioaccumulation and metabolism (e.g. in the chloragogen tissue)

rate of certain organic pollutants in these biostructures [71, 154]. However, because of the high organic carbon content and quality (humification) in casts and burrow linings, pollutants may result immobilised. The conceptual model in Fig. 4 explains how earthworms could participate in the bioremediation of API-contaminated soils. Such a bioremediation would consist in two complementary processes [130]: (1) external earthworm-depending inactivating processes and (2) earthworm gut-associated inactivating processes. Here, inactivating processes refer to biodegradation and immobilisation of APIs in soil, both actions rendering them unavailable to edible crops, thus reducing the risk of API exposure to consumers.

External earthworm-depending inactivating processes are mainly driven by microorganisms and mesofauna (e.g. nematodes, springtails, enchytraeids, mites and millipedes) associated with the structures created by earthworms (biostructures)

such as middens, casts and the burrow system (Fig. 4). The high nutrient content of these structures boosts microbial proliferation. Moreover, the presence of cutaneous mucus (burrow linings and middens) and gastrointestinal mucus (casts and middens) also provide a C-labile source for microfauna and mesofauna foraging. Many studies have examined the organic carbon dynamic and microbial community structure of earthworm casts [139, 140], burrow linings [72, 139] and middens [54, 55, 68]. All them conclude that these biostructures are hotspots of organic matter decomposition, displaying higher microbial and enzymatic activities respect to undisturbed soil [70, 71, 141]. Therefore, it can be assumed that earthworm biostructures are also microenvironments for API biodegradation. However, because of the organic matter content of biostructures, API may also be immobilised by binding to organic ligands, thus reducing their bioavailability and transport in soil [47, 142, 143]. Extracellular enzymes or exoenzymes represent also a pivotal mechanism of API inactivation. Enzymes such as phenol oxidases (laccases) and peroxidases (manganese peroxidase and lignin peroxidase) are actively involved in the oxidative metabolism of organic contaminants including APIs [144, 145]. For example, laccase from the white-rot fungi (lignin degraders) *Trametes versicolor* removed 100%, 95% and 85% of diclofenac, trimethoprim and carbamazepine, respectively, from aqueous enzymatic preparations [146]. Similarly, peroxidases from multiple biological sources are also able to degrade (>80%) many APIs such as triclosan, carbamazepine, naproxen and antibiotics [145]. Many other white-rot fungi species degrade anticancer drugs via oxidative reactions catalysed by laccases and peroxidases [147]. These enzymes are produced and excreted to the environment by soil microorganisms [148], and the presence of lignocellulosic-rich organic matter induces their production [149]. Furthermore, laccase activity requires molecular oxygen, so earthworm burrowing activity should facilitate laccase-mediated degradation of organic pollutants [150] because of soil aeration increase. Therefore, API dissipation by these exoenzymes should be a potential biodegradation process, particularly in earthworm biostructures.

Earthworm gut-associated inactivating processes involve gut microbiota and the enzymes secreted by the earthworm gut epithelium (Fig. 4). Many digestive enzymes have been measured in the gastrointestinal content of earthworms such as lipases, esterases, chitinases and cellulases [151–153]. Furthermore, laccase activity has also been found in the gastrointestinal content of epigeic and endogeic earthworms, although its activity level is low respect to other digestive enzymes [154], an expected finding if one considers that the earthworm alimentary canal is anoxic [155] and laccases require molecular oxygen. However, laccase activity has been measured in the casts of some earthworm species [141], suggesting that microbial-mediated oxidative metabolism occurs in these biostructures. Carboxylesterases are other group of enzymes with potential to metabolise pharmaceuticals and illicit drugs containing the ester bond such as capecitabine, cilazapril, clopidogrel, cocaine, dabigatran etexilate, enalapril, heroin, imidapril, irinotecan, meperidine, methylphenidate, olmesartan, orlistat, oseltamivir, quinapril, ramipril, temocapril and trandolapril [156]. Some of these compounds are detected in reclaimed wastewater, surface water and groundwater [157, 158]. Interestingly, carboxylesterase activity

has been found in the gastrointestinal tract of several earthworm species [153, 159] and in soil disturbed by earthworms [160]. However, it has not been demonstrated if the earthworm-induced carboxylesterase activity hydrolyses ester-containing APIs as mammalian carboxylesterases do [156, 161].

The persistence of exoenzymes largely depend on the organomineral complexes of soil [162]. Binding of exoenzymes to clays and organic matter protect them from physic stress (soil desiccation or high temperature) and microbial foraging [163]. With this premise, biochar technology has been proposed as an environmentally compatible approach to stabilise exoenzymes and concentrate their activity in soil for agronomic and remediating purposes [164]. The next section discusses how biochar may synergistically improve the earthworm-assisted bioremediation of contaminated soils.

7 Biochar-Improved Vermiremediation

In the last decade, biochar technology has emerged as a remediating strategy to eliminate a wide range of both organic and inorganic pollutants from water and soil [165–168]. Biochar is simply charcoal, but it is used as a soil conditioner instead of being used for energy generation [169]. This carbonaceous material is produced by pyrolysing solid organic feedstocks (e.g. manure, wood chips, pine needles, spent coffee grounds, municipal biosolids, nut shells, corncob, rice straw, switchgrass, and many others) under anoxic environment and temperatures between 250 and 700°C [169, 170]. Biochar has been used in the remediation of API-contaminated wastewater [171, 172]. Some studies even suggest that biochar may be an ideal material in filtering drinking water because of its excellent capacity to adsorb many inorganic and organic pollutants, including APIs [173]. However, the remediation capacity of biochar depends on the type of feedstock and the pyrolysis temperature which, in turn, have a strong influence on the physicochemical and structural properties of biochar [174]. Pyrolysis temperatures above 450°C generally produce biochar suitable to be used in bioremediation of contaminated soils because of its higher specific surface area, open porosity, alkalinity, hydrophobicity, density of aromatic groups and lower oxygenated functional groups on the surface compared to biochar produced at temperatures below 450°C [175]. For example, wheat straw-derived biochar produced at 700°C had a higher adsorption capacity for ketoprofen, atenolol and carbamazepine than biochar produced at 300°C [176]; a marked difference in the specific surface area between both biochars explained the biochar-specific adsorption of these APIs (605 m^2/g for 700°C-biochar versus 6.47 m^2/g for 300°C-biochar). Moreover, physicochemical properties of biochar other than the specific surface area seem to be involved in API adsorption. For instance, a laboratory study that compared the sorption behaviour of sulfamethoxazole in eight types of biochar (bamboo, Brazilian pepper wood, sugarcane bagasse and hickory wood, produced at both 450 and 600°C) evidenced that only the biochars derived from sugarcane bagasse and bamboo at 450°C had the highest capacity for retaining

sulfamethoxazole [177]. This high sorption ability was corroborated in soil column tests (2% w/w biochar), which led to propose those biochars as soil amendments to reduce API leaching potential. Researchers of that study also postulated that the occurrence of functional groups on the biochar surface would explain the high sorption capacity of the biochars produced at 450°C.

The pH is another environmental variable that facilitates API sorption onto biochar surface. Sorption of triclosan and ibuprofen significantly increased in solution of pH between 4.0 and 7.0 [178]. Furthermore, the occurrence of humic substances in the aqueous phase reduced the sorption of APIs to biochar because of two reasons: the binding of APIs to the dissolved humic substances and/or blockage of the open pores of biochar by humic substances, thus hampering the interaction between biochar and APIs [178]. These observations suggest that in alkaline soils or soil with a high organic matter content, biochar may fail in its capacity of binding APIs. Despite these interfering factors, what it seems clear is that pH <7.0 favours adsorption of APIs to biochar surface, irrespectively of the soil type [179].

Biochars produced at low pyrolysis temperatures (<450°C) are more appropriated for soil fertilisation. They generally contain non-pyrolysed organic matter susceptible to be foraged by soil microorganisms; therefore its application causes an increase of soil microbial activity and biomass [175]. This type of biochars has a low specific surface area and porosity, which reduces its capacity to retain agrochemicals such as herbicides [180], therefore not compromising the agronomic purpose of pesticide treatment [181].

The scope of adding biochar to API-contaminated soils is decreasing API bioavailability and toxicity to plants. Indeed, bioaccumulation of APIs by plants is substantially reduced in biochar-amended soils. For example, the application of biochar produced at 700°C to soil (5% w/w) reduced a 86% and 63% the uptake of 5 and 50 mg/kg sulfamethazine, respectively, by lettuce (*Lactuca sativa*) [182]. Similarly, carbamazepine and propranolol concentrations were markedly lower in *Lolium perenne* grown in API-spiked soils amended with biochar produced at 450–520°C than plants grown in biochar-free, API-spiked soils [183]. However, the adsorption of APIs on the biochar surface could have two side-effects: (1) an enhanced toxicity on soil microorganisms because of progressive accumulation of APIs on the biochar surface [177] and (2) the failure of API biodegradation because of limited bioaccessibility for microbial degradation [80]. One strategy that could partially solve these biochar-linked side-effects could be the co-application of earthworms and biochar.

Past studies have reported no clear synergistic effects from co-application of earthworms and biochar on soil microbial communities [184] or soil enzyme activities and plant growth [185]. However, a recent investigation evidenced beneficial effects of the co-application of *A. caliginosa* and willow chip-derived biochar on the abundance of springtails and soil fungal biomass after 6 months of incubation (1% w/w biochar in 2.65 L of soil holding 4 adult earthworms), although such positive interactions depended on the soil type [186]. Moreover, some studies have shown that incubation of earthworms (*L. terrestris* and *A. caliginosa*) in the presence of pine needle- or spent coffee ground-derived biochar caused a significant increase of soil

extracellular enzymes linked to C-, P-, and S-cycling, which were bound onto biochar surface [187]. The earthworm mucus produced by the skin mucous cells and the gastrointestinal epithelium was postulated as the main mechanism of enzymatic activation of biochar [73]. The functional system created by the co-application of earthworms and biochar was proposed as a strategy for removing organic pollutants from contaminated soils and feedstocks [164]. We propose an identical model for the in situ degradation or immobilisation of APIs in agricultural soils (Fig. 4). Whether or not this bioremediation strategy is viable will depend mainly on the following variables, which require further investigation:

1. *Earthworm species and exotic species.* Figure 2 illustrates the feeding strategies of epigeic, anecic and endogeic earthworms. Both anecic and endogeic species are soil engineering organisms because of their intensive burrowing activity [39]. Moreover, some laboratory experiments have shown that anecic and endogeic species can co-exist in a limited volume of soil. For example, the burrowing activity of *L. terrestris* was not affected by the presence of *A. caliginosa*, although the depth of the burrow system created by the anecic species was shorter than the burrow structure created when the species was incubated alone [188]. Moreover, the burrowing activity of *A. caliginosa* was favoured by the presence of the anecic earthworm *Aporrectodea giardi*; the organic matter-rich walls of the burrows created by *A. giardi* served as a food source to *A. caliginosa* [188]. These examples suggest that co-application of earthworms of different ecological strategies to soils contaminated with APIs could be the best option for obtaining the maximal benefit from earthworm activity on API dissipation. Environmental fate of APIs should be, therefore, investigated in soils holding a wide representation of the most common earthworms found in agricultural soils [92–94], ideally covering the three ecological groups of earthworms (Fig. 2). In our model of in situ vermiremediation, particular concern should be put on the introduction of earthworm exotic species (the term refers to not naturally occurring species, so-called alien species, in the location in which it is found [189]). Indeed, one of the objectives of the United Nations Sustainable Development Goal no. 15 (Life on land, www.undp.org) is "to prevent the introduction and significantly reduce the impact of invasive alien species on land and water ecosystems...". Therefore, care must be taken when we chose in situ vermiremediation. Endogenous and exogenous features of earthworms such as feeding behaviour (epigeic, endogeic and anecic), tolerance to environmental changes (phenotypic plasticity), reproductive characteristics, morphological characteristics and locomotion as well as environmental variables (edaphic and climatic conditions, presence of predators, and substantial and continue surface litter layers, among others) are important invasiveness traits to be considered before adding earthworms to agricultural soils [70].
2. *Earthworm tolerance to biochar.* Many studies have investigated the potential toxicity of biochar upon earthworms. Doses of biochar $\leq$2.0% (w/w) generally are tolerated by different earthworm species as indicated by the absence of significant avoidance response to biochar-amended soils [190]. However, signs

of oxidative stress are frequently found at those biochar doses in *E. fetida* [191] and *L. terrestris* [192], although some studies have reported no oxidative damage in *E. fetida* exposed at doses of biochar >2% [193]. Despite these contrasting results, further research is still needed to know long-term effects of earthworm inhabiting biochar-amended soils. For example, a 6-month mesocosm study with *A. caliginosa* incubated in two different soils evidenced that the synergistic effects of earthworms and biochar (1% w/w) increased the abundance of other soil organisms such as springtails and fungi, beside to improve soil fertility and plant growth [186]. Similarly, a 2-year field experiment examined the impact of biochar applied on topsoil (10 cm depth) at application rates of 10, 25 and 50 t/ha (corresponding to 0.6, 1.5 and 3% w/w, respectively) on both soil macrofauna and mesofauna [194]. The study revealed that, although the abundance of earthworms decreased as the concentration of biochar increased, biochar did not cause a significant impact on earthworm community structure, and the dose of 0.6% did not alter earthworm species richness compared to that of control (biochar-free) soils. Conversely, it was found a significant increase in the abundance of enchytraeids, mites and collembolans at the highest doses of biochar. In other field study, researchers observed that biochar applied at 5 and 10 t/ha was no toxic to macrofauna and also caused an attraction effect to earthworms after 2 years of application [195], thus recording a twofold density of earthworms in the soils that received 10 t/ha biochar respect to control (biochar-free) soils. Factors such as type of biochar and application rate, type of soil, climatic conditions, time of exposure and microbial community generally modulate the earthworm response to biochar-amended soils. Taken together these studies encourage biochar application rates of around 1% (w/w) on topsoil to be compatible with fauna diversity and abundance, and to exploit the potential synergistic effects of earthworms and biochar to immobilise or degrade APIs.

3. *Pharmaceutical toxicity and accumulation in earthworms.* To date, most of toxicity tests with APIs have been performed using *E. fetida* and *E. andrei* as model organisms (discussed in Sect. 4 of the chapter), and data show that these earthworm species tolerate high API concentrations compared with other soil organisms (e.g. [87]). Therefore, the use of epigeic earthworms in the vermicomposting of API-contaminated feedstocks could be a workable strategy. However, the sensitivity of anecic and endogeic earthworms (Fig. 2) to APIs should be explored in order to apply them in the in situ vermiremediation strategy (Fig. 4). In addition, API toxicity has been generally evaluated using a single chemical, and API mixture or even API molecules mixed with other environmental contaminants commonly detected in agricultural soils have not been investigated. As discussed in previous sections, a wide variety of APIs is generally found in reclaimed wastewater and biosolids, so exposure of soil fauna to an API mixture is probably the most real scenario. Similarly, API biodegradation should be also studied in the context of multiple environmental contaminants co-existing in agricultural soil.
4. *Toxicity of API metabolites*. Biodegradation of APIs in soil not necessarily lead to full mineralization. For example, a laboratory study reported that mineralisation

of triclosan (1, 10 and 100 mg/kg) in soils varied between 5.8 and 6.5% (cumulative recovery of $^{14}CO_2$) over a period of 42 days [80]. The finding suggests that metabolites may persist in soil with potential toxicity on soil organisms and soil function. For example, triclosan is photochemically decomposed into the toxic metabolites 2,8-dichlorodibenzo-p-dioxin (2,8-DCDD) and 2,4-dichlorophenol (2,4-DCP), which are very unstable in aqueous solutions [196], but their organic carbon-adsorption coefficients (K_{OC}) suggest a high affinity for the soil organic matter (log $K_{OC} = 3.2$ for 2,8-DCDD and log $K_{OC} = 2.8$ for 2,4-DCP; estimated values generated using the EPISuite™ software, USEPA, www.chemspider.com).

5. *Synergistic effects of APIs and other environmental contaminants*. A vast variety of organic and inorganic pollutants may occur in agricultural soils. For example, PAHs, PCBs, polybrominated diphenyl ethers and phthalates are frequently detected in agricultural soils irrigated with reclaimed wastewater or fertilised with biosolids or municipal composts [197–200]. Additionally, chemical control of agricultural pests may lead to accumulation of pesticides in soil. Therefore, toxic effects and degradation of APIs should be investigated in a context of pollutant mixture, which is the most realistic scenario in the agroecosystem. Furthermore, the high capacity of biochar to retain environmental pollutants, including APIs [176, 201], may result in toxic biochar at long term because of high concentrations of pollutants onto its surface. Therefore, this concern must be clarified in detail to know whether biochar could behave as a secondary source of soil pollution under specific soil conditions (e.g. changes in pH, moisture or biodiversity).
6. *Life cycle assessment for earthworm-biochar bioremediation technology.* Life cycle assessment (LCA) consists of a set of standardised and robust tools for appraising the efficiencies of methodologies and processes aimed to attend the decision-making related to environment protection and efficiency of the process (ISO14040:2006, ISO 2006). In the case of bioremediation of contaminated sites, LCA has been used to identify adverse impacts from the application of remediation strategies and consequently to take alternative remediation actions [202]. LCA can be used before initiating the remediation action (predictive) to select the best option according to technical, economic and environmental variables or when the remediation action is completed (prospective LCA). In the latter case, the scope of LCA is to know the environmental impacts derived from the applied remediation technology. For example, an LCA study of systems for biochar production revealed that some issues such as costs related to the pyrolysis process as well as feedstock selection, management and transportation hampered the economic viability of biochar technology, therefore compromising its affordability as a strategy for climate change mitigation [203]. The systematic review by Matustík et al. [204] on LCA of biochar technology evidenced that although the application of biochar to agricultural soils provides important environmental and economic benefits, there are still some issues that require further understanding and improvements such as the mechanisms underpinning the biochar effects on soil quality and crop yield and the use of low-tech pyrolysis systems

(e.g. Kon-tiki flame curtain kilns [205, 206]) accessible to small-scale rural farming. A detailed step-by-step description of LCA is beyond the scope of this chapter but can be found in the handbook by Hauschild et al. [207], and several reviews [202, 208] in which cases study are discussed.

8 Conclusions

Crops need healthy soils, but their fertility is under permanent threat of degradation by multiple environmental stressors (e.g. high agrochemical input, nutrient imbalance, loss of soil biodiversity, salinisation and decrease of organic matter). Additionally, water consumption for crop irrigation is a serious challenge in the coming years because of the global climate change, particularly in areas of arid and semiarid climates. The use of by-products derived from wastewater treatment plants such as biosolids and treated wastewater seems an affordable solution to alleviate the water and organic matter demands in the agriculture. However, both biosolids and treated wastewater contain significant amounts of APIs that pose a serious threat to soil functioning and human health.

One of the strategies for removal APIs at the source or in agricultural soils is the vermiremediation (i.e. use of earthworms to remove environmental pollutants). Earthworms provide multiple ecosystem benefits, from improve soil quality and fertility up to be used in the recycling of solid organic waste (vermicomposting). All these ecosystem services require the intervention of microorganisms. Indeed, microbes, earthworms and their interactions are proposed as a vermiremediation strategy to remove APIs. Many ecotoxicological studies with earthworms indicate that these organisms may contribute to contaminant degradation by stimulating microbial degraders, or may reduce contaminant mobility and bioavailability by facilitating sorption of contaminants to soil organic-mineral complexes. Likewise, certain earthworm species (epigeic earthworms) are commonly used in the aerobic composting of solid organic residues to produce organic fertilisers (vermicompost). Data in the literature reveal that vermicomposting may be also a viable strategy for removing organic contaminants occurring in raw materials such as biosolids and manure. Based on this knowledge, we propose two bioremediation strategies to reduce the risk of API uptake by plants and the potential adverse effects on soil microorganisms. The first system consists of vermicomposting of API-contaminated biosolids and manure (ex situ vermiremediation), whereas the second one involves the inoculation of agricultural soils with earthworms (in situ vermiremediation). In the last decade, biochar has emerged as an eco-friendly strategy for fighting against soil pollution. Because recent studies indicate that the co-application of earthworms and biochar improve soil quality in terms of microbial proliferation and soil detoxification, the in situ vermiremediation system considers also the synergistic effects of soil-dwelling earthworms and biochar in the removal or immobilisation of APIs. Main advantages, drawbacks and uncertainties in the use of earthworms in API

inactivation are summarised in Table 2 in an attempt to encourage future research in this field of bioremediation.

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Constructed Wetlands and Phytoremediation as a Tool for Pharmaceutical Removal

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Contents

Abstract Constructed wetlands are one of the most often applied nature-based solutions for water management. This ecotechnology is widely accepted due to its robustness to treat wastewater. The assessment of organic carbon and nutrients removal for conventional wastewater treatment has been documented for nearly 70 years. In the recent decade, interest has increased in regard to their performance to treat water contaminated with pharmaceuticals. In 2020 we have passed 200 publications on the latter. Therefore, there is a fair amount of knowledge available to

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discuss the applicability of constructed wetlands to control the emission of pharmaceuticals. The current chapter aims to (1) provide an insight to the performance of constructed wetlands under a variety of configurations and design options for the removal of pharmaceuticals; (2) discuss removal processes, namely, plant and biological-driven biodegradation, the challenges in its application and reproducibility, the knowledge gaps and the future trends; and (3) link constructed wetland usage and developments with the recent trends of nature-based solution and phytoremediation implementation towards a green transition.

Keywords Biofilters, Combined sewage overflow, Contaminants of emerging concern, Nature-based solutions, Organic micropollutants, Wastewater, Water reuse and reclamation

1 Introduction

Pharmaceutical release into the environment is one of the largest researched topics of the past decades within the environmental field [1, 2]. More than 15,000 publications are available on the topic, among which, 2,773 are review papers (accessed March 2020 on Scopus). Naturally, several subtopics emerged [3], e.g. from effect directed analysis of these compounds to answer questions on their toxic effects; non-target screening analytical analysis to understand what other compounds, human metabolites and transformation products are occurring; and development of suitable treatment technology or the potential impact to crops and consequently to humans and animals due to consumption of potentially contaminated crops.

Compounds like diclofenac (an anti-inflammatory drug) and erythromycin (an antibiotic) are already listed as candidate contaminants by the European Union (EU) and the United States Environmental Protection Agency (EPA). However, only a small number of compounds are currently covered by legal regulations, namely, with reference to water contamination. Current policy approaches to manage pharmaceutical residues are considered inadequate for the protection of water quality and freshwater ecosystems [4]. In EU, the creation of the NORMAN Network was a stepping stone to support the monitoring of emerging environmental substances [5], and the ongoing implementation of the Water Framework Directive has increased the debate around the list of priority substances and substances of concern. The Organisation for Economic Co-operation and Development (OECD) has called for a global discussion on moving towards proactive policy action to curb pharmaceutical pollution [4].

Earlier on, two waste streams have been considered the major source of pharmaceuticals to the environment: wastewater [6–8] and biosolids [9, 10]. A major research effort has been placed on wastewater [11], not only in the occurrence but also in the treatment technology – *how can these compounds be effectively removed?*

However, while more efficient treatment technology is still being developed [12], there is also increasing interest on water reuse and water reclamation for crop irrigation [13], as well as biosolids (either as sludge [14] or manure [15]) application on land for resources recovery. Therefore, there is a raising concern with the overall fate of pharmaceuticals, and the topic of manure application raises added concern due to the contamination with antibiotics and the issues with antibiotic resistance [16].

Water treatment technology is in itself a very broad topic. The present chapter will focus on constructed wetlands (CWs). Constructed wetlands are a widely accepted and robust wastewater treatment technology, which enables many different kinds of wastewater to be treated in a cost-efficient way [17]. CWs are one of the most often applied nature-based solutions for water management [18], namely, for the treatment of domestic and agricultural wastewaters, coal mine drainage and stormwater run-off; mainly because of a set of beneficial features, including environmental quality preservation, landscape conservation and economic convenience [19]. In addition, CWs have been successfully implemented for diverse agriculture and industrial sectors [20], such as seafood-processing industry, olive mill industry, dairy, alcohol fermentation industry and abattoir industry. High-pollutant loading rates and toxic substances can be effectively treated with CWs; thus, they have great potential for implementation in low-income countries and rural areas [21]. More recently, CWs have been also proposed for the treatment of greywater in urban areas, including by coupling CW with disinfection units such as ultraviolet radiation disinfection and chlorination to reliably meet the standards for reuse [22]. However, further concerns arise when it comes to the potential contamination by pharmaceuticals and other emerging contaminants.

In recent years, CWs application as an advanced treatment unit to address contamination by emerging organic micropollutants, including pharmaceuticals has been attracting increasing attention [23]. An unsupervised search on Scopus in March 2020 for "constructed wetland" *AND pharmaceuticals* retrieved 210 publications, 26 of each review papers. The earliest publication dates from 2004 – indicating quite an active topic of the research in CWs for the past 16 years. From the review papers covering the topic, some contain just brief mentions to CWs within broader topics [24, 25], while others provide extensive overview on the potential phytotoxic effect of pharmaceutical to plants [26] or are totally dedicated to mitigation of pharmaceutical contamination by CWs [27, 28]. There is, therefore, an interesting amount of information available providing a good overview of the overall performance of these systems, the removal processes and their limitations. The current chapter aims to:

1. Provide an insight to the application of constructed wetland technology under a variety of configurations and design options in regard to pharmaceutical removal
2. Discuss removal processes, namely, sorption, photodegradation, plant and biological-driven biodegradation, the challenges in its application and reproducibility, the knowledge gaps and the future trends

3. Link CWs usage and developments with the recent trends of nature-based solution and phytoremediation implementation towards a green transition

2 Constructed Wetlands for Pharmaceuticals Removal

2.1 The Basics of the Ecotechnology

First of all, it is important to clarify that "constructed wetland" is currently a broad ecotechnology name that encompasses a range of different technical solutions. A review of CW technology is outside the present scope, thus a good starting point for learning the basics of these systems would be the most cited text book on the topic by Kadlec and Wallace [29]. For the present chapter, one needs to take into consideration the three classical designs (based on hydrological characteristics – the water position and flow direction):

- Surface flow (SF; usually 0.3 m shallow beds with only a small layer of substrate at the bottom (few cm) for plants to root; water flows horizontally above substrate; fed continuously; Fig. 1a)
- Horizontal subsurface flow (HSSF; usually 0.5 m depth bed filled with substrate, sand and/or gravel; water flows horizontally below the surface; fed continuously; Fig. 1b),
- Vertical flow (VF; usually 1 m depth bed, filled with substrate, sand and/or gravel; water flows vertically top to bottom; fed by pulses; Fig. 1c).

For a broader overview of the different CWs designs and subdivisions in the classification, one can refer to Fonder and Headley [30]. For a question of systematization, the present chapter follows the most common grouping used in the published reviews within the field, the three classical designs: SF, HSSF and VF.

In the recent years, among other alternatives and intensified wetland designs, aeration at the bottom of the beds has been one of the most well accepted approaches to increase performance and decrease area requirements [31, 32]. Aeration has been introduced mostly in HSSF and VF designs and studies exist regarding its performance with respect to pharmaceuticals, further detailed. Other types of intensification (e.g. fill and drain, or usage of specific sorbing media) are not covered in this chapter.

The three classical designs, SF, HSSF and VF are the ones most exhaustively used for water treatment, while aerated CWs have been gaining popularity for the past decade [23, 33]. It is very important as well to mention the hybrid systems, which combine more than one type of CW to make use of different processes (e.g. VF + HSSF, for aerobic followed by anaerobic processes [34]). In addition, it is important to consider that any given design can be employed for different types of water (e.g. stormwater, wastewater, surface water). Specific designs are preferred for certain types of water and pollutant loadings (e.g. VF or aerated systems when nitrification is demanded); more details can be found in traditional CW literature and design guidelines [29, 35, 36]. The key point is that depending on pollutants loading

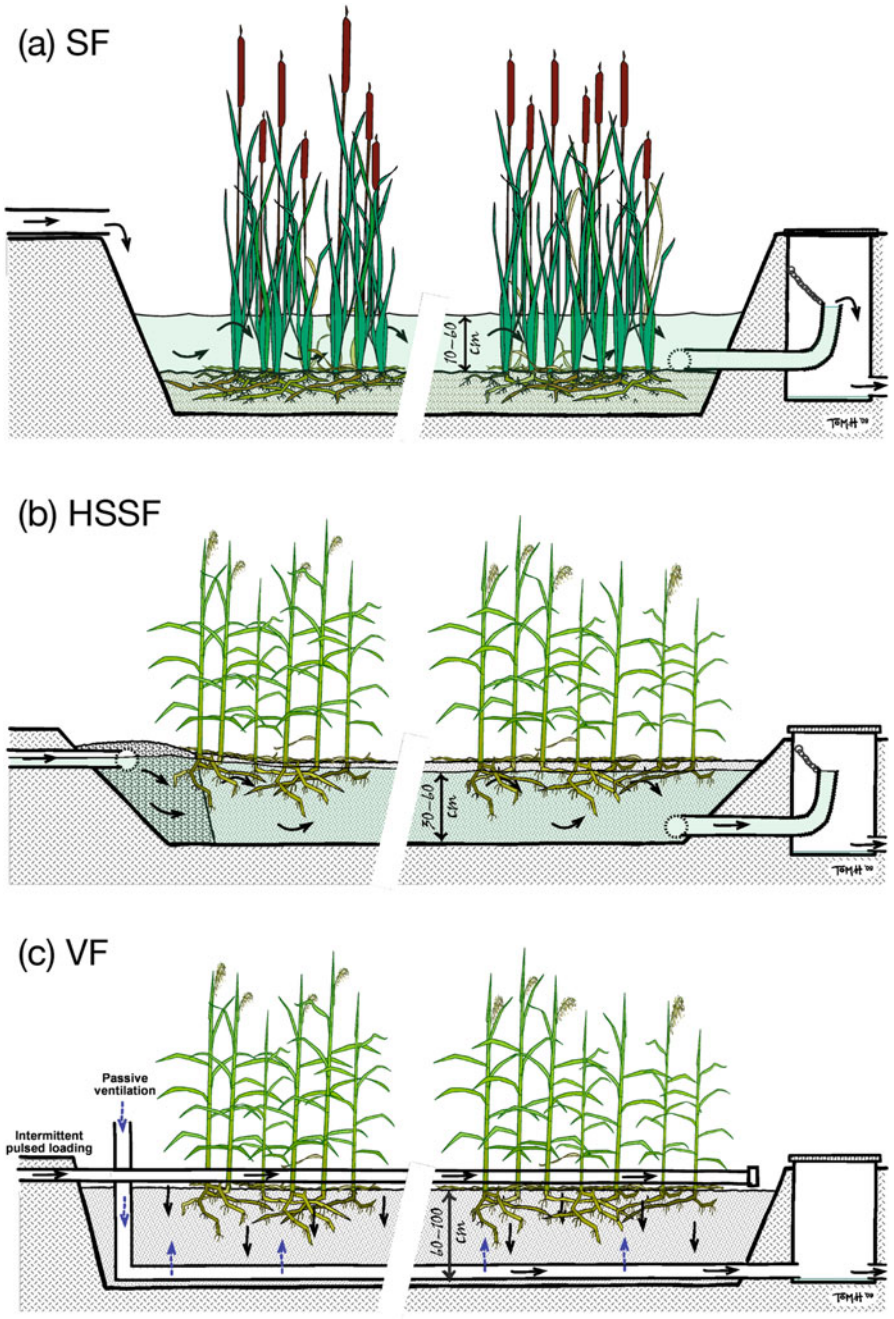

Fig. 1 The three classical types of constructed wetlands (**a**) surface flow (SF), (**b**) horizontal subsurface flow (HSSF) and (**c**) vertical subsurface flow (VF). Reprinted from Ecological Engineering (51), Fonder and Headley, The taxonomy of treatment wetlands: A proposed classification and nomenclature system, 203–211, Copyright (2013), with permission from Elsevier [30]

and operation strategy, the different designs SF, HSSF, VF, aerated or hybrid designs can behave very differently not only hydraulically but also in terms of pollutant removal, due to different removal/transformation processes.

The research on CWs for pharmaceuticals phytoremediation has been mostly focused in wastewater, the major source of pharmaceuticals to the environment. Combined sewer overflows (CSOs) can also be a relevant source of pharmaceuticals and CWs can be an effective technology to treat CSOs, including to control pharmaceuticals emission [37], but literature is scarce for this specific application. Agricultural run-off is also of importance when manure or sludge is applied to land, and pharmaceutical compounds might leach from the soil. However, the use of CWs to control agricultural run-off has been mostly studied for pesticides [38–40] and not for pharmaceuticals. Therefore, lessons learned from wastewater treatment systems are the most valid when considering CWs to treat CSO and agricultural run-off for pharmaceutical compounds.

When treating wastewater, CWs can be used alone as a decentralized solution, either as single-house application or for small housing agglomerates, or as centralized solution employed in rural areas or urban areas up to around 1,000 persons equivalent (PE). Typically, a sedimentation tank is used for primary treatment, while the CW ensures the secondary treatment. When hybrid systems are used, sometimes tertiary treatment is also achieved [29]. In basic wastewater treatment terms, primary treatment ensures removal of solid material; secondary treatment deals with the removal of dissolved and suspended organic material, as well as potentially the nutrients (nitrogen and phosphorus); and tertiary treatment are the polishing methods used following a traditional wastewater treatment plant (WWTP) [41]. The exception to the common CWs configuration is the so-called French system that consists of a hybrid design containing multiple beds and operated to perform both primary and secondary treatment using planted beds [42]. However, CWs can also be used as part of larger classical WWTPs (conventional activated sludge), in this case usually as a polishing step (tertiary treatment). It is therefore important to study CWs performance for pharmaceuticals treatment, not only by CW type but also wastewater strength (e.g. secondary vs tertiary treatment). As for classical pollutants, design and operational factors (area, depth, hydraulic loading rate, organic loading rate and hydraulic retention time) and physicochemical parameters (dissolved oxygen, temperature and pH) are critical for the performance of the systems. The geographical and environmental inherent variability, for instance plants used (that should be native) or temperature (that is linked with microbial activity and evapotranspiration) makes systematization and comparison of systems a complex task.

2.2 Historical Developments

A good starting point to understand the potential of CWs to control pharmaceutical contamination from wastewater is to study the relevant review papers on the topic (Table 1). The first mini-review work by White, Belmont [43] provided an earlier

Table 1 Overview of the relevant review papers covering the topic pharmaceuticals and constructed wetlands (CWs)

Main focus	Type of CW[a, b]	Compounds reported[b]	Number of publications revised	Removal	Kinetics	Removal mechanisms	Design and/or operation considerations	Reference
Pharmaceuticals in wastewater and treatment wetlands	SF, HSSF	Carbamazepine, cotinine, cyclophosphamide, fenoprofen, gemfibrozil, ibuprofen, others ns	3	Yes	No	Yes	No	[43]
Phytoremediation and wastewater treatment	VF, others ns	Ns	5	No	No	No	No	[44]
Emerging pollutants	SF, HSSF, VF	Carbamazepine, clofibric acid, diclofenac, ibuprofen, ketoprofen, naproxen, others ns	5	Yes	No	No	No	[25]
Emerging pollutants and treatment	HSSF, others ns	Caffeine, diclofenac, fluoxetine, ibuprofen, ketoprofen, naproxen, salicylic acid, others ns	8	Yes	No	Yes	No	[45]
Organic and metallic pollutants and wetlands	SF, others ns	Carbamazepine, clofibric acid, fluoroquinolones antibiotics, ibuprofen, others ns	9	Yes	No	No	No	[48]
Emerging pollutants and treatment	SF, HSSF, others ns	Caffeine, carbamazepine, ceftiofur, ciprofloxacin, clofibric acid, diclofenac, enrofloxacin, ibuprofen, ketoprofen, naproxen, norfloxacin, salicylic acid, tetracycline, others ns	17	Yes	No	Yes	No	[46]

(continued)

Table 1 (continued)

Main focus	Type of CW[a, b]	Compounds reported[b]	Number of publications revised	Removal	Kinetics	Removal mechanisms	Design and/or operation considerations	Reference
Natural and onsite treatment processes	SF, others ns	Caffeine, carbamazepine, clofibric acid, diclofenac, ibuprofen, ketoprofen, monensin, naproxen, narasin, salinomycin, salicylic acid, others ns	4	Yes	No	Yes	No	[47]
CWs and pharmaceuticals	SF, HSSF, VF and hybrid	115 compounds in total. More common: acetaminophen, atenolol, caffeine, carbamazepine, clofibric acid, diclofenac, gemfibrozil, ibuprofen, ketoprofen, metoprolol, naproxen, salicylic acid, sulfamethoxazole, trimethoprim	38	Yes	No	Yes	Yes (brief mention)	[49]
Aquatic plant-based systems and pharmaceuticals	SF, HSSF, VF and hybrid	137 compounds in total, including personal care products. More common pharmaceuticals: atenolol, caffeine, carbamazepine, clofibric acid, diclofenac, ibuprofen, ketoprofen, naproxen, salicylic acid	37	Yes	No	Yes	Yes	[50]
CWs and pharmaceuticals	SF, HSSF, VF, hydroponic gravel bed and others	137 compounds in total, including personal care products. More common pharmaceuticals: atenolol, caffeine, carbamazepine,	47	Yes	Yes (brief mention)	Yes	Yes	[27]

		clofibric acid, diclofenac, fluoxetine, gemfibrozil, ibuprofen, ketoprofen, naproxen, salicylic acid, sulfamethoxazole, trimethoprim						
Wetlands and wastewater treatment	HSSF, VF, hybrid and others ns	Caffeine, carbamazepine, ibuprofen, sulfamethazine, tetracycline, others ns	12	Yes	No	Yes (brief mention)	Yes (brief mention)	[51]
Trace organic contaminants and different treatment technology	Ns	17β-estradiol, bisphenol A, caffeine, carbamazepine, diclofenac, estriol, estrone, ethinylestradiol, galaxolide, gemfibrozil, ibuprofen, ketoprofen, metoprolol, naproxen, nonylphenol, propranolol, sulfamethoxazole, tonalide, triclosan, trimethoprim	Ns	Yes	No	No	No	[56]
Emerging pollutants and watersheds	HSSF and others ns	Ns	4	Yes (brief mention)	No	Yes (brief mention)	Yes (brief mention)	[52]
Priority substances + contaminants of emerging concern and CWs	SF, HSSF, VF, hybrid and others	17β-estradiol (E2), 17-α-ethinylestradiol (EE2), azithromycin, clarithromycin, diclofenac, erythromycin, estrone (E1), imidacloprid	46	Yes	No	Yes (brief mention)	No	[55]
CWs and pharmaceuticals	SF, HSSF, VF and others	17β-estradiol, acetaminophen, carbamazepine, diclofenac, naproxen, estrone	15	Yes	No	Yes	Yes (brief mention)	[57]

(continued)

Table 1 (continued)

Main focus	Type of CW[a, b]	Compounds reported[b]	Number of publications revised	Removal	Kinetics	Removal mechanisms	Design and/or operation considerations	Reference
Chiral pharmaceuticals	Ns	Ns	16	No	No	Yes (brief mention)	No	[58]
Lemna minor and phytoremediation	Ns	Caffeine, cefadroxil, cisplatin, diclofenac, fluoxetine, ibuprofen, metronidazole, naproxen, sulfamethoxazole, trimethoprim, others ns	10	Yes	No	No	No	[54]
Adsorbents and non-steroidal anti-inflammatory drugs	Ns	Diclofenac, ibuprofen, naproxen	5	Yes	No	No	No	[53]
Phytoremediation % plant-microbe interaction	SF, HSSF, VF and others ns	Most common: caffeine, carbamazepine, diclofenac, ibuprofen, ketoprofen, naproxen, others ns	80 (not only about CWs)	Yes	No	Yes (mostly focused on microbial degradation)	Yes	[60]
CWs + algae and pharmaceuticals	SF, HSSF, VF and others ns	39 compounds. Most common: caffeine, carbamazepine, diclofenac, ibuprofen, ketoprofen and naproxen	21	Yes	No	Yes (brief mention)	No	[59]
Antibiotics + antibiotic-resistant genes and CWs	SF, HSSF, VF, hybrid and others	Sulfonamide antibiotics, quinolone antibiotics, tetracycline antibiotics, macrolactone antibiotics, chloramphenicol antibiotics, polyether antibiotics and beta-lactam antibiotics	39	Yes	No	Yes	No	[61]

CWs and pharmaceuticals, design and operational factors	SF, HSSF, VF and hybrid	26 compounds selected: acetaminophen, atenolol, caffeine, carbamazepine, clarithromycin, codeine, diclofenac, erythromycin, furosemide, gemfibrozil, ibuprofen, ketoprofen, lincomycin, metoprolol, monensin, naproxen, ofloxacin, oxytetracycline, salicylic acid, sulfadiazine, sulfamethazine, sulfamethoxazole, sulfapyridine, tramadol, trimethoprim, venlafaxine	65	Yes	No	Yes	Yes (major focus)	[62]
CWs and pharmaceuticals, performance comparison	SF, HSSF, VF, hybrid and aerated	29 compounds selected (and 19 transformation products): acetaminophen, atenolol, bezafibrate, caffeine, carbamazepine (10,11-dihydro-10,11-dihydroxycarbamazepine; 10,11-dihydro-10-hydroxycarbamazepine; 2-hydroxycarbamazepine; 3-hydroxycarbamazepine; carbamazepine 10,11-epoxide), clarithromycin, clofibric acid, codeine, diclofenac (4-hydroxydiclofenac), diltiazem, doxycycline,	63	Yes (major focus)	No	Yes	Yes (brief mention)	[28]

(continued)

Table 1 (continued)

Main focus	Type of CW[a, b]	Compounds reported[b]	Number of publications revised	Removal	Kinetics	Removal mechanisms	Design and/or operation considerations	Reference
		fexofenadine, gemfibrozil, ibuprofen (1-hydroxyibuprofen; 2-hydroxyibuprofen; carboxyibuprofen), ketoprofen (3-Ethylbenzophenone; Dihydro Ketoprofen), metoprolol, mirtazapin, naproxen (O-desmethylnaproxen), ofloxacin, ranitidine, salicylic acid, sotalol, sulfadiazine, sulfamethazine, sulfamethoxazole (N-acetylsulfamethoxazole), sulfapyridine, tramadol (O-desmethyltramadol; N-desmethyltramadol; N, O-didesmethyltramadol), trimethoprim, venlafaxine (O-desmethylvenlafaxine; N-desmethylvenlafaxine; N, O-didesmethylvenlafaxine)						

[a]Surface flow (SF), horizontal subsurface flow (HSSF), vertical flow (VF), hybrid configurations of any type (e.g. VF + SF)
[b]Ns stands for non-specified, is used associated with the type of wetland and compounds list

overview of the first laboratorial and pilot tests accounting in total for less than five published works. Already by that time, both White, Belmont [43] and Schröder, Navarro-Aviñó [44] considered wetlands (both natural and CWs) promising to remove pharmaceutical compounds from wastewater.

Early in 2010s, a couple of review papers started to include some mentions to natural and water treatment wetlands for the removal of emerging pollutants, including pharmaceuticals [25, 45–47]. However, the second overview of the topic on a review paper dedicated to CWs was performed by Haarstad and Bavor [48] within a vast revision of organic and metallic pollutants. By then, not more than nine publications were available. As publication rate kept increasing, the three first larger review works specifically focused on pharmaceuticals, and CWs were published in 2014 [27, 49, 50]. By then, around 40 publications were available and a careful systematization of the data was produced. These works, for the first time, compared CWs design as well as different levels of treatment (primary to tertiary) being still a reference in the field.

Li et al. [49] focused on the application of CWs for secondary wastewater treatment and as a wastewater polishing treatment. Removal efficiencies published started to point to the inherent variability associated with (1) *compound*: some compounds are easily removed, typically because they are easily biodegraded, while others tend to be recalcitrant (poor biodegradability); and (2) *CW design*: different removal mechanisms can be promoted/inhibited due to specific configurations (e.g. aerobic, anaerobic, photodegradation). However, the studies were few, and the majority limited to lab-scale studies. Therefore, the authors carefully phrased a "consensus towards the potential of CWs for pharmaceuticals removal" [49]. This review paper also provided a simple overview of design parameters and description of substrate, plants and microbes role on removal, as well as of the research gaps at the time. Complementarily, Zhang et al. [50], from the same research group, provided a smaller overview on removal efficiency and placed more emphasis on removal mechanisms (photodegradation, sorption, plant uptake and phytodegradation and microbial degradation), design and operational parameters (bed depth, vegetation, hydraulic retention time), as well as attempted to establish correlations with compound physicochemical properties, namely, the octanol water partition coefficient [50]. More details in Sect. 3.

Verlicchi and Zambello [27] had a broader scope, so besides the secondary and tertiary treatment, also covered CWs used for primary treatment. In addition, they limited their review to pilot and full-scale systems (operated outdoors in "real environmental conditions") treating urban wastewater. The paper also includes a large discussion section on removal mechanisms, modelling and design parameters. The potential of CWs to remove a wide spectrum of pharmaceuticals was described and linked to the coexistence of anoxic-aerobic-anaerobic microenvironments within surface flow, as well as subsurface flow systems that favour the different mechanisms involved in their removal. More specifically, SF and HSSF CWs used as primary treatment revealed removals above 40% for 14 compounds (acetaminophen, atenolol, caffeine, diclofenac, diphenhydramine, ibuprofen, ketoprofen, metoprolol, naproxen, salicylic acid, sulfamethoxazole, sulfapyridine, triclocarban and

triclosan), two compounds (nadolol and sotalol) below 20%, and three compounds (carbamazepine, gemfibrozil and trimethoprim) showed both positive and negative removals depending on the original study. The amount of results was limited to allow a proper comparison of CW designs. None of the systems reported was a French system nor, to the best of my knowledge, a French system has been studied for the removal of pharmaceuticals. Overall, Verlicchi and Zambello [27] concluded that CWs provide comparable efficiencies at the secondary treatment level to conventional WWTPs for the removal of many common pharmaceuticals, including caffeine, ibuprofen, naproxen and salicylic acid. One of the knowledge gaps identified was the need to optimize the removal of the most critical compounds, mainly antibiotics and some analgesics and anti-inflammatories [27].

Other brief mentions to pharmaceuticals and CWS can also be found in a couple of later reviews [51–55]. Melvin and Leusch [56] provide an interesting comparison of CWs with other wastewater treatment technology, where "ponds and CWs" continued to be considered at least as efficient as classical activated sludge systems. However, results were not statistically better than other alternative technologies, oxidation ditch and membrane bioreactor, probably due to generalization of the datasets (grouping of efficiency for different compounds and independently of specific technology designs). Vo et al. [57] aimed at going beyond removal efficiency and focused on the removal mechanisms of pharmaceutical and personal care products in CWs. Zhou et al. [58] have reviewed chiral pharmaceuticals in the environment and remediation technologies applied for their treatment, including a small section dedicated to CWs. In spite of the interesting title, the work does not provide details about chiral processes. Rabello et al. [59] reviewed the combination of CWs and algae systems. The latter were considered more efficient than CWs in removing the most commonly studied compounds, i.e. caffeine, carbamazepine, diclofenac, ibuprofen, ketoprofen and naproxen, than the CWs. Nevertheless, the combination of both systems was proposed as an effective alternative for removing pharmaceuticals from domestic wastewater [59]. Nguyen et al. [60] surveyed literature investigating plant-based remediation practices, especially CWs, to remove pharmaceuticals and personal care products. They have expanded their overview towards the plant-bacteria synergism: the microbes (both rhizo- and endophytes) in CWs not only degrade the compounds directly but also accelerate plant growth by producing growth-promoting enzymes and hence increasing the remediation potential [60]. More details in Sect. 3.

Liu et al. [61] are the only review specifically dedicated to antibiotics and antibiotic-resistant bacteria, covering a good amount of literature. The removal efficiency of CWs for antibiotics showed good performance (average value above 50%), especially VF (average value of 80%). The removal efficiencies of sulfonamide and macrolide antibiotics were lower than those of tetracycline and quinolone antibiotics, stressing again compound-dependency of the performance. Regarding the antibiotic resistance genes, HSSF had better performance (above 50%) than VF, especially for sulfonamide resistance genes [61].

Most recently, two review works have been published [28, 62] making use of the, so far, most complete data collection on the topic (more than 60 papers). Their earlier

work [62] focused on the role of CW design (SF, HSSF, VF and hybrid systems) and respective operational factors, making use of correlation analysis to establish links with pharmaceuticals removal efficiency. It should be mentioned that these latest review papers [28, 62] pre-filtered the 25–30 compounds for which the number of data points was sufficient to provide statistically significant results. Therefore, one cannot find updated information for the more than 100 different pharmaceuticals that have been reported at least once in a paper about CWs [27, 50]. To find metadata on specific compounds, the review papers from 2014 are a better reference.

Ilyas and van Hullebusch [28] compared the performance of the different CWs, SF, HSSF, VF and hybrid systems (Table 2). In addition, besides looking into the removal of the 29 more common pharmaceuticals, the authors included as well information about 19 transformation products (for the specific name of the compounds see Table 2). This provided the first overview on the dynamics of transformation products, including formation and removal, in CWs. Hybrid designs were considered to perform better, followed by VF, HSSF and SF by Ilyas and van Hullebusch [28]. Results provide an interesting overview of the differences among designs. However, one should bear in mind the large standard deviation (Table 2) due to the compromise of including for each CW design, results from lab to full-scale systems and from primary to tertiary treatment.

2.3 Performance

A careful look at Table 2 shows that for 17 of the 29 common compounds, differences among designs were not significant. VF seems to perform better for caffeine, ibuprofen and naproxen, while hybrid designs have shown better removal for diclofenac and trimethoprim. Overall, the differences pinpoint that removal can be compound-specific and that part of the efficiency can be explained by the removal mechanisms associated with each design. A very good example is the different usage of VF (for oxidation of pollutants) and HSSF (explored as anaerobic of facultative systems) that can result in different removal efficiencies for the same compound (e.g. ibuprofen and diclofenac). Thus, compounds removed more efficiently by VF tend to be mainly driven by aerobic degradation, while anaerobic degradation prevails for HSSF. Hybrid systems, and the coexistence of aerobic and anaerobic conditions, seem to favour other compounds, such as diclofenac [63, 64]. However, other factors such as the retention time can play a role. More on processes is detailed in the next section.

Regarding specifically CWs for secondary wastewater treatment and tertiary treatment, a wide range of variability for several compounds is consistently seen in all 2014 revisions. Further reading [27, 49] is recommended to ascertain detailed comparisons between compounds, designs and treatment stages. Here the best performances from Table 2 and major conclusions from the last-mentioned reviews are listed per removal range:

Table 2 Comparison of removal efficiency (mean % and standard deviation) of selected 29 pharmaceuticals in different types of CWs and classical activated sludge (CAS)

Pharmaceutical	CAS (n)	SF (n)[a]	HSSF (n)[a]	VF (n)[a]	Hybrid (n)[a]	Statistical results[a]
Acetaminophen	96 ± 6 (5)[b]	99 (1)	70 ± 24 (12)	97 ± 1 (2)	83 ± 25 (11)	Non-significant differences in removal efficiency
Atenolol	54 ± 25 (9)[b]	57 ± 24 (5)	82 ± 19 (7)	79 ± 20 (4)	73 ± 37 (2)	Non-significant differences in removal efficiency
Bezafibrate	64 ± 32 (13)[b]	48 ± 28 (3)	45 ± 13 (4)	56 ± 11 (2)	NA	Non-significant differences in removal efficiency
Caffeine	83 ± 21 (23)[c]	62 ± 29 (17)	84 ± 16 (63)	97 ± 2 (4)	77 ± 25 (26)	The removal efficiency with VF was significantly higher compared with SF, HSSF and hybrid; the removal efficiency with SF was significantly lower compared with HSSF
Carbamazepine	18 ± 28 (48)[c]	31 ± 22 (23)	30 ± 24 (38)	40 ± 20 (11)	27 ± 20 (18)	Non-significant differences in removal efficiency
Clarithromycin	49 ± 29 (5)[b]	41 ± 21 (6)	45 ± 20 (10)	49 ± 57 (2)	46 ± 9 (2)	Non-significant differences in removal efficiency
Clofibric acid	40 ± 22 (11)[b]	30 ± 9 (4)	49 ± 24 (14)	NA	NA	The removal efficiency with SF was significantly lower compared with HSSF
Codeine	68 ± 26 (5)[b]	64 ± 26 (5)	59 ± 18 (4)	95 ± 1 (2)	NA	Non-significant differences in removal efficiency
Diclofenac	35 ± 32 (28)[c]	42 ± 24 (22)	39 ± 24 (45)	50 ± 17 (13)	56 ± 32 (25)	The removal efficiency with hybrid was significantly higher compared with HSSF
Diltiazem	73 ± 23 (4)[b]	66 ± 25 (4)	68 ± 12 (3)	NA	NA	Non-significant differences in removal efficiency
Doxycycline	71 ± 49 (3)[b]	73 ± 18 (4)	73 ± 2 (2)	NA	61 ± 15 (3)	Non-significant differences in removal efficiency
Fexofenadine	18 (1)[d]	18 ± 10 (3)	39 ± 26 (3)	NA	NA	Non-significant differences in removal efficiency
Gemfibrozil	57 ± 34 (17)[c]	12 ± 2 (4)	58 ± 23 (8)	45 ± 9 (4)	95 (1)	The removal efficiency with SF was significantly lower compared with HSSF and VF
Ibuprofen	86 ± 22 (39)[c]	57 ± 28 (27)	53 ± 27 (61)	79 ± 24 (10)	62 ± 29 (32)	The removal efficiency with VF was significantly higher compared with SF, HSSF and hybrid
Ketoprofen	57 ± 21 (25)[c]	48 ± 30 (22)	47 ± 35 (27)	50 ± 3 (6)	45 ± 28 (17)	Non-significant differences in removal efficiency
Metoprolol	18 ± 16 (13)[c]	33 ± 23 (5)	60 ± 32 (15)	74 ± 9 (2)	99 (1)	Non-significant differences in removal efficiency
Mirtazapine	[e]	55 ± 23 (3)	8.3 ± 5.0 (3)	NA	NA	The removal efficiency with SF was significantly higher compared with HSSF

Naproxen	72 ± 23 (34)[c]	50 ± 22 (28)	63 ± 26 (42)	75 ± 17 (8)	64 ± 24 (24)	The removal efficiency with VF was significantly higher compared with SF, HSSF and hybrid
Ofloxacin	60 ± 27 (13)[b]	NA	98 ± 4 (13)	87 ± 10 (3)	NA	Non-significant differences in removal efficiency
Ranitidine	62 ± 37 (4)[b]	79 ± 20 (3)	36 ± 8 (4)	NA	NA	The removal efficiency with SF was significantly higher compared with HSSF
Salicylic acid	98 (2)[f]	76 ± 19 (15)	79 ± 21 (20)	98 (1)	86 ± 17 (15)	Non-significant differences in removal efficiency
Sotalol	35 ± 34 (7)[b]	15 ± 8 (3)	18 ± 12 (3)	NA	82 (1)	Non-significant differences in removal efficiency
Sulfadiazine	92 ± 10 (5)[b]	61 ± 35 (10)	46 ± 30 (6)	52 ± 22 (12)	NA	Non-significant differences in removal efficiency
Sulfamethazine	72 ± 48 (3)[b]	48 ± 48 (9)	45 ± 27 (21)	35 ± 30 (12)	74 (1)	Non-significant differences in removal efficiency
Sulfamethoxazole	52 ± 29 (24)[c]	54 ± 29 (13)	43 ± 24 (10)	54 ± 29 (14)	61 ± 31 (7)	Non-significant differences in removal efficiency
Sulfapyridine	52 ± 44 (4)[b]	79 ± 4 (6)	84 ± 3 (6)	84 ± 5 (12)	99 (1)	The removal efficiency with SF was significantly lower compared with HSSF and VF
Tramadol	23 ± 27 (2)[b]	23 ± 22 (5)	58 ± 42 (11)	46 ± 42 (2)	NA	The removal efficiency with SF was significantly lower compared with HSSF
Trimethoprim	32 ± 31 (26)[c]	70 ± 21 (15)	65 ± 31 (12)	69 ± 27 (12)	96 ± 5 (3)	The removal efficiency with hybrid was significantly higher compared with HSSF
Venlafaxine	20 ± 11 (1)[g]	43 ± 26 (5)	5.1 ± 3.6 (3)	40 ± 21 (2)	63 ± 4 (2)	The removal efficiency with SF was significantly higher compared with HSSF

n number of observations; NA not available

[a]Values and statistical results reprinted from Environmental Science and Pollution Research, Ilyas and van Hullebusch 2020, Performance comparison of different types of constructed wetlands for the removal of pharmaceuticals and their transformation products: a review, https://doi.org/10.1007/s11356-020-08165-w, Copyright (2020), with permission from Springer Nature [28]

[b]Calculated from metadata from [82]

[c]Calculated from metadata from [56]

[d]From [114]

[e]Negative removal reported by [113]

[f]From [115]

[g]From [116]

- Highly removed compounds (>70%): acetaminophen, alfuzosin, alprazolam, atenolol, atorvastatin, azithromycin, caffeine, codeine, dipyridamole, famotidine, fenofibrate, furosemide, ibuprofen, levomepromazine, metronidazole, metoprolol, mianserin, nadolol, naproxen, nifuroxazide, ofloxacin, paroxetine, ranitidine, salbutamol, salicylic acid, sulfadimethoxine, sulfapyridine and verapamil
- Moderately removed (40–70%): bezafibrate, clarithromycin, clofibric acid, diltiazem, diclofenac, doxycycline, gemfibrozil, ketoprofen, sulfadiazine, sulfamethazine, sulfamethoxazole, tramadol, trimethoprim and venlafaxine
- Poorly removed (<40%): carbamazepine, cimetidine, clenbuterol, clindamycin, fexofenadine, glibenclamide, glimepiride, irbesartan, lorazepam, maprotiline, mefenamic acid, memantine, mirtazapine, oxazepam, perphenazine, phenobarbital and sotalol

CWs are promising to effectively remove (>70%) the pharmaceutical compounds included in the EU watch list by the Commission Decision 2015/495/EU, diclofenac and the macrolide antibiotics (erythromycin, clarithromycin and azithromycin), 17-beta-estradiol and oestrone (respectively E2 and E1) and the synthetic hormone 17-alpha-ethinylestradiol (EE2) [55]. However, except for diclofenac that has been extensively studied, all other compounds were only reported in less than ten or even five publications [55].

Additionally, making use of the data compiled from Ilyas and van Hullebusch [28] and adding results from classical activated sludge (CAS) systems (Table 2), it is possible to establish gross comparisons of removal efficiency between CWs and conventional WWTPs. As it has been reported in the first studies, CWs can provide similar (acetaminophen, caffeine, clarithromycin, clofibric acid, diltiazem, doxycycline, fexofenadine, gemfibrozil, ibuprofen, ketoprofen, naproxen, ranitidine, salicylic acid, sotalol, sulfamethoxazole) or even better removal (atenolol, carbamazepine, codeine, diclofenac, metoprolol, sulfapyridine, tramadol, trimethoprim and venlafaxine) than conventional technology for domestic and urban wastewater treatment. Only bezafibrate, sulfadiazine and sulfamethazine are better removed by CAS than CW, probably the cases were sorption plays a major role as removal mechanisms.

Regarding transformation products, it is known from other technologies, including other biological processes, that pharmaceuticals removal are rarely mineralized, but rather a high variety of transformation products are formed [65]. In CWs, studies reporting transformation products are scarce when compared with the overall data on parent compounds. Not more than three papers report on the same transformation product, while several of the transformation products in CWs are only reported once [28]. Overall, results point the formation of some (e.g. 4-hydroxydiclofenac), while others (e.g. 1-hydroxyibuprofen or carboxyibuprofen) are removed. There is a clear knowledge gap on dynamics (formation/removed) of pharmaceutical transformation products in CWs.

Moreover, Ilyas and van Hullebusch [28] compared for the first time the performance of different aerated systems. Aeration, as an operational option to increase the efficiency of aerobic processes in CWs (higher BOD removal or nitrification) can

also be of relevance to overcome oxygen transfer limitation and enhance the removal of pharmaceuticals. Results from a total of six publications, covering aerated variations of SF, HSSF and hybrid systems, showed that the removal of the compounds diclofenac, ibuprofen, naproxen, caffeine, atenolol and metoprolol was favoured by the higher availability of dissolved oxygen in aerated systems, thus pointing towards the upregulation of aerobic biodegradation pathways [64, 66–70]. Acetaminophen is normally easily biodegraded and no differences to non-aerated controls were observed [70].

By now it is clear that removal efficiency depends on design, pointing towards the need to better understand removal mechanisms and operational factors that condition the removal.

3 Removal Mechanisms and Processes

3.1 Mechanisms and Processes Overview

Constructed wetlands are known for their complex ecosystem allowing for a multitude of biological and physicochemical processes. By selecting the most adequate design (or combination of designs in case of hybrid systems) and operational conditions, one has the possibility to shape the water treatment as a function of the quality/strength of the incoming water to the CW. Therefore, the removal processes in CWs are pollutant and design dependent.

The main removal processes in CWs are photodegradation, sedimentation, volatilization, sorption and biological degradation [29]. Nitrogen processes comprise volatilization, ammonification, nitrification, nitrate-ammonification, denitrification, fixation, plant/microbial uptake (assimilation), ammonia adsorption, organic nitrogen burial, anaerobic ammonia oxidation (anammox). Nevertheless, the major processes just change nitrogen speciation, only few processes ultimately remove total nitrogen from the wastewater [71]. Degradable carbon compounds are rapidly utilized in wetland carbon processes. At the same time, a variety of wetland decomposition processes produce carbon [29]. The assessment of organic carbon removal, usually documented as COD and BOD, for conventional wastewater treatment has been documented for 70 years [72]. When it comes to organic chemicals (e.g. PCBs, PAHs, pesticides) studies only started in the early 2000s. A comprehensive analysis of the removal processes of organic micropollutants was prepared by Imfeld and Braeckevelt [73]. More recently, pharmaceuticals removal processes have been discussed by several of the available review papers mentioned in the previous section [27, 28, 50, 57, 60, 62]. Phosphorus transformations include peat/soil accretion, adsorption/desorption, precipitation/dissolution, plant/microbial uptake, fragmentation and leaching and mineralization and burial [71]. However, removal of phosphorus in all types of CWs can be limited, sometimes requiring additional measures, e.g. usage of special substrates or inclusion of a chemical precipitation step. Regarding particles, normally assessed as total suspended solids

(TSS), CWs when effectively designed can provide reliable removal without any clogging problems for more than 20 years [74].

Plants stabilize the surface of the beds, provide good conditions for physical filtration, prevent vertical flow systems from clogging, insulate the surface against frost during winter, and provide a huge surface area for attached microbial growth [75]. Plants metabolism affects the treatment processes to different extents depending on the CW design. The most active reaction zone of constructed wetlands is the rhizosphere (the root zone). This is where physicochemical and biological processes take place that are induced by the interaction of plants, microorganisms, the substrate and pollutants [76], including pharmaceuticals degradation [60]. Plant uptake of nutrients is only of quantitative importance in low-loaded systems – surface flow system (SF) [75]. However, for organic contaminants, plant uptake and phytovolatilization, as well as contaminant accumulation and metabolic transformation, can be relevant for different plants and compounds [77], as well as different CW designs.

The relative importance of a particular removal process can vary significantly, depending on the organic contaminant being treated, the wetland type (e.g. SF, SSF, VF) and operational design (e.g. retention time), the environmental conditions, the type of vegetation within the system, as well as the substrate [29]. Anaerobic processes predominate in HSSF (apart from in the proximity of the macrophyte roots), facultative or aerobic processes usually prevail in SF, while VF and aerated systems are dominated by aerobic processes. Hybrid systems are an effective way to sequence these processes by including different designs/beds in a single treatment system. CWs support a large spectrum of biogeochemical reactions and various environmental conditions within a single bed. In fact, CWs are complex bioreactors characterized by considerable fluxes of material and energy governing chemical reactions over spatial and temporal gradients [73]. From an engineering perspective, they are also attractive biofilm reactors in which the biofilm can be manipulated, depending on the CW design and operation. Thus, pollutant removal depends on two processes simultaneously occurring at different scales: (1) the various and coexisting redox processes within a bed, and (2) the processes driven by the plant, namely, those occurring at the rhizosphere scale. Two parameters can be helpful to understand the status of a given reactor/CW, the dissolved oxygen and the oxidation reduction potential (ORP), including for pharmaceuticals removal [27, 28, 50]. Moreover, one should bear in mind that CWs are not a homogenous reactor, but in fact zonation occurs in terms of pollutant concentration (inflow area > outflow area) and with bed depth. The hydraulic retention time (HRT), including the length of time the water is in contact with the substrate, biofilm and the plant roots, affects the extent to which the removal or biotransformation of pollutants can occur. Typical HRT is in the order 7–10 days in SF, 2–5 days in HSSF and minutes to hours in VF. There is the general idea that longer retention times result in better performance, which is in general true for SF and HSSF [29]. However, such comparison should only be performed for the same design type, thus, CWs sharing the same removal processes. A clear example is the higher nitrification capacity of VF versus HSSF, including a minor area requirement for the same pollutant load, but indeed with a

much lower retention time [32]. Whereas plants significantly affect the removal of pollutants in HSSF with long hydraulic retention times, their role is minor in pollutant removal in periodically loaded VF [76].

Photodegradation is only a relevant process in SF, but not for subsurface systems (HSSF and VF), where water is kept below the surface of the substrate. Moreover, photodegradation in SF will be dependent on the depth of the bed and type/density of vegetation used. It should be noted that SF can have plants rooted in the bottom that can be emergent, submerged, or floating plants, as well as free-floating plants. In addition, the biofilm system responsible for the transformation of the pollutants is distinct between SF and subsurface systems. In SF, the substrate layer is reduced, and water flows on top of it, thus the majority of the biodegradation processes occur in the water column by action of the biofilm and periphyton [78] attached to the plant surfaces (e.g. leaves, stems, roots in case of free-floating plants) [79]. For the subsurface systems (HSSF and VF), biofilm is attached to the substrate, usually sand or gravel as in classical sand filters and other type of biofilters, but in CWs the plant have a critical role shaping the microbial community of the biofilm [80].

Removal processes are also dependent on physicochemical properties of the pollutants, most notably evaporation, sorption and consequently sedimentation (when compounds are sorbed to particles/solids that are trapped by sedimentation). Different organic chemicals can show specific characteristic for a wide range of physicochemical properties such as water solubility, vapour pressure, octanol-water partition coefficient (Log K_{ow}), organic carbon partition coefficient (K_{oc}), Henry's law constant [73], acid dissociation constant (pKa) and the partition coefficient (Log K_d) [27]. The relationship between these physicochemical characteristics and the fate of pharmaceuticals in CWs is integrated in the next mechanisms description.

From the main CWs mechanisms, volatilization and phytovolatilization of pharmaceuticals can be excluded due to their low Henry's law constant values; pharmaceuticals are not volatile at the normal environmental temperatures. Therefore, pharmaceuticals are removed in CWs by the combination of (1) sorption and sedimentation; (2) plant uptake combined with translocation and further phytodegradation and microbial degradation within the plant; and (3) microbial degradation in the water-substrate interface (biofilm and/or periphyton) (Fig. 2). In the following paragraphs, each of the relevant processes are detailed. It should be denoted that sorption and sedimentation, as well as plant uptake and phytoaccumulation are non-destructive processes. They contribute for reducing the concentration of the contaminants simply by relocating them, which might imply that the contaminant can become available in the future (e.g. plants die off) or pose as hazardous (e.g. disposable of substrate by the end-life of the treatment system) [73]. The other processes imply the degradation of the compounds, also the photodegradation in the SF systems. Pharmaceuticals degradation rarely occurs by mineralization, thus a high variety of transformation products are formed, not only by photodegradation [27] but also in biological systems [65].

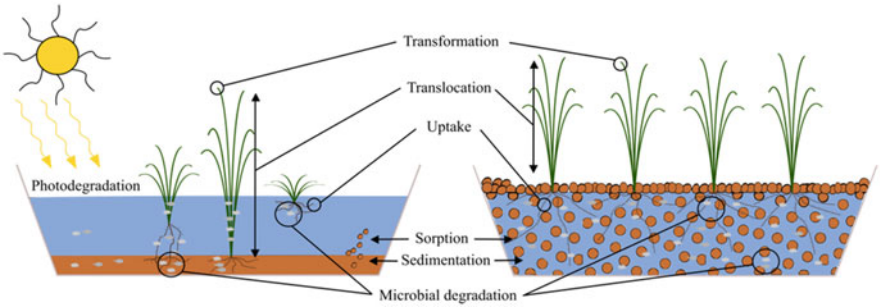

Fig. 2 Schematic representation of the processes by which pharmaceuticals are removed and/or degraded in surface flow CWs (SF, left side), in horizontal subsurface flow (HSSF) and vertical flow (VF) CWs (right side). Green elements are plants, grey elements represent microorganisms and brown elements represent substrate. Transformation comprises both phytodegradation and microbial degradation within the plant

3.2 Photodegradation

The removal efficiency of this degradation mechanism is decided by season and SF CW design. The seasonal variation relates with the light intensity and cycle, while the design controls the level of light penetration, reflection, and refraction through the wetland cell [50, 57]. Direct photolysis and indirect photodegradation can be important processes for most pharmaceuticals, as they generally contain aromatic rings, heteroatoms and/or other functional groups [27] that can either directly absorb solar radiation or react with photoinduced reactive intermediates ($^1O_2$, HO•, •OOR, ^{3}DOM*). The number of studies focused on the photolytic degradation in wetlands is moderate compared to those in the surface water (i.e. lake and river) [57].

Photodegradation has been considered key in removing diclofenac, ibuprofen, ketoprofen, naproxen, propranolol and triclosan in SF. In addition, the elimination of some recalcitrant compounds in SF, including clofibric acid and carbamazepine was considered to be correlated with high HRT and exposure to sunlight. Other pharmaceuticals are recalcitrant to photolytic degradation, namely, sulfamethoxazole, sulfathiazole, sulfamethazine, and trimethoprim [27, 50, 57]. It should be remembered that high plant coverage can block the light radiation and consequently reduce photodegradation. On the other hand, plant activity can increase the amount of organic matter available to produce intermediate reactive species. Thus far, there is no reliable rule of thumb for predicting the photodegradation behaviour of pharmaceuticals [27].

3.3 Sorption and Sedimentation

Sorption of a chemical to soil or sediment results from physical and/or chemical adhesion of molecules to the surfaces of solids, or from dissolved molecules partitioning between the aqueous phase and soil organic matter [73]. Since suspended particles from wastewater are retained in a CW bed, sorption of dissolved organic contaminants on soil, organic carbon, mineral surfaces and biofilms coating the substrates can be a significant mechanism for their removal [50].

Modelling sorption of organic compounds in sediments, soils and biosolids typically relies on the sorption coefficient (K_d). According to [81], a pharmaceutical with $K_d < 500$ L kg^{-1} or Log $K_d < 2.7$ implies poor capacity for sorption onto solids. Moreover, the organic carbon partition coefficient (K_{oc}), i.e. the ratio of contaminant mass adsorbed per unit weight of organic carbon in the soil to the concentration in solution, is also a commonly used proxy to estimate sorption of pharmaceuticals [50]. Nevertheless, pharmaceuticals can also sorb to the inorganic component of the substrate, convoluting predictions of the total sorption in CWs. The octanol-water partition coefficient (Log K_{ow}) has been adopted as the standard measure of hydrophobicity of a chemical compound [50]. Sorption may occur due to hydrophobic interactions of the aliphatic and aromatic groups of an organic compound with the lipophilic cell membrane of the microorganisms, or the lipid fractions of the suspended solids [82]. Hydrophobic compounds can be easily adsorbed onto the organic matter coming with the wastewater and built up within the CW substrate due to filtration and sedimentation. However, pharmaceuticals can also sorb due to electrostatic interactions with the organic matter located on the surface of soil and/or sediment that exhibit a negative charge because of the functional groups present (i.e. carboxylic and phenolic groups). Thus, the electrostatic interaction with positively charged pharmaceuticals provides a suitable removal mechanism for the latest [62]. While neutral molecules partition to solid phases via relatively weak van der Waals and electron donor-acceptor interactions, charged species can interact with charged sorbents (e.g. organic matter, clays, metal oxides and oxyhydroxides) through stronger electrostatic mechanisms, such as cation-exchange, cation-bridging and complexation [50]. The most common example are antibiotics, moderately water soluble and ionizable, that exist as either neutral or charged species depending on pH conditions, for which ionic interactions are possible sorption mechanisms. This stresses that not only hydrophobicity but other compound's properties (e.g. chemical structure, water solubility, acid/base properties, etc.) and substrate characteristics (e.g. composition of organic matter, redox potential, temperature, pH, ionic strength, cations, anions, etc.) govern pharmaceuticals removal by sorption. It should be stressed that many of the methodologies and relationships suggested for determining sorption capacity have been derived from studies with neutral or hydrophobic compounds, namely, classical organic pollutants such as PAHs or PCBs. The fact that pharmaceuticals are often ionized at typical wastewater pH adds to the complexity of predicting their behaviour.

Recalcitrant pharmaceuticals such as carbamazepine are known to be removed by sorption from the water phase in CWs [50]. Higher retention in gravel beds was observed for carbamazepine (97 ng g^{-1}; 77%) in relation to clofibric acid (14 ng g^{-1}; 11%) and ibuprofen (15 ng g^{-1}; 12%), which was explained by carbamazepine higher hydrophobicity and electrostatic interactions between the acidic compounds charged negatively and negatively charged biofilm covering the gravel bed. In contrast, sorption of polar compounds such as caffeine was considered of minor importance in CW systems, because of their high water solubility and low hydrophobicity [83]. For ionizable pharmaceuticals, pH-dependent octanol/water partition coefficient (Log D_{ow}) has been considered more useful than Log K_{ow} [27]. However, it has also been observed that pharmaceuticals that are moderately hydrophilic (with Log D_{ow} ranging from −2.3 to 3) tend not to bind significantly to organic matter [63].

The majority of the HSSF and VF systems in the different studies revised by Verlicchi and Zambello [27] have been filled with gravel (generally with a particle size of 8–15 mm and porosity 30–40%) and in some cases LECA (light expanded clay aggregate). Gravel and sand are naturally more inert; thus, sorption will be very much dependent on the organic matter that builds up in time within the systems. However, optimization may be attempted by selecting materials with high sorption capacity. Dordio and Carvalho [84] provide an in-depth analysis of the role of the CW substrate on the removal of xenobiotics, including pharmaceuticals. Less common media such as LECA or activated carbons and more recent innovations like kaolinite, diatomite, cork, perlite and zeolites have been tested. However, the majority of those experiments were carried in the lab with synthetic wastewater (tap water spiked with a few compounds, not always at realistic conditions), thus raising concerns with the sustainability and applicability of these new approaches in the long term [27]. It should be stressed that LECA has been consistently shown in different studies to provide extensive sorption of carbamazepine and atenolol [62].

One should bear in mind that sorption can be seen not only as an end mechanism in the form of sedimentation but also a factor affecting the bioavailability of compounds for plant uptake and for biodegradation processes. Sorption provides different reaction times for the plants, biofilm and respective communities to interact with the pollutants. Hijosa-Valsero et al. [85] observed very limited adsorption of the musk fragrances galaxolide and tonalide (present in personal care products) despite their high hydrophobicity and strong affinity to media adsorption (Log K_{ow} 6.26 and 6.35, respectively). In fact, it was due to the easy uptake by plant rhizosphere, rather than being adsorbed by media. Moreover, the substrate itself not only conditions sorption but also affects the biofilm microbial community. Vesuvianite (or idocrase, a silicate mineral) had better removal efficiency than gravel and zeolite for treating, sulfonamides in spite of the limited sorption of these compounds. Yet, vesuvianite conditioned the growth of the microbial consortium and lengthened HRT via its larger porosity, thus contributing for an overall better removal efficiency [86]. A similar behaviour was observed for a study with six commonly used materials in CWs (sand, zeolite, blast iron slag, petroleum coke, polonite (natural calcium silicate) and crushed autoclaved aerated concrete). Laboratorial batch tests with

ibuprofen and iohexol revealed that the adsorption capacity of these materials was low (at the level of μg g^{-1}) compared to well-known sorbents such activated carbon (at the level of mg g^{-1}). Columns packed with the six materials showed an increase of 2–58% in pharmaceutical removal over 66 days and attributed to microbial degradation. Furthermore, community-level physiological profiling analysis indicated that materials shaped the microbial community metabolic function not only in the interstitial water but also in the biofilm. Although the adsorption capacity of the common materials was low, they may be a driver to improve the removal of OMPs by altering microbial community function in CWs [87].

Overall, the role of adsorption in the removal of pharmaceuticals has been considered fairly moderate [57].

3.4 Plant Uptake, Translocation, Phytodegradation and Microbial Degradation Within the Plant

Phytoremediation is a technology relying on plants, and their associated rhizosphere microorganisms to remove, transform or contain toxic chemicals located in soils, sediments, groundwater, surface water and even the atmosphere [77]. The main mechanisms that constitute phytoremediation include phytostabilization, phytoextraction, phytoaccumulation, phytovolatilization, phytotranspiration, phytodegradation/transformation, endophytic degradation and rhizosphere remediation [60, 88]; these can occur simultaneously and to varying degrees.

Phytostabilization is used to minimize migration of contaminants in soils, mainly associated with the precipitation and immobilization of heavy metals. Phytoextraction and phytoaccumulation exploit the ability of plants to remove contaminants from soil or water into harvestable plant biomass. Plants uptake pollutants through their roots (phytoextraction), which can be translocated to the aboveground parts, such as stems, leaves or even fruits where it can be accumulated (phytoaccumulation) or further transformed/degraded (phytodegradation/transformation, endophytic degradation). Phytotranspiration and phytovolatilization aim to exploit plants' capacity of "pumping" high volumes of water and converting a contaminant into a volatile form, thereby removing the latter from the soil or water into the atmosphere [77].

Interestingly, studies on the uptake of pharmaceuticals by plants used in phytoremediation started much later than on crop plants and where driven by the interest to understand the potential phytotoxic effects [26]. Thus, considering the concentration levels that pharmaceuticals normally present in the environment, either in wastewaters or reclaimed water, it is not expected that phytotoxic effects will occur in phytoremediation systems, including CWs.

Since then, research on plant uptake and processes, in spite of its developments, is still not so well understood, as for example sorption. Part of the reason has been the need to perform hydroponic studies to better understand plant-driven processes. Four

wetland plant species commonly used in CWs (*Typha latifolia*, *Phragmites australis*, *Iris pseudacorus* and *Juncus effusus)* were capable of removing ibuprofen and iohexol from spiked culture solutions. The pharmaceuticals were taken up by the roots and translocated to the aerial tissues. However, at the end of the experiment, plant accumulation constituted only up to 1.1 and 5.7% of the amount ibuprofen and iohexol spiked initially [89]. In addition, these observations have been confirmed at mesocosm scale: Hijosa-Valsero et al. [85] observed that plants removed pharmaceuticals not only by uptake but also by the adsorption to plants roots. Pharmaceuticals, such as ibuprofen, salicylic acid and caffeine, could be attached on the root of *P. australis* and *Typha angustifolia*. These compounds were also present in plant biomass. Therefore, there has been the interest in predicting the uptake of pharmaceuticals. In this regard, the role of Log K_{ow} has also been explored to predict both uptake and phytodegradation efficiency [57]. However, contrary to classical organic contaminants like PAHs or PCBs, for which, Log K_{ow} is a good proxy, it does not work as predictor for pharmaceuticals [50]. Compounds with Log K_{ow} in the range (1.8–3.1) are expected to be uptake by plants. For ionizable pharmaceuticals, pH-dependent Log D_{ow} can be calculated, but still fails to support predictions [26]. Pharmaceuticals Log K_{ow} can vary widely, e.g. −3.05 for iohexol (x-ray contrast media), 3.5 for ibuprofen or 4.5 for diclofenac. However, the corresponding Log D_{ow} at pH 8 is −3.11, 0.5, and 0.7, respectively [73, 89], falling outside the optimal uptake range. For ionizable organic compounds, ionization is expected to reduce their uptake to the shoots owing to a decrease in their membrane permeability. Nevertheless, these compounds have been documented to be uptake by different plants. The best modelling efforts are being used to understand the underlying processes of uptake and transport in plant, as well as for the analyses of the most relevant parameters and processes, but cannot be expected to predict exact concentrations for ionisable compounds [90].

Micropollutants are thought to be simply driven by diffusion since no specific transporters for synthetic organic micropollutants exist in the cell membranes of plants [76]. Therefore, phytotranspiration is of relevance for pharmaceuticals removal. Some studies have investigated the amount of water loss during their campaigns, finding that evaporation and evapotranspiration may greatly influence the results. In planted beds this water loss is greatly increased by plant transpiration, and evapotranspiration rates depend heavily on the plant type and their vegetative stage. Naturally, loss of water due to evapotranspiration can cause artefacts in determining the removal efficiency due to the "up concentration" of compounds in the effluent, while on a mass flow calculation such error will be avoided. In addition, evapotranspiration will also affect the water balance and can result in increased HRT instead of the expected theoretical HRT [27]. Moreover, ibuprofen removal for different CW mesocosms was positively correlated with the rate of evapotranspiration indicating that plant uptake may be an important process for the ibuprofen removal [91]. Even though the majority of evidence for uptake and translocation has been found in laboratorial conditions, these were recently confirmed for full-scale systems. Analysis of plant tissues, from *P. australis* from HSSF systems, evidenced

uptake, metabolism and accumulation of recalcitrant micropollutants such as ketamine and carbamazepine [92].

There are, therefore, also evidences of pharmaceutical's metabolization within the plant tissue. That biotransformation can occur either through phytodegradation (or phytotransformation) or endophytic degradation. Phytodegradation (or phytotransformation) is defined in this context as the metabolic degradation or breakdown of organic contaminants by plant enzymes [77]. In this process, micropollutants may be mineralized but are most often partially transformed into stable intermediates and stored in the plants [50]. He et al. [93] found that *P. australis* can metabolize ibuprofen to hydroxyibuprofen, 1,2-dihydroxyibuprofen, carboxyibuprofen and glucopyranosyloxy-hydroxyibuprofen. In addition, cytochrome P450 monooxygenase was found to be involved in the production of the two hydroxy intermediates. A few more examples of phytodegradation of pharmaceuticals by CWs plants are provided by Vo et al. [57]. Endophytic degradation of pharmaceuticals has also been reported in the literature. Endophytic bacteria isolated from *P. australis* were shown to promote growth of their host and to contribute to carbendazim metabolism. Sauvêtre et al. [94] presented strong evidence that xenobiotic metabolism and degradation pathways in plants can be modulated by interactions with their endophytic community. A few more examples of this plant-bacteria synergism can be found in Nguyen et al. [60].

In spite of the recent advances, studies on the metabolic transformation of pharmaceuticals in plant tissues and their intermediate transformation products are rather limited. Apart from a very recent and interesting study that used mass spectrometry imaging to identify xenobiotics, including pharmaceuticals and respective transformation products in plant leaves from a CW [95], the usage of advanced mass spectrometry in studies with CWs is scarce. Moreover, there is little quantitative evaluation of the contribution of uptake, translocation, accumulation and metabolization for the overall removal efficiency of pharmaceuticals in CWs [50, 57].

3.5 Microbial Degradation (or Rhizosphere Remediation)

Removal efficiency for pharmaceuticals, as for other pollutants, is higher in planted than unplanted CWs [62]. Hijosa-Valsero et al. [96] found that root-related biofilms, plant exudates and microenvironment modifications near plant tissues could play a role in the removal of tetracycline. Moreover, the presence of plants improved the degradation of naproxen, ibuprofen, diclofenac and caffeine, while *P. australis* displayed better performance than *T. angustifolia* [97]. Tai et al. [98] reported that root sorption and rhizobacterial activities made the most important contribution in macrolide removal. However, some exceptions are also known, for instance, clarithromycin and trimethoprim exhibit higher removal in non-planted systems [96]. Overall, it is well established that rhizosphere remediation, through microbial degradation, is of major importance for pharmaceutical removal in CWs. However,

the ability of specific plants species and plant-microbes interactions in improving the removal efficiency of pharmaceuticals in CWs is considered unclear [27, 60].

In previous studies, indirect approaches like mass balance estimations without detailed process identification have been applied to assess microbial degradation [50]. Thus, little information is currently available on integrating microbial community structure with pharmaceutical pathways of metabolism in CWs [60]. One of the key questions posed by the scientific community was whether pharmaceuticals, namely, antibiotics, could induce toxic effects to the microbial community. Weber et al. [99] findings indicated that the presence of ciprofloxacin had an adverse effect on the bacterial communities in CW systems, initially reducing their ability to assimilate anthropogenic carbon-based compounds, but the bacterial communities returned to normal functioning after a 2–5 week acclimation period. Fernandes et al. [100] saw that microbial communities in CWs treating livestock wastewaters with veterinary antibiotics (enrofloxacin and tetracycline) were able to adapt without significant changes. Overall, it seems that CWs microbial community may be affected by short pulses of higher levels of antibiotics but should be able to recover easily. For other pharmaceuticals, e.g. ibuprofen and iohexol, no effects to the microbial community have been noted [101].

Regarding the microbial degradation itself, there is overall (not only for CWs) a lack of studies on the specific organisms and genes involved in the biodegradation of pharmaceuticals. For instance, it is reported that polar-acidic pharmaceutically active compounds such as ibuprofen can be degraded by indigenous microbial communities in the wetland ecosystem, as well as *Serratia quinivorans*, *Corynebacterium segmentosum*, and *Escherichia coli* (species commonly found in municipal wastewater) [60]. Despite the wide consortium of microorganisms present in inflowing wastewaters and in biofilm communities, only a few microorganisms are expected to transform organic micropollutants. Studies in other types of biofilm reactors have suggested that only around 0.1% of the biomass was actually contributing to the degradation of this type of compounds [102]. First, the relatively low concentration of pharmaceuticals compared to other pollutants in wastewater may be insufficient to induce enzymes that are capable of degrading the pharmaceuticals. Thus, it is unlikely that pharmaceuticals will be favourable energy or carbon sources for microorganisms [50]. So, co-metabolism has been suggested as a possible mechanism for the biotransformation of pharmaceuticals and other organic micropollutants. It is known that autotrophic ammonia oxidizing bacteria and/or heterotrophic microbes plays a role in pharmaceuticals degradation activity [57, 60]. Balcom et al. [103] found that the relative abundance of sequences associated with ammonia monooxygenase, which was able to hydroxylate ibuprofen, was highest in biofilm samples from planted tanks. A study focused on removal of ibuprofen by CWs found that both the interstitial water and biofilm microbial community metabolic function were influenced by CW design, plant presence and species, but design had a greater influence than plants. Moreover, canonical correlation analysis indicated that biofilm microbial communities in three designs (mimicking HSSF, VF and aerated HSSF) played a key role in ibuprofen degradation. The

enzymes associated with co-metabolism of L-arginine, L-phenylalanine and putrescine were found to be potentially linked to ibuprofen transformations [104].

Overall, there are clear evidences of the importance of microbial biodegradation for pharmaceuticals removal in CWs. However, there is a big knowledge gap in relation to the microbial communities in CWs biofilms and their role and genetic information for the removal/biotransformation. Mostly, there is a need to disassemble all the experimental systems [50] after testing removal and further sequence the microbial communities. Furthermore, increased sequencing effort is also needed for full-scale systems.

From what is known, microbial degradation is in general favoured by warmer conditions. Higher removal in the summer of different pharmaceuticals, e.g. salicylic acid and caffeine, has been consistently associated with higher activity of the microbial communities (also when using biodegradation constants corrected for biomass) [57]. Moreover, we can cycle back to CW design and concepts of potentially favouring specific reactions types. For example, anaerobic environments favour the biodegradation of naproxen and diclofenac, while aerobic environments favour the removal of ibuprofen or carbamazepine [28, 57]. In addition, it should be stressed that the nature and extent of microbial degradation of organic chemicals within a CW is also expected to strongly depend on the physicochemical properties of the contaminant. Indeed, the biological degradability or recalcitrance of organic compounds may often be explained by its chemical structure, for instance, the presence of secondary, tertiary or quaternary carbon atoms as well as functional groups [73]. Nevertheless, pharmaceuticals cannot be treated as groups with respect to chemical behaviour. Small changes in chemical structure can have significant impact on solubility and polarity [50]. Matamoros and Bayona [105] reported that in HSSF, the removal of diclofenac was below 45%, while ibuprofen (80%) and ketoprofen (69%) showed greater removal. These compounds are all in the therapeutic class of non-steroidal anti-inflammatory, are aryl derivatives of propionic acid and are deprotonated and negatively charged (pH 5.3); only differing in the aryl group. However, biodegradation involves enzymatic reactions specific to chemical structures, which may result in varying rates of biodegradation [50]. Thus, the efforts placed in trying to use Log K_{ow} also for the prediction of biodegradation have failed [27]. During biodegradation, pharmaceuticals may undergo (1) mineralization; (2) transformation to more hydrophobic compounds, which partition onto the solid phase; and (3) transformation to more hydrophilic compounds, which remain in the liquid phase. To date, few published studies on microbiological degradation of pharmaceuticals in CWs are available, and these studies only use indirect methods to identify possible biodegradation pathways for pharmaceutical removal [50]. Since only a few compounds and studies have reported on transformation products [28], information on transformation pathways is severely lacking.

One last point to be mentioned is enantiomeric processes. Several pharmaceutical compounds are chiral [58]. Thus, enantiomeric fractionation of chiral compounds can be used as an indicator of enzyme-compound interactions and, together with mass balance approaches, an indicator of biodegradation [106], including for phytoremediation mechanisms [107]. Very little literature data was (and still is)

available regarding the dynamics of enantiomers of pharmaceuticals in CWs. Only a limited number of chiral drugs have been investigated to date [27]: atenolol, citalopram, fluoxetine, ibuprofen, metoprolol, nadolol, pindolol, propranolol, salbutamol and sotalol. Different removal efficiencies were observed for the S- and R-enantiomers of most of these compounds, but little else is known.

In general, even though microbial degradation is a key process driving the removal of pharmaceuticals in CWs, detailed process understanding is lacking. Answers to questions such as: which species (degraders) degrade which compounds; which transformation products are being formed and what are the biotransformation pathways; and are the processes driven by co-metabolism and/or catabolism are expected in the future.

4 Constructed Wetlands a Nature-Based Solution and Important Ecotechnology for a Green Transition

CWs have been used for decades as a valid alternative to treat wastewater for small (rural or urban) communities. Their historical application and development has followed different trends in different parts of the world [29]. Currently, we are observing a potential new phase. As climate change and environmental degradation are an existential threat to Europe and the world, a European Green Deal has recently been proposed [108]. This deal provides a road map with actions to (1) boost the efficient use of resources by moving to a clean, circular economy; and (2) restore biodiversity and cut pollution. For that, investment in environmentally friendly technology is expected, namely, in nature-based solutions. CWs are one of the identified nature-based solutions that can contribute to water purification and waste treatment, as well as for resources recovery [18, 109]. Naturally, as OECD calls for better policy approaches to manage pharmaceutical residues [4], we can expect an increased pressure on the usage of CWs to address not only classical pollutants but also pharmaceuticals and other contaminants of emerging concern [55].

Even though CWs require a higher footprint than conventional systems (roughly CWs are designed with 1–20 m^2 per person equivalent), these nonetheless represent a suitable toolbox to address pharmaceuticals contamination. But it is also perceived from different works, namely, performed in the laboratory, that there is potential to optimize the ecotechnology to achieve better performances for pharmaceuticals treatment. Potentially for its application as polishing step to conventional systems, or even as the final step in treatment tailored to specific users, such as healthcare or hospital facilities [27]. In spite of the potential, CWs application for such purpose is rather limited. This implies that the knowledge gathered in these review papers assess how CWs built to treat different types of wastewater perform in regard to pharmaceuticals. We are missing more studies on designs dedicated to improve pharmaceutical removal. Nevertheless, there is potential to apply optimized CWs

designed specifically to remove pharmaceuticals and other organic micropollutants. Even though data on removal efficiency is widely available, the small sample size on kinetic parameters, namely, areal removal rates [64, 91, 110], might be hindering the design of dedicated CWs for pharmaceuticals removal. In addition, we cannot forget the role that CWs play on the challenges and opportunities in wastewater reuse [111]. Ultimately further research is needed to optimize the removal of the most "critical" compounds. The scientists are working on the "critical" compounds, those that current knowledge indicates that are less effectively removed by CWs, like antibiotics and some analgesics and anti-inflammatories. Practitioners and water utilities are waiting for legislation that will dictate which "critical" compounds they need to meet on their emission targets.

As for other technologies, research on CWs needs to be coupled with the risk assessment of residual pharmaceuticals and transformation products to the aquatic environment. There is a clear knowledge gap with respect to the dynamics of pharmaceutical transformation products in CWs, also in the application of suspect-screening and non-target screening methodologies based on high-resolution mass spectrometry [112] to support the characterization of pharmaceuticals removal in CWs.

5 Conclusions

Constructed wetlands, by its main designs SF, HSSF and VF, but also hybrid systems and most recently aerated systems have demonstrated a good capacity to remove different pharmaceuticals from wastewater and treated wastewater. Various processes are involved in pharmaceuticals removal: photodegradation in SF and sorption, plant uptake and several transformation processes within the plant and microbial degradation in all different CW designs. Biodegradation is pointed as proposed the major removal process, but the true extent of plant-driven processes has not yet been properly quantified.

Processes are not only controlled by CW design, but also limited by pharmaceuticals' chemical properties. Photodegradation can be explored by using SF wetlands. Though, removal is limited to sensitive compounds and its implementation may encounter regulatory limitations (e.g. prohibition of open wastewater). Sorption occurs in all systems, depending on compound, type of substrate and build-up of particles and organic matter in the system. Plants can uptake and translocate pharmaceuticals; against earlier expectations of phytoaccumulation, it is now clear that both the plant and the endophytic bacteria can biotransform pharmaceuticals. Biodegradation, namely, by the concerted synergy of plants and microorganisms, has proven a key process. There is, though, limited process understanding at the microbial community level, as well as the biochemical degradation pathways. More efforts are needed in the application of multi-omics (metagenomics, metatranscriptomics, proteomics, metabolomics) as well as in the characterization of transformation products to CW studies. Aerobic biodegradation tends to be more efficient than

the anaerobic; therefore, VF and aerated systems have shown the best performances. However, hybrid designs that allow for multiple processes and extended treatment time have shown benefits for increasing the removal of certain compounds. Operational parameters have been widely studied, but their role cannot be disconnected from the specific designs tested and the environmental conditions of geographical area where the systems are tested/built. Several of these parameters are important to understand the observed processes but are also independent variables that may prove hard to control. In addition, pharmaceutical removal has been assessed in CWs designed and built to removal carbon, nitrogen and phosphorous. It is yet to be proposed which key mechanism can and should be optimized for improving the removal of pharmaceuticals in CWs.

The political agenda is currently favourable for increasing the efforts on controlling pharmaceuticals pollution by implementation of CWs as nature-based solutions. Thus, further developments of the technology are expected for the future.

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Part IV
Current Status of Analytical Methods

Development of Methods for the Determination of PhACs in Soil/Earthworm/Crop System Irrigated with Reclaimed Water

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Contents

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Abstract Pharmaceuticals have been becoming a major concern of environmental pollution since the beginning of the century. The ways in which these contaminants are introduced into the environment are very different, but almost always associated with wastewater. In fact, current wastewater treatment plants are not designed for the removal of pharmaceutical products. Indeed, the problem of water scarcity has played an important role in the introduction of pharmaceutical products into the environment, particularly in the agricultural sector. Because of the drought, more and more countries are resorting to the use of treated wastewater to irrigate vegetables for human consumption. Consequently, the reuse of wastewater in agriculture constitutes a continuous introduction of these molecules into the soil.

The effects of this practice are not entirely clear. However, the probability that these compounds can enter the food chain directly is high. In fact, through radical absorption, plants could uptake pharmaceuticals from soil and water, leading to the accumulation of drugs in the tissues.

The development of analytical methods of solid matrices such as soil or plant tissues requires substantial work due to the great complexity of the matrices and the differences between the physico-chemical properties of analytes of interest. Several multi-class methods have recently been developed to determine a large number of pharmaceutical products in soil or plants using different extraction techniques.

This chapter addresses to list all the analytical procedures published so far used for the extraction and analysis of pharmaceutical products from plant tissues and from the soil irrigated with treated wastewater.

Keywords ASE, Crop uptake, Pharmaceuticals, QuEChERS, Soil contamination, USE

1 Introduction

Treated wastewater is an incredible resource to cope with the increasing demand of water to meet the agricultural sector, undermined by the continued lack of water and frequent water shortages. In fact, the use of wastewater in irrigation as well as stabilizing the nutrient content in the soil can lead to an increase in the production of crops in arid areas due to the constant input of organic and mineral components into the soil through the wastewater. Despite the high content of nutrients, especially nitrogen immediately available for plant growth [1], irrigation of crops with recycled water can lead to the spread of pharmaceutical active compounds (PhACs) in agricultural land and waters. These substances are part of the products of daily use

and are not completely removed from the activated sludge treatment plants. Hence, they are a source of concern due to the harmful effects that these substances can cause in a variety of aquatic and terrestrial organisms due to their continuous entry into the environment.

The main drawback arising from the application of wastewater effluents is that vegetables could uptake PhACs from soil and water, leading to their accumulation at trace level in fresh products for human consumption [2]. In fact, the main disadvantage of wastewater reuse in agriculture is the potential contamination of soil, crops, and water sources and the inherent risk of harmful effects that contamination poses to exposed organisms. However, the PhAC concentrations in crops and vegetables should be much lower than the dosage for an effective therapy.

Every day, due to the constant development of the pharmaceutical industry, new compounds are approved and marketed. However, there is a general lack of knowledge of the effects that these substances cause on the environment. This is explained in part due to the reduced number of studies or the intrinsic difficulty of extracting and isolating and quantifying organic compounds at trace levels in soil and plants. Moreover, the analytical techniques used for this type of pollutants are relatively expensive.

Although several analytical methods have been developed to extract PhACs from water and soil samples, one of the greatest efforts of recent years is to try to develop robust analytical methods for the analysis of PhACs in plant tissues. Given the small number of these publications, it is evident how this issue represents a difficult challenge to overcome. Furthermore, the use of an inappropriate or low sensitive method with relatively high limits of quantification could contribute to give unreliable results as a consequence of the low concentrations with which these compounds are present in water or in the soil.

Due to the complexity of the sample matrix, the analysis of environmental samples involves several difficulties. The analyses of solid or semi-solid matrices such as soil, sludge, or sediment or of biological samples (animal, vegetable, or plasma tissues) are more complex than liquid samples, which usually require fewer pretreatment phases, due to their liquid form. In the case of wastewater, for example, an initial filtration is sufficient to remove the particulates followed by a solid-phase extraction using cartridges with different absorbent resins. The latest technological advances also allow for the analysis of surface or wastewater by direct injection avoiding all the problems related to the handling of the sample [3, 4].

Sample preparation has simply the purpose of transferring the analytes in a measurable form [5]. The first step for the success of an analytical method is sampling. In fact, the determination of pollutants in soil or plants initially requires specific sampling techniques that take into account the heterogeneity of the soil or plant matrix [6].

Furthermore, the sample preparation for the analysis of pharmaceutical residues is a very critical aspect since the analytes of interest are intimately bonded to the components of the matrix to be studied. Indeed, prior to the analysis, specialized extraction techniques are needed that can effectively isolate the analytes without any component of the matrix potentially interfering with the detection of the analyte. The

goal is to develop the most selective extraction method, optimizing the extraction conditions to exploit the chemical differences between analytes and matrix components. In the event that part of the matrix is co-extracted with the analytes, it is often advisable to carry out a further step consisting of techniques for cleaning the matrix from interfering co-extractives with the compounds of interest.

In fact, to obtain a selective method, it is necessary to consider in particular the removal of the matrix components such as organic matter, lipids, waxes, sugars, or pigments. The removal of these co-extracts must be controlled to minimize any adverse effects that could affect the detection of the compounds of interest. Therefore, several post-extraction cleaning procedures have been developed for the removal of the co-extracted matrix components.

Another often overlooked difficulty is the optimization of the extraction conditions of a new analytical method; it is necessary to use a relevant matrix containing the analytes of interest. For persistent organic contaminants or for pesticides, certified reference materials (CRM) are commercially available. However, as far as PhACs are concerned, these materials are not available, and the development must be based on internally produced materials. In most cases, producing internally contaminant-free material means growing vegetables until fully harvested (60–90 days in the case of a lettuce). This may lead to a delay in the development of the analytical method which is not always feasible. Very often then, we resort to the use of vegetables from organic farming [7].

This reference material thus obtained is then doped with known concentrations of the compounds of interest in order to study the recovery tests and thus guarantee the ability to perform a quantitative analysis of the compounds with the developed method.

In an attempt to obtain a rapid and efficient extraction of the analytes from solid matrices, different extraction techniques such as microwave-assisted extraction (MAE), ultrasound solvent extraction (USE), pressurized liquid extraction (PLE), or the most recent QuEChERS method have recently been the subject of in-depth study, due to good efficiency and reliability. In fact, these are the extraction techniques currently most used for the determination of PhACs in soil or plants. They are preferred to traditional extraction methods such as Soxhlet, as they guarantee greater contact between the solvent and the soil particles, with consequent greater coverage of the analytes and a lower consumption of organic solvents. In fact, the Soxhlet method involves very long extraction times with the consumption of large quantities of organic solvents.

The analysis of PhACs is commonly performed using liquid or gas chromatography techniques, although liquid chromatography is preferred as it is more suitable for the analysis of polar compounds.

The purpose of the chapter is to discuss all the analytical procedures used for the extraction and analysis of pharmaceutical products and their related compounds from plant tissues and from the soil irrigated with treated wastewater. This chapter will describe all that is known about the analytical procedures published so far. In the coming section, we will discuss the most popular previously reported methods for pharmaceuticals extractions (USE, PLE, MAE, QuEChERS) from soil and plant

tissues. The different cleanup techniques of the extracts will also be addressed, while the last section will be dedicated to separation and detection techniques. Given the lack of analytical methods in the literature, the determination of drugs in earthworms will be only partially treated at the end of the chapter.

2 Most Common Extraction Techniques

2.1 Assisted Solvent Extraction (ASE)

Assisted solvent extraction (ASE) is also known as pressurized liquid extraction, (PLE), pressurized fluid extraction (PFE), enhanced solvent extraction (ESE), or high-pressure solvent extraction (HPSE). ASE and PLE are the most popular names.

It is considered as an advanced, reasonably uncomplicated extraction technique, time saver, and easy to learn. Briefly, the samples are mixed with a proper cell matrix and filled in the cell. Then the cell is placed in a carousel; the latter rotates automatically in a way to put the cell in the oven chamber. After preheating the oven chamber, the cell is filled with the extraction solvent and kept for an optimized static time, under the desired temperature and pressure. Finally, a solvent containing the extracted analytes is obtained and collected in a vial, while the cell is then washed and purged with a nitrogen flow [8]. All of those steps constitute one cycle and can be repeated several times. The number of cycles is also optimized during method development.

It is usually employed at temperature above the boiling point of the used extraction solvent, along with high pressure to keep the solvent in liquid state during the extraction process. Further, solvent's viscosity decreases as the temperature increases, which enhances its ability to wet the matrix and solubilize the target analytes [9]. Therefore, besides its rapid extraction process, ASE has the advantage of less solvent consumption, less toxicity, more environmentally friendly, and possibility automation. All of these criteria have made ASE popular in pharmaceutical extraction field. It has been successfully applied to a wide range of analytes and pharmaceuticals since polar and non-polar solvents, or a mixture of solvents, may be used [10], though many parameters need to be optimized in order to obtain the highest analyte recoveries, such as solvent selection, temperature, pressure, extraction time, and other parameters such as extraction mode, extraction time, number of cycles, cell matrix, and flush volume.

2.2 Ultrasound Solvent Extraction (USE)

Ultrasound solvent extraction is based on cavitation effect generated by ultrasound radiations in a water bath. Usually solid samples and the extractant are mixed in a centrifuge tube and placed into tube rack in the ultrasonic water bath. The ultrasound

radiations generate a great number of tiny bubbles in liquid media (the extraction solvent added to the sample in the centrifuge tube) and mechanical erosion of solids (soil and crops samples); thus, particles rupture [11]. However, USE does not always reach high efficiency as efficiencies reached by other methods. Additionally, ultrasonic irradiation enhances the release of matrix components simultaneously with the analytes, hence an important matrix effect.

For a maximum pharmaceuticals' recovery, several parameters should be optimized such as type of solvent, irradiation conditions (temperature and amplitude of sonication), sonication time, number of cycles, and sample amount. Generally, to obtain the highest extraction efficiency with the lowest matrix interferences, several extraction cycles are employed, each with a fresh and small solvent volume [12]. Thus, solvents from the different extraction cycles are combined and processed to the following step, usually clean-up step, unless it is skipped to analysis.

Several ultrasound devices are available, such as the water bath, probes, sonireactors, or microplate horn [13]. Yet, an ultrasonic water bath was always employed for soil and crop analysis, which is also known to be the cheapest and the most available [14–16]. An important aspect is that these types of devices are almost always available in every laboratory. Finally, USE is an environment-friendly technique, less solvent consumer, and energy and time saving.

2.3 Microwave-Assisted Extraction (MAE)

MAE is also called microwave-assisted solvent extraction (MASE). In this technique, microwave energy is used to directly heat the solvent in contact with the sample, thus achieving the partition of target analytes from the sample to the solvent and accelerating the speed of extraction. The microwave energy released is nonionizing radiation that causes molecular motion by migration of ions and rotation of dipoles [12]. The efficiency of MAE depends strongly on the nature of the solvent and the matrix [17]. Solvents used for MAE should be able to absorb microwaves. In other words, solvent should have dipole leading (polar). However, a combination of polar and non-polar solvents has extended MAE usage [10].

It is an automated green extraction technique offering many advantages such as less solvent consumption and short extraction times, even less than USE technique [17, 18]. Additionally, several samples can be extracted simultaneously, hence increasing the number of samples analyzed daily. It also offers protection for thermolabile compounds. Further, the equipment for MAE is relatively expensive, which probably is the main reason why a small number of studies are dealing with MAE. Finally, several factors should be optimized in order to obtain the best efficiency, such as solvent selection, temperature, and extraction time.

2.4 *QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe)*

QuEChERS (acronym of quick, easy, cheap, effective, rugged, safe) extraction technique, as its name tells, is known to be fast, easy, and cheap. It consists of two consecutive steps, the extraction/partitioning step usually using acetonitrile as extractant and salts for partitioning and a clean-up step using dispersive solid-phase extraction (d-SPE). Additionally, QuEChERS is one of the widely known green extraction methods. It requires small amount of low toxic, non-halogenated solvents and reagents, as well as laboratory equipment and no external energy supply. Furthermore, this method has also introduced the concept of d-SPE for clean-up purposes. Also, the low cost and short time allow the extraction of a reasonable number of samples. It was first introduced for the determination of pesticides in vegetables [19]; later it was successfully employed for other compounds (e.g., pharmaceuticals, hormones, chlorinated compounds, etc.) in different matrices (eggs, blood, earthworms, and environmental matrices) [20–24].

Nowadays, three main QuEChERS methods are officially known and widely used. Briefly, the original method (OR) [19] is the first method developed by Anastassiades and coworkers in 2003, who are known as the fathers of QuEChERS. They employed acetonitrile as extractant and $MgSO_4$ + NaCl as partitioning salts. Later in 2007, Lehotay and coworkers [25] introduced a modified QuEChERS, in which they employed acidified acetonitrile with 1% acetic acid as extractant and the acetate-buffered salts ($MgSO_4$ + CH_3COONa) for partitioning; it is adopted as an American official method (AOAC). Finally, Anastassiades and coworkers modified their method in 2007 [26] to employ the citrate-buffered salts ($MgSO_4$ + $C_6H_5Na_3O_7{\bullet}2H_2O$ + $Na_2C_6H_6O_7{\bullet}1.5H_2O$) for partitioning, and it is adopted as a standard method of the European Committee for Standardization (CEN). Lastly, $MgSO_4$ + PSA (primary secondary amine) were used as d-SPE salts in all three previously mentioned methods.

However, many authors have introduced own modifications according to their needs, including the extractant and d-SPE salts. Details concerning extraction and clean-up steps developed in the last couple decades are reported in Sects. 4 and 5 for soil and crops, respectively. At last, several QuEChERS commercial kit versions are now available and sold by many vendors, which played an important role in the usage expansion and wide application of this method, since salts are already precisely weighed and mixed, avoiding the extra effort and time loss for this step.

Thereupon, QuEChERS and modified QuEChERS methods have reached extraction yields for multiresidue analysis in the same order or even better than the three previously reported techniques, without the need of the sophisticated expensive equipment. However, to date and according to our literature survey, only 6 studies have reported multiresidual extraction methods from soil using QuEChERS technique, whereas more than 30 studies are reported for crop extraction.

2.5 *Other Extraction Methods (Soxhlet and Solid-Liquid Extraction)*

Soxhlet extraction is known for being a traditional extraction procedure. The sample is placed in a thimble-holder, and fresh extraction solvent from a distillation flask fills the sample. When solvent completely fills the thimble-holder, a siphon takes the solvent, with the extracted compounds, returning it back to the distillation flask [27]. This operation is repeated several times as extraction is completed according to the criteria of the study. The complexity, the high amounts of solvent needed and the great time of run required, has caused Soxhlet to be relegated to the background (at least in the pharmaceutical area), giving way to the latest techniques. Moreover, extraction methods such as USE have the ability to penetrate deep into the solid matrix, usually by means of the creation of cavities, which allows to obtain a good extraction performance that Soxhlet is not available to achieve [27].

For liquid matrices such as the analysis of surface water or wastewater, solid-liquid extraction (SLE) is the most effective and easiest to use technique. It does not need major pretreatments. Normally, prior filtration helps remove particulate matter from the samples. It is very effective and used to clean the matrix in combination with other extraction techniques. In order to extract the target compounds of the matrix, an organic solvent or water is mixed with the sample until equilibrium, and the liquid is removed from the mixture (extract). Commonly, heat is applied in order to enhance the extraction efficiency, as well as buffer compounds to have control of the pH. SLE has advantages such as simplicity and few amounts of solvent; however, this extraction procedure is little selective, and that is the main reason that modern extraction techniques have broader paths than SLE.

Only one report showed the capability of SLE to extract acetaminophen from *Brassica juncea* using 1 mL of HCl 0.1 M and nitrogen [28]. A comparative analysis of the most current techniques in terms of costs/speed of execution is reported in Table 1.

Table 1 Comparison between the extraction methods used in terms of time, instrumentation required, solvent volume (green chemistry), and total cost per sample

Extraction method	Time (minutes)	Specific instrumentation	Solvent volume (mL)	Cost
ASE	10–30	Yes	15–100	$$$
USE	30–60	Yes	8–30	$$
MAE	5–20	Yes	5–10	$$$
QuEChERS	20	No	10	$
Traditional methods (Soxhlet)	360	Yes	220	$$$ $

3 Sample Preparation

Sample preparation concept is to convert a field matrix to a sample suitable for analysis. It is a crucial and important step especially for food and environmental matrices such as crops and soil, which are characterized by their complexity, besides the presence of pharmaceuticals in these matrices in trace amounts [29]. This step also allows to weaken the interactions established between pharmaceuticals and matrices, which is highly dependent on the physical and chemical properties of both pharmaceuticals and matrices. Further these interactions affect the applicability of different extraction methods, their factor conditions, as well as their efficiency and reproducibility.

Additionally, sample pretreatment is needed to assure its homogeneity and a good contact with the solvent during the extraction process. On the other hand, most of the analytical devices are unable to handle those matrices directly, which requires a pretreatment step before extraction for any matrix. In general, soil preparation includes three steps [12]: the first step is drying of sample, either by the use of the oven, by air-drying, or by lyophilization. However, sample exposure to elevated temperature may risk analyte degradation and alteration. Whenever lyophilization is employed, analytes are neither degraded nor evaporated, and the drying time is shorter. Generally, lyophilization is the most advantageous drying technique [30]. The second step consists in the homogenization and finely grounding of the soil samples generally using a mortar. The third and last step is soil sieving at 2 mm to remove coarse particles to increase sample homogeneity. Thereafter, soil samples are stored at −20°C or +4°C in the dark until analysis.

To perform ASE, from 0.5 to 20 g (Table 2) of soil are placed in a stainless-steel extraction cell (33 mL extraction cell volume is the most commonly used) and capped with filters at both ends. Soil sample is usually mixed with a cell matrix (dispersant) before loading in the cell, to enhance the extraction efficiency. Finally, the extraction cell is placed in the oven, and the system automatically starts setting the desired temperature and pressure. For MAE, 0.2–3 g (Table 4) were used, and no common step was found between the reported studies; they differ largely according to the MAE procedure employed. Finally, USE and QuEChERS are the easiest, where soil samples in both methods were introduced in centrifuge tubes. For USE 0.5–5 g (Table 3) of soil were used, the extraction solvent is therefore added, and the tube is placed in the sonication bath. While from 1 to 10 g (Table 5) of soil were used for QuEChERS, the extraction solvent and salts were added and the tube was immediately vortexed and hand shacked.

Plants are normally harvested when they have reached commercial size. When treating branched root plants, care should be taken, while their extraction from soil is done in order to preserve the root part completely. Once samples are recollected, they are handwashed and rinsed with tap water in order to remove residues such as the remaining soil and other interferences [7]. When plant samples are composed of a root part and a vegetative part or fruits, normally each part is treated differently due to its great difference between them [31]. Plant sample pretreatments also consist of

Table 2 ASE extraction and analysis conditions in soil

	ASE extraction conditions							Analysis				
Pharmaceuticals	Soil mass (g)	Solvent	T (°C) P(psi or bar)	Static extraction time (min) × number of cycles	Flush volume (%)	Cell matrix (dispersant)	Clean-up	Recovery (%)	RSD (%)	LOD	Instrument	References
Diclofenac, allopurinol, verapamil, carbamazepine, spironolactone, salbutamol, omeprazole, atenolol, bendroflumethiazide, diltiazem, acetylsalicylic acid, furosemide, prednisolone, cyproterone acetate, erythromycin, enalapril, metoprolol, paracetamol, citalopram, cimetidine, diazepam, zopiclone, oxazepam, amoxicillin, ramipril, terbutaline, penicillin G, ibuprofen, morphine, gestodene, losartan potassium, amlodipine besylate, simvastatin	20 g	Methanol/aqueous ammonia solution (0.1 mol L^{-1}) (1:1, v:v)	80°C 1,500 psi	5 min × 5	50%	Ottawa sand	Tandem MAX-HLB SPE cartridges	66–114%	1–14%	0.1–<2.5 ng/g	LC-MS/MS	[64]
Chlortetracycline, oxytetracycline, sulfadiazine, erythromycin, and tylosin (degradation products: TYL A, B, C, and D)	10 g	Methanol/0.2 M citric acid buffer (pH 4.7 adjusted with NaOH) (1:1, v:v)	Room T °C 1,500 psi	10 min × 1 and 3 min × 1	100%	Ottawa sand	Tandem SAX-HLB SPE cartridges	50–100%	Not reported	0.6–5.6 g/kg	LC-MS/MS	[58]
Ciprofloxacin and norfloxacin	0.5 g	50 mM aqueous phosphoric acid (pH 2.0) and acetonitrile mixture (1:1, v/v)	100°C 100 bar	15 min × 6	150%	Quartz sand	MPC disk cartridge	75–92%	8–11%	0.05 mg/kg of dry matter	LC-FLD	[68]
Paracetamol, salicylic acid, propranolol hydrochloride, clofibric acid, ketoprofen, diclofenac sodium salt, bezafibrate, warfarin, flurbiprofen, indomethacin, ibuprofen sodium salt, meclofenamic acid sodium salt, gemfibrozil, atenolol, salbutamol, sulfamethoxazole, sulfamethazine sodium	2.7 g	MeOH:H2O (50:50, v/v)	60°C 1,500 psi	5 min × 2	100%	Sea sand	HLB cartridges	>60% (for 20 analytes)	Not reported	<20 ng/g (for 20 analytes)	LC-MS/MS	[55]

salt, furosemide, pravastatin sodium salt, carbamazepine, nimesulide, (±)-metoprolol, (±)-tartrate salt, clotrimazole, trimethoprim, caffeine, S(±)-2-(6-methoxy-2-naphthyl)-propionic acid (naproxen), and irgasan (triclosan)												
Acetaminophen, diclofenac, fenoprofen, ibuprofen, indomethacin, ketoprofen, mefenamic acid, naproxen, phenazone, bezafibrate, clofibric acid, gemfibrozil, pravastatin, caffeine, carbamazepine, famotidine, ranitidine, atenolol, metoprolol, nadolol, pindolol, propranolol, timolol, furosemide, hydrochlorothiazide, glyburide, albuterol, chloramphenicol, clindamycin, flumequine, lincomycin, metronidazole, novobiocin, ornidazole, sulfadiazine, sulfadimethoxine, sulfaguanidine, sulfamethazine, sulfamethizole, sulfamethoxazole, sulfamethoxypyridazine, sulfanilamide, sulfathiazole, sulfisoxazole, trimethoprim	4 g	Milli-Q water at neutral pH	100°C 1,500 psi	5 min × 3	60%	Hydromatrix	HLB cartridges	50–140%	Not reported	<0.01–0.83 ng/g	UPLC-MS/MS	[53]
Sulfapyridine, sulfamerazine, sulfadimethoxine, tetracycline, chlortetracycline, oxytetracycline, doxycycline, erythromycin, roxithromycin	3 g	50% phosphate buffer and 50% acetonitrile with pH = 3	70°C 1,500 psi	10 min × 1	60%	Diatomite	Tandem SAX-HLB SPE cartridges	60.2–97.6%	<10%	0.2–1.7 μg/kg	HPLC-MS/MS	[70]
Sulfapyridine, sulfamerazine, sulfadimoxine, tetracycline, chlortetracycline,	3 g	50% methanol and 50% 0.2 M citric acid with	70°C 1,500 psi	10 min × 1	60%	Sea sand and diatomite	Tandem SAX-HLB SPE cartridges	67.3–97.4%	<9%	0.2–1.1 ug/kg	HPLC-MS/MS	[71]

(continued)

Table 2 (continued)

Pharmaceuticals	ASE extraction conditions						Clean-up	Analysis				References
	Soil mass (g)	Solvent	T (°C) P(psi or bar)	Static extraction time (min) × number of cycles	Flush volume (%)	Cell matrix (dispersant)		Recovery (%)	RSD (%)	LOD	Instrument	
oxytetracycline, doxycycline, erythromycin, roxithromycin		pH adjusted to 4.7 by NaOH										
Chlortetracycline and oxytetracycline, and their TPs: 4-epitetracycline, 4-epianhydrotetracycline, 4-epichlorotetracycline, epioxytetracycline, α-apo-oxytetracycline, and β-apo-oxytetracycline	5 g	Methanol/water (3:1, v/v) containing 25 mM EDTA and 0.6 M sodium chloride (pH 8.0)	Room T°C 10 MPa	20 min × 2	60%	HydromatrixVR diatomaceous earth	Tandem SAX-HLB SPE cartridges	41%–110%	0.12–0.34%	0.08–0.3 mg/g	HPLC-MS	[59]
Methylparaben, methyl protocatechuate, propylparaben, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, 1,3,7-trimethylxanthine (caffeine), 1,7-dimethylxanthine, carbamazepine, 3-hydroxycarbamazepine, 10,11-dihydro-10-hydroxycarbamazepine, carbamazepine-10,11-epoxide, diclofenac, 4-hydroxydiclofenac, ibuprofen, 1-hydroxyibuprofen, 2-hydroxyibuprofen, carboxyibuprofen, sulfamethoxazole, and N4-acetylsulfamethoxazole	2 g	MeOH (0.5% v/v, formic acid)	50°C 1,500 psi	5 min × 2	60%	Diatomaceous earth	In-cell clean-up with C18 and PSA	60–103%	<11%	0.04–4.31 ng/g	HPLC-MS/MS	[69]
Sulfisomidine, sulfamerazine, sulfacetamide, sulfadoxine, sulfabenzamide,	5 g	MeOH-water (90:10, v/v)	100°C 1,500 psi	5 min × 3	100%	Hydromatrix	HLB cartridges	60–130%	< 23%	0.01–4.19 ng/g	HPLC-MS/MS	[61]

succinylsulfathiazole, sulfaquinoxaline, sulfadiazine, sulfadimethoxine, sulfamethazine, sulfamethizole, sulfamethoxazole, sulfamethoxypyridazine, sulfapyridine, sulfathiazole, sulfisoxazole, sulfanitran and N4-acetylsulfamethazine, and the acetylated metabolites N4-acetylsulfamethoxazole, N4-acetylsulfapyridine, N4-acetylsulfadiazine, and N4-acetylsulfamerazine												
Clofibric acid, ibuprofen, salicylic acid, 2,4-D, gemfibrozil, naproxen, ketoprofen, diclofenac, carbamazepine, 4-nonylphenols, triclosan, bisphenol A, di-n-butyl phthalate, butyl benzyl phthalate, bis-2-ethylhexylphthalate, estrone, 17-estradiol, 17-ethinylestradiol	10 g	Acetone/hexane/acetic acid (50:50:2; v/v/v)	100°C 1,000 psi	5 min × 2	50%	Diatomaceous earth	HLB cartridges	54–118%	< 10%	0.25–2.5 ng/g	GC-MS	[54]
Avermectin, abamectin, doramectin, emamectin benzoate, eprinomectin, ivermectin, moxidectin, and selamectin	5 g	Methanol/water (1:1, v/v)	40°C 100 bar	5 min × 3	75%	Sea sand	HLB cartridges	63–80%	9–15%	LOQ: 0.5–2.5 ng/g	LC-MS/MS	[72]
Sulfadiazine, sulfadimethoxine, sulfamethazine, sulfamethoxazole, and sulfathiazole	4 g	0.1 M Tris (pH 8.8) and acetonitrile (85:15)	200°C 100 bar	5 min × 1		Diatomaceous earth	No clean-up	62–93%	< 10%	<15 g/kg	LC-MS/MS	[56]
Oleandomycin, erythromycin, tylosin, roxithromycin, tiamulin fumarate, monensin, ivermectin, salinomycin	30 g	1% (v/v) aqueous ammonia in methanol	80°C 140 bar	10 min × 2	70%;	Ottawa sand	Diol SPE cartridges	43–118%	19–29%	0.2–1.6 mg/kg	LC-MS/MS	[73]
Acetaminophen, codeine, carbamazepine,	3 g	Water	90°C 500 psi	7 min × 3	100%	Sea sand	Tandem SAX-HLB	59–119%	<16%	0.1–6.8 ng g^{-1}	LC-MS/MS	[9]

(continued)

Table 2 (continued)

Pharmaceuticals	ASE extraction conditions						Clean-up	Analysis				References
	Soil mass (g)	Solvent	T (°C) P(psi or bar)	Static extraction time (min) × number of cycles	Flush volume (%)	Cell matrix (dispersant)		Recovery (%)	RSD (%)	LOD	Instrument	
ciprofloxacin, clofibric acid, diazepam, diclofenac, fenofibrate, ibuprofen, metoprolol, norfloxacin, ofloxacin, oxytetracycline, sulfamethoxazole, tetracycline, propranolol, trimethoprim, and 4-epitetracycline hydrochloride, 4-epioxytetracycline							SPE cartridges					
Chlortetracycline, doxycycline, oxytetracycline, and tetracycline antibiotics	5 g	5% (w/v) sodium acetate, 100 mM EDTA, in a 50:50 solution with methanol, adjusted to pH 8 with sodium hydroxide	60°C 104 bar	5 min × 2		Hydromatrix	Strata-X SPE cartridges	22–99%			LC-MS	[50]
17R-Ethinylestradiol, 17-β-estradiol, 17R-estradiol, sulfamethazine, sulfadimethoxine, sulfameter, sulfamethizole, sulfamerazine, sulfachloropyridazine, sulfadiazine, sulfamethoxazole, tetracycline, and oxytetracycline. Estrone and 4-epitetracycline. 17-β-Estriol and sulfathiazole. Conjugated estrogen metabolites, estrone-3-sulfate, estrone-3-glucuronide, 17R-ethinylestradiol-3-glucuronide, 17β-estradiol-3-sulfate, 17R-estradiol-3-	5 g	Water/methanol/acetone (50/25/25, v/v/v) containing 25 mM EDTA, 2% ammonium hydroxide, and 0.6 M sodium chloride	Room T °C 100 bar	10 min × 2	60%	Hydromatrix	HLB SPE cartridges	21–105%	4–24%	0.01–0.1 ng/g	LC-MS/MS	[60]

sulfate, 17β-estradiol-17-sulfate, and 17β-estradiol-3-glucuronide. Anhydrotetracycline hydrochloride, anhydrochlorotetracycline hydrochloride, 4-epichlorotetracycline hydrochloride, and chlortetracycline hydrochloride												
Chlortetracycline, doxycycline, oxytetracycline, and tetracycline	5 g	Deionized water (pH 7.0–6.8)	70°C 1,500 psi	10 min × 1	100%	Sea sand	SAX and Strata-X cartridges	71–96%	8–15%	<1–3 μg kg^{-1}	LC-MS/MS	[52]
Flubendazole, erythromycin, erythromycin (–H2O), dicloxacillin, ciprofloxacin, sulfamethoxazole, oxytetracycline, carazolol, diclofenac, meclofenamic acid, carbamazepine, clofibric acid, natamycin, enilconazole, ketoconazole, fluconazole, clotrimazole, miconazole, itraconazole, griseofulvin, voriconazole, thiabendazole, difenoconazole, hexaconazole, penconazole, propiconazole, paclobutrazol, prochloraz, tebuconazole, bromuconazole, cyproconazole, epoxiconazole, fenbuconazole, fluquinconazole, flusilazole, flutriafol, metconazole, prothioconazole, terconazole, myclobutanil, triticonazole, carbendazim, metalaxyl	5 g	Acetone/citric acid 0.2 M (50:50) (pH adjustment at 4.5 with sodium hydroxide)	50°C 1,500 psi	5 min × 2	50%	Diatomaceous earth	Strata-X SPE cartridges	>70%		≤10 μg kg − 1	UHPLC-MS	[74]

Table 3 USE extraction and analysis conditions in soil

	USE extraction conditions					Analysis				
Pharmaceuticals	Soil mass (g)	Solvent	Time (min) × number of cycles	Temp. (°C)	Clean-up	Recovery (%)	RSD (%)	LOD	Instrument	References
Tetracycline, chlortetracycline, oxytetracycline, trimethoprim, sulfadimidine, sulfadiazine, sulfadoxine, sulfathiazole, sulfamethoxazole, ciprofloxacin, enrofloxacin	3 g (for tetracyclines, sulfonamides, and trimethoprim) 1 g (for ciprofloxacin and enrofloxacin)	MeOH:EDTA-McIlvaine buffer pH = 6 (90:10)	20 min × 3		SPE-C18 cartridge (for tetracyclines, sulfonamides, and trimethoprim) n-Hexane (for ciprofloxacin and enrofloxacin)	61–105%			LC-MS/MS	[76]
4-Epioxytetracycline, 4-epitetracycline, acetylcysteine, allopurinol, alprazolam, atorvastatin, azithromycin, bezafibrate, bisphenol A, butylparaben, carbamazepine, chloramphenicol, clofibric acid, ciprofloxacin, clarithromycin, codeine, diazepam, diclofenac, ethylparaben, etoricoxib, fenofibrate, flufenamic acid, gemfibrozil, ibuprofen, indomethacin, metformin, methylparaben, metoprolol, naproxen, norfloxacin, ofloxacin, oxytetracycline, paracetamol, propranolol, propylparaben, salicylic	1 g	Mix of methanol, distilled water, and of McIlvaine-EDTA buffer	30 min × 1		Strata-X 33 U polymeric reversed phase SPE cartridges	38–104%	<30%		UHPLC-MS/MS	[77]

acid, sulfamethoxazole, telmisartan, tetracycline, thiamphenicol, triclocarban, triclosan, trimethoprim, vildagliptin, warfarin, tetrahydrocannabinol, 11-nor-9-carboxy-9-tetrahydrocannabinol										
Quinolones (cinoxacin, oxolinic acid, nalidixic acid and flumequine) and fluoroquinolones (norfloxacin, enrofloxacin, enoxacin, ciprofloxacin and danofloxacin)	1 g	Aqueous solution of MgNO3 50% (w/v) containing 4% of ammonia	30 min × 1	Room temperature	No clean-up	> 82%	< 12%	0.04–0.08 μg g^{-1}	HPLC-UV	[78]
Norfloxacin, ciprofloxacin, enrofloxacin, tetracycline, Chlortetracycline, oxytetracycline, sulfacetamide, sulfachloropyridazine, sulfadimethoxine, sulfamerazine, sulfadimidine, sulfamethoxazole, sulfathiazole, sulfamethizole, sulfamethoxypyridazine, sulfapyridine and sulfisoxazole, ofloxacin	2 g	Mixture of potassium phosphate buffer and acetonitrile (ACN) (1/1, v/v, pH 3.2) and 0.4 g Na2EDTA	10 min × 3		Tandem SAX-HLB SPE cartridges	56–97%	1–20%	8.9 μg/kg	LC-MS/MS	[75]
Clofibric acid, ibuprofen, naproxen, ketoprofen, diclofenac, triclosan, 4-tert-octylphenol, 4-n-nonylphenol, bisphenol A, and estrone	5 g	Mix of acetone and ethyl acetate	15 min × 4		C18 cartridge	63.8–110.7%			GC-MSD	[79]

(continued)

Table 3 (continued)

Pharmaceuticals	USE extraction conditions				Clean-up	Analysis				References
	Soil mass (g)	Solvent	Time (min) × number of cycles	Temp. (°C)		Recovery (%)	RSD (%)	LOD	Instrument	
Acetylsalicylic acid, ibuprofen, paracetamol (acetaminophen), flurbiprofen, naproxen, diflunisal, ketoprofen; diclofenac sodium salt, valproic acid; primidone, nadolol, propranolol (hydrochloride), diethylstilbestrol, estrone, 17-β-estradiol, 17-α-ethinylestradiol, estriol, amitriptyline, imipramine, and clomipramine in the form of hydrochlorides	5 g	Ethyl acetate/formic acid (50:1, v/v)	15 min × 3		Silica gel columns	> 80%	1.1–10.0%	0.3–1.7 ng g^{-1}	GC-MS	[80]
4-tert-Octylphenol, benzo[a]pyrene, bisphenol A, carbamazepine, indole, p-cresol, phenanthrene, triclosan	2 g	80:20 IPA:H2O	10 min × 2	Room temperature	HLB cartridges and Florisil cartridges	46.1–110%	4–48%	7.6–60.7 µg/g	GC-MS	[81]
Difloxacin, sarafloxacin, enrofloxacin, ciprofloxacin, enoxacin, norfloxacin, chlortetracycline, oxytetracycline, doxycycline, tetracycline, sulfaquinoxaline, sulfaclozine, sulfamethoxydiazine,	5 g	ACN:Na2EDTA-McIlvaine buffer (pH 4.0, 5:5, v/v) and 0.2 M NaOH	10 min × 2		HLB cartridge	> 60%	< 20%	0.01–2 µg/kg	LC-MS/MS	[82]

sulfamonomethoxine, sulfadimidine, sulfamethoxazole, tylosin, roxithromycin, kitasamycin, erythromycin, tilmicosin, clindamycin, valnemulin, and tiamulin										
Carbamazepine, diclofenac, cis-diltiazem, lamotrigine, methadone, midazolam, oxcarbazepine, sulfamethoxazole, trimethoprim, valsartan, cocaine, acridone, 4-′-hydroxydiclofenac, and valsartan acid	0.5 g	ACN:MeOH (1:1, v/v) and 0.5% formic acid in ACN:MeOH (1:1, v/v)	20 min × 3		HLB cartridge	71–122%	< 10%	0.03–1 ngg^{-1}	LC-MS/MS	[83]
Oxytetracycline, sulfachloropyridazine, and tylosin	4 g	MeOH:0.1 M EDTA:McIlvaine buffer, 50:25:25	10 min × 3		SAX-HLB SPE cartridges	27–105%		18–40 μgkg^{-1}	HPLC-UV	[84]
Clofibric acid, ibuprofen, salicylic acid, allopurinol, paracetamol, gemfibrozil, fenoprofen, amitriptyline, metoprolol, naproxen, mefenamic acid, ketoprofen, carbamazepine, diclofenac, fenofibrate	5 g	ACN containing 2% of NH4OH and ACN containing 2% of formic acid	15 min × 2	Room temperature	No clean-up	80–115%	8–12%	0.04–0.24 ng g^{-1}	GC-MS	[85]
Doxycycline, erythromycin, progesterone, tylosin tartrate, amoxicillin, norfloxacin, sulfadiazine, trimethoprim, enrofloxacin, flumequine, tilmicosin	1 g	MeOH:ACN:0.1 M EDTA:McIlvaine buffer (pH 4), 30:20:25:25	10 min × 3		HLB cartridges	63–121%	≤ 20%	0.5–3 μg/kg	LC-MS/MS	[86]

(continued)

Table 3 (continued)

	USE extraction conditions					Analysis				
Pharmaceuticals	Soil mass (g)	Solvent	Time (min) × number of cycles	Temp. (°C)	Clean-up	Recovery (%)	RSD (%)	LOD	Instrument	References
Acetylsalicylic acid, ibuprofen, paracetamol (acetaminophen), flurbiprofen, naproxen, diflunisal, ketoprofen, diclofenac sodium salt, valproic acid, primidone, nadolol, propranolol (hydrochloride), diethylstilbestrol, estrone, 17-β-estradiol, 17-α-ethinylestradiol, estriol, amitriptyline, imipramine, and clomipramine in the form of hydrochlorides	5 g	Ethyl acetate/formic acid (50:1, v/v)	15 min × 3		Silica gel columns	52–112.6%	≤ 9.0%	0.3–1.7 ngg^{-1}	GC-MS	[80]

Table 4 MAE extraction and analysis conditions in soil

Pharmaceuticals	MAE extraction conditions				Clean-up	Analysis				References
	Soil mass (g)	Solvent (power in watt)	Time (min) × number of cycles	Temp (°C)		Recovery (%)	RSD (%)	LOD	Instrument	
Acetylsalicylic acid, carbamazepine, chloramphenicol, clofibric acid, diclofenac, florfenicol, flunixin, ibuprofen, ketoprofen, mefenamic acid, metoprolol, naproxen, niflumic acid, paracetamol, phenylbutazone, propranolol, pyrimethamine, triclosan and thiamphenicol, 17α-ethinylestradiol, 17-β-estradiol, and estrone	1 g	3:2 methanol/water (500 W)	6 min × 1		HLB cartridges	91–101%	< 6%	0.8–5.1 ng/kg	GC-MS	[18]
Acetylsalicylic acid, ibuprofen, paracetamol, flurbiprofen, naproxen, diflunisal, ketoprofen, diclofenac sodium salt, diethylstilboestrol, estrone, 17β-estradiol, 17-α-ethinylestradiol, and estriol	5 g	Acetonitrile (400 W)	15 min × 1	115°C	HLB cartridges	> 50%		0.3–5.7 ng/g	GC-MS	[87]
Oxolinic acid and flumequine		1 M phosphoric acid buffer at pH 2 and dichloromethane	22 min × 1	90°C	1 M aqueous sodium hydroxide	79–94%	3–7%		LC-FLD	[88]
Norfloxacin and ciprofloxacin	0.2 g	Water (120 W)	5 min × 1		No clean-up	98%	5.21%		HPLC-photometric detector	[89]

(continued)

Table 4 (continued)

Pharmaceuticals	MAE extraction conditions				Clean-up	Analysis				References
	Soil mass (g)	Solvent (power in watt)	Time (min) × number of cycles	Temp (°C)		Recovery (%)	RSD (%)	LOD	Instrument	
Caffeine, 17-estradiol, ibuprofen, ketoprofen, musk ketone, naproxen, triclosan, and epicoprostanol	3 g	2:1 (v/v) methylene chloride/methanol	15 min × 3	115°C	Pasteur pipettes were filled with pre-extracted silica gel (1.11 ± 0.01 g) and further packed with anhydrous sodium sulfate (~0.5 cm) and activated (2 N HCl) copper granules (~0.5 cm) in hexane to serve as clean-up columns	89.6%	2.89%		GC-MS	[90]
Sulfadiazine, sulfapyridine, sulfamerazine, sulfameter, sulfadimidine, sulfamethoxazole, sulfamonomethoxine, sulfaphenazole, sulfaquinoxaline, and sulfanitran	2 g	Triton X-114 (1.5%, v/v) (800 W)				69.7–102.7%	< 7%	0.42–0.68 ngg^{-1}	LC-UV	[91]
Methylparaben, ethylparaben, propylparaben, butylparaben, isopropylparaben,	0.5–2 g	MeOH (350 W)	3 min × 1		PTFE column	92–102%	5.4–6.9%	0.5–4.5 ng kg^{-1}	GC-MS	[92]

isobutylparaben, benzylparaben, 2-phenylphenol, 4-phenylphenol, nonylphenol, 4-tert-octylphenol, bisphenol A, and triclosan										
Norfloxacin and danofloxacin	1 g	Aqueous 20% (w/v) Mg(NO3) 2.6 H2O and 2% (v/v) NH3 solution	20 min × 1	80°C	No clean-up	70–130%	1–6%	Few nano-grams per gram	HPLC-FD	[93]
1H-Benzotriazole, 5-methyl-1H-benzotriazole, benzothiazole, 2-hydroxybenzothiazole, 2-methylthiobenzothiazole, benzenesulfonamide, and toluenesulfonamide	3 g	Methanol	10 min × 1 (200 W)	120°C	No clean-up	70–110%	< 11%		HPLC-UV	[94]
Abamectin, doramectin, and ivermectin	1 g	ACN/water (90/10)	15 min × 1	120°C	SPE cartridge				UHPLC-MS/MS	[95]
Sulfadiazine, sulfamerazine, sulfamethoxydiazine, sulfamonomethoxine, sulfadimethoxine, sulfa-methoxazole, and sulfaquinoxaline	2 g	Triton X-114 (5.0%, v/v)			No clean-up	81.2–93.7%	4.6–9.5%	3.2–5.7 ng g^{-1}	HPLC-UV	[96]
Sulfadiazine, sulfameter, sulfamonomethoxine, and sulfaquinoxaline	2 g	ACN (320 W)			On line SPE	82.6–93.7%		1.4–4.8 ng g^{-1}	LC-MS/MS	[97]
Sulfadiazine and its two major metabolites, N-acetylsulfadiazine and 4-hydroxysulfadiazine	10 g	Acetonitrile/water 1:4 (v/v)	15 min × 1	150°C						[98]

(continued)

Table 4 (continued)

Pharmaceuticals	MAE extraction conditions				Clean-up	Analysis				References
	Soil mass (g)	Solvent (power in watt)	Time (min) × number of cycles	Temp (°C)		Recovery (%)	RSD (%)	LOD	Instrument	
Sulfaguanidine, sulfacetamide, sulfadiazine, sulfathiazole, sulfapyridine, sulfamethazine, sulfamerazine, sulfamethoxazole, and sulfadimethoxine	1 g	Methanol	45 min × 1	110°C				Sub- to low ng per g	LC-MS/MS	[99]
Tetracycline, deoxytetracycline, oxytetracycline, chlortetracycline	1 g	Methanol (400 W)	20 min	60°C	μ-SPE device consisted of copper (II) isonicotinate	70.6–110.5%	<15.1%	0.1–6.3 ng/g	HPLC-UV	[100]

Table 5 QuEChERS extraction and analysis conditions in soil

	QuEChERS extraction conditions				Analysis				
Pharmaceuticals	Soil mass (g)	Solvent	Extraction salts	Clean-up salts	Instrument	Recovery (%)	RSD (%)	LOD	References
Bentazone, atrazine, carbamazepine, phenytoin, and its metabolite 5-(p-hydroxyphenyl)-5-phenylhydantoin	5 g	CH3CN:H2O 70:30, 5% CH3COOH	Original salts: anhydrous MgSO4 and NaCl	d-SPE: C18 and anhydrous MgSO4	HPLC-UV	88–113%	≤14%	4–493 μg/kg	[102]
14 veterinary products, 11 hormonal steroids, 6 human contaminants (paracetamol, sulfamethoxazole, fluvoxamine, carbamazepine, ibuprofen, bisphenol A)	5 g	Milli-Q water and ACN	AOAC salts	SAX and Strata-X SPE cartridge	LC-MS/MS	20–90%	≤30%		[106]
Ibuprofen and its metabolites (hydroxyibuprofen and carboxyibuprofen)	5 g	Acidified purified water (pH 2.5, hydrochloric acid) and for sonication acidified ACN (1% HCOOH)	EN salts: MgSO4, NaCl, 1 g of sodium citrate dehydrate (NaCit), and 0.5 g of sodium citrate sesquihydrate (Na2Cit)	No clean-up	LC-FLD	>80%	<5%		[101]
Ceftiofur, clopidol, florfenicol, monensin, salinomycin, sulfamethazine,	10 g	Distilled water and CAN acidified with acetic acid	Sodium acetate	C18	LC-MS/MS	60.25–120.3%	<13% except for		[103]

(continued)

Table 5 (continued)

Pharmaceuticals	QuEChERS extraction conditions			Clean-up salts	Analysis				References
	Soil mass (g)	Solvent	Extraction salts		Instrument	Recovery (%)	RSD (%)	LOD	
sulfathiazole, sulfamethoxazole, tiamulin, and tylosin							clopidol (25.2%)		
Sulfacetamide, sulfisomidine, sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, sulfameter, sulfadimidine, sulfamethizole, sulfadoxine, sulfamethoxazole, sulfamoxole, sulfamonomethoxine, sulfisoxazole, sulfabenzamide, sulfaquinoxaline, sulfadimethoxine, erythromycin, roxithromycin, clarithromycin	5 g	McIlvaine buffer	Na2EDTA and NaCl	d-SPE: PSA, C18, and MgSO4	LC-MS/MS	61.4–118.9%	<20%		[105]
73 target compounds (pharmaceuticals and TPs)	1 g	Milli-Q H2O and 1% acetic acid in ACN	Anhydrous MgSO4 and sodium acetate	Anhydrous MgSO4 and C18	LC-MS/MS	70–120% (this approach achieved to extract a total of 53 over 73 compounds)	≤20%		[104]

the removal of the water content and also a crushing process in order to obtain fine powder samples [32]. Although not all the studies have decided to eliminate the sample moisture, water content removal could provide more stabilization to the sample when stored [33, 34]. Besides, working with wet matrices makes it necessary to work with a higher amount of sample than working with dry samples. Commonly, freeze-drying process is performed ensuring a complete water removal. On the other hand, the crushing process is performed to ensure homogenization of the sample, but it also improves the subsequent extraction process. Depending on the extraction method performed and the use of dry or wet samples, the quantity of sample required varies. In general, 0.1 to 15 g of samples are used for analysis. Small quantities up to 5 g are used for dry samples [35], while larger amounts are required when wet samples are analyzed [36]. Freeze-drying samples, apart from preserving the stability of compounds of interest, allows to store in a small space originally voluminous samples. Lyophilization is the most common freeze-drying technique used [37] due to its rapidity but also the certainty of not wasting sample during the process [38].

As before-mentioned, sample quantity strongly depends on the extraction method used. Similar sample weights are used for the extraction of pharmaceutical products from plant tissues. From 0.1 to 8 g samples [39, 40] were used to perform ASE as its methodology is mainly focused on parameters such as solvent, temperature, and pressure, while sample amount plays a secondary role. In the case of USE, weights between 0.1 and 10 g sample were used [41–44]. However, compared to ASE, less quantity of sample is needed when USE was employed. Little amount of sample enhances the extraction efficiency due to the need to create cavities inside the matrix that are easily formatted when lower amount of sample is analyzed. On the other hand, ASE is not as sensitive to this factor owing to other parameters such as temperature and pressure which could help in the creation of these cavities inside the sample. Finally, as regards the QuEChERS method, several authors used weight ranges from 0.5 to 15 g [7, 36, 45, 46]. Looking at the original method developed by Anastassiades et al. [19], the established weight was 10 g of wet sample. This quantity was established on the basis of the amount of salts used by the QuEChERS methodology. It has to be noted that, depending on the nature of the analyzed sample (low-fat samples, high-fat samples, low water amount samples, etc.), different weights and hydration volumes should be applied in order to achieve a successful extraction performance. When 10 g of fruits between 25 and 80% of water content are analyzed, the amount of hydration water added varies depending on its water content. For example, for fruit with 60% water content, 4 mL of water should be added. On the other hand, 5 g of cereals and honey requires 10 mL of hydration water, while fruits with a water content above 80% do not need any amount of water hydration [47]. All these considerations are based on pesticide extraction though; extraction variations could be observed when PhACs are the target analytes.

4 Extraction Procedures for Pharmaceuticals from Soil

The fate of pharmaceuticals in soil depends strongly on their physico-chemical properties and soil texture; they might be sorbed on soil particles and its organic matter content, degraded by soil microbial community, volatilized, or leached to groundwater [48]. In order to better understand the fate of pharmaceuticals in soil, accurate and reliable analytical methods are needed as in to identify and quantify these molecules at low environmental concentrations ranging from a few μg/kg up to g/kg [48].

Soil is a very complex matrix, and complex interactions between pharmaceuticals and soil particles and organic content are established. Besides, in solid environmental matrices, pharmaceuticals are present at very low concentrations with a large number of potentially interfering compounds. Due to this, analytical method development is a challenging task that requires adequate extraction and clean-up procedures. Extraction techniques should be selective and highly effective. For example, target analytes should be better recovered when co-extracts (matrix impurities) are fewer. Further, they should be fast, easy, and cheap and require minimal organic solvent use. A following clean-up step is essential in these methods since soil is very complex, and a co-extracted matrix contaminant is involuntarily co-extracted (such as humic and fulvic substances), especially for USE, PLE, and MAE, which they employ energy to extract pharmaceuticals [49]. Moreover, this step helps in sustaining analytical devices and to improve limits of detection and quantification as pharmaceuticals are present at low concentrations in soil. Solid-phase extraction (SPE) is the most commonly used clean-up technique for the extraction techniques discussed in this section. Notwithstanding the foregoing, few studies have omitted this step since it allows the loss of the analytes along with the removal of matrix interferences. Despite everything, all of those modern analytical techniques include decreased sample amounts, less solvent consumption, time saving, high recoveries, good reproducibility, repeatability, and detection limits.

4.1 Assisted Solvent Extraction (ASE)

The selection of the extractants for trace residues from soil is crucial. One advantage of ASE is the possibility to choose a wide range of solvents. Several extraction solvents and buffer solutions have been employed to extract pharmaceuticals simultaneously from soil, with varying degrees of target compound recoveries [50, 51]. Acetonitrile (ACN), methanol (MeOH), hexane, and water are the most commonly used solvents for the soil and recognized as safe and environmentally friendly. Among these, the use of water as a solvent represents the most ecological extraction method and has already been successfully used with recoveries of over 50% [9, 52, 53]. Generally, a mixture of solvents of different polarity is more successful for the ASE application. This mixture allows to extract the majority of

the analytes covering the entire polarity range of the compounds, reducing the presence of other components of the matrix in the final extracts. An instance for this, Durán-Alvarez and coworkers [54] obtained improved recoveries when the mix of solvents acetone/hexane (1:1, modified with 2% acetic acid) was employed instead of single solvent, with which acetone is the polar solvent and hexane is the non-polar solvent. The addition of some buffers such as ammonia, citric acid, phosphoric acid, formic acid, sodium acetate, and ethylenediaminetetraacetic acid (EDTA) has also shown an improvement in recoveries. The commonly used mixtures are generally methanol/water or acetonitrile/water with some of the previously mentioned buffers. In some cases, those mixtures were also used unbuffered [54, 55].

The use of EDTA in the ASE method has not been recommended in most cases for several reasons. An example is the greater co-extraction of the matrix with consequent lower sensitivity and clogging of the ASE apparatus (cells and device tube) [56]. However, in some cases, its addition did not significantly improve analyte recoveries [52]. In contrast, EDTA has been successfully employed in the techniques discussed below (USE, MAE, and QuEChERS).

In addition to the selection of the solvent, several other parameters must be carefully considered in the development of the ASE method, such as temperature, pressure, extraction time and extraction mode, number of cycles, cell matrix, and flush volume. Those parameters are related and should be balanced to obtain acceptable recoveries for all target analytes. By far, temperature has proven to be the most important parameter. Theoretically, the high temperature helps to stop the strong interactions between pharmaceutical products and soil components; therefore, the higher the temperature, the higher the extraction yield [51, 57]. Furthermore, the temperature influences the physico-chemical properties of the solvents, therefore viscosity, density, and polarity. For example, the dielectric constant of pure water drops from 79 to 35 when the conditions are changed from room temperature and pressure to 200°C and 1.5 MPa, obtaining a water solvent similar to methanol at room temperature, with low density and polarity [57]. In other words, under high temperature conditions, the properties of the solvents are modified so as to reduce the viscosity and surface tension and greater diffusivity, which improves the wetting of the matrix and improves the mass transfer from the matrix to the solvent. All in all, the reduced interactions between analytes and matrix and the greater diffusion of the solvent in the high temperature matrix, in addition to the increasing solubility of the analytes, allow a faster mass transfer and a complete extraction process. On the other hand, in addition to all these strengths of the high temperature used in the ASE, some weaknesses are also presented, and it is worth mentioning them: (1) the co-extraction of other unwanted compounds from the matrix due to the bonds which break under high temperature conditions, thus converting the ASE into a less selective method; (2) the degradation of the thermolabile analytes at high temperature; (3) and, finally, the formation of toxic compounds due to chemical reactions that occur at high temperature. However, these weaknesses can be avoided by minimizing the extraction time and the number of cycles. Overall, the extraction temperature should be optimized to extract the analytes of interest with less matrix interference, loss of

selectivity, degradation, and toxic components. The temperature range used for ASE soil extraction ranges from 40 to 200°C, with 100°C being the most used temperature. A good temperature choice is 100°C as it exceeds the boiling point of most of the organic solvent and is low enough to avoid the degradation of the analytes, the excess of co-extracts, and the formation of toxic compounds. Since tetracyclines can undergo unwanted transformations at high temperature (they can be converted into their epi or anhydro form), some studies have performed their extraction at room temperature [58–60]. Tetracyclines and fluoroquinolones are extracted with lower temperatures from ambient to 100°C [58], whereas sulfonamides showed the necessity of higher temperature ranging from 50 to 200°C [61]. Many studies [62–64] have demonstrated that the extraction pressure has no influence on the recoveries and extraction efficiency. However, an elevated pressure is important to maintain the solvent in its liquid state. Additionally, it helps to wet the sample, resulting in an improved extraction efficiency [65]. Pressures from 500 to 1,500 psi were usually employed, with 1,500 psi the most common pressure.

The extraction time is the time in which the solvent and the matrix are in contact with the desired temperature and pressure and depends on the matrix and the target analyte characteristics [57]. It should be as short as possible but also adequate for mass transfer. Moreover, extraction time depends on the extraction mode (static or dynamic). Static mode was always chosen for pharmaceutical extraction from soil rather than the dynamic mode. It is worthy to mention that different instruments are designed for each mode. In the static mode, the solvent and the sample are maintained for a specific time at constant temperature and pressure, and the solvent is only replaced partly or completely when another cycle is employed [66]. If this time is longer than necessary, thermal degradation may occur for the extracted analytes resulting in a slower and less efficient extraction procedure. In addition, an equilibrium point of the analytes is established in the matrix and solvent in static mode, and beyond this point, the extraction efficiency will not increase. Hence, static extraction time should be optimized carefully. Most often, a static extraction time of 5 min (2 × 5 min cycles) or 10 min (1 × 10 min cycle) is employed.

The volume of the solvent can be introduced in several cycles or in one cycle. Fractioning the solvent in several cycles can improve extraction efficiency by avoiding the analyte equilibrium point to take place. However, it could also provide lower recoveries due to high co-extraction of matrix interferences [67]. Hence, a moderate number of cycles should be employed. Mainly 1–3 cycles are used.

The soil samples are generally mixed with a cellular matrix (dispersant) to prevent clogging of the cell and improve soil contact with the solvent. Among the different cellular matrices available, Ottawa sand [64], quartz sand [68], sea sand [55], diatomaceous earth [69], and Hydromatrix diatomaceous earth [59] are usually the dispersants commonly used with the soil. Lastly, flush volume is the least mentioned in studies, even though it is important to know the final volume of the extract. It is expressed as the percentage of the cell volume, and it ranges from 50 to 150% in all studies dealing with soil as matrix. This interval is sufficient to extract most of the analytes suitable for quantitative analysis, in a minimum volume of solvent, reducing the solvent consumption and concentrating the analytes as much as possible. Finally,

in a comparison with conventional methods such as Soxhlet, ASE is favored by time and by the reduction of the volume of solvent. However, compared to other more sustainable techniques such as MAE, USE, and QuEChERS, ASE may no longer be considered in terms of time or cost savings, but in some cases, it can provide better efficiency, reproducibility, and robustness. Therefore, ASE is a choice of interest for the development of analytical methods to determine pharmaceutical products from the soil (Table 2).

4.2 Ultrasound Solvent Extraction (USE)

Different extraction solvents were employed for pharmaceutical extraction from soil with ultrasonication, such as acidified aqueous solutions, EDTA solutions, and phosphate and McIlvaine buffers, mixed with organic solvent. Acetonitrile, methanol, and ethyl acetate are the main organic solvents employed with sonication methods (solvent selection depends on its viscosity). Na_2EDTA (disodium ethylenediaminetetraacetate) is a chelating agent and is used extensively to prevent pharmaceutical products such as tetracyclines and fluoroquinolones from forming chelated complexes with soil matrix (such as metal ions present in the soil). Sulfonamides are amphoteric and are best extracted in an acid medium, while tetracyclines are better extracted with a McIlvaine buffer. However, a combination of buffer and organic solvent has been used for the simultaneous extraction of multiple pharmaceutical residues. Hu and colleagues used potassium phosphate buffer with ACN (1/1, v/v, pH = 3.2) with 0.4 g of Na_2EDTA in order to obtain the best recoveries for fluoroquinolones, tetracyclines, and sulfonamides [75] (Table 3).

The sonication time and the number of cycles are important parameters and should be optimized so as to obtain the maximum recovery of the target analytes with a minimum matrix that interferes with the compounds (this can be explained by the distribution coefficients of pharmaceutical products rather than by the kinetics of desorption process). The sonication time ranged from 10 to 30 min, and the number of cycles ranged from 1 to 4 cycles at maximum. The temperature of the ultrasonic bath was not controlled in most studies. However, USE extraction was always performed at room temperature.

4.3 Microwave-Assisted Extraction (MAE)

In MAE, extraction parameters such as time, power, solvent (type and volume), pressure, temperature, number of cycles, and sample size are important parameters that require vigorous optimization to obtain the maximum recovery yield. The extraction solvents for MAE should be capable of absorbing microwaves, with permanent dipole leading (polar), although the combination of solvents with and

without dipole has extended the MAE application to a wide variety of analytes (polar and non-polar) (Table 4). Methanol, acetonitrile, and water are the most commonly used as a solvent for green extraction. The presence of water in the extraction solvent has been shown to increase extraction rates. This can be explained by the absorbance of the microwave energy by the water, resulting in efficient heating of the samples.

High pressure and temperature include the risk of degradation of pharmaceutical products and should be carefully optimized. The temperature is generally set between 60 and 120°C, while the extraction time is generally very short when applying MAE, since the solvent is heated directly with microwave energy and can last from 3 to 20 min.

4.4 QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe)

The selection of the extraction solvent is a crucial parameter. Acetonitrile (ACN) has been shown to provide the best extraction efficiency of a wide range of compounds, including pharmaceutical ones, with the lowest matrix interference. Furthermore, it was used by the QuEChERS fathers when the method was first introduced [19]. ACN was used as extraction solvent in all the studies dealing with soil extraction by QuEChERS technique, besides water or McIlvaine buffer as hydration solvent of the sample [101–106]. Hydration of the soil with water is important to weaken the interactions of the target analytes with the adsorption sites of the soil humic substance, promoting desorption and allowing the ACN to obtain better access to the soil pores. Likewise, the McIlvaine buffer is able to prevent the complexation of analytes with cations such as Mg^{2+} or Ca^{2+}. To the greatest extent, the hydration step allows the reconstitution of the dry samples with a high water percentage, for which the QuEChERS method was originally designed [107]. However, satisfactory results were obtained with water in almost all the abovementioned studies. Only Meng and coworkers employed McIlvaine buffer in their extraction procedure [105]. Besides, the acidification of the hydration solution and/or ACN with hydrochloric acid or acetic acid has shown to improve analyte recoveries [101–104].

Three QuEChERS salts are known which are used intensively: (1) the original non-buffered method ($MgSO_4$ + NaCl), (2) the AOAC method with acetate buffer ($MgSO_4$ + CH_3COONa), and (3) the EN method with citrate buffer ($MgSO_4$ + $C_6H_5Na_3O_7 \cdot 2H_2O$ + $Na_2C_6H_6O7 \cdot 1.5H_2O$). The AOAC and EN buffers have the ability to acidify the medium. The extraction pH is a very important parameter, and a small variation can affect extraction efficiency, especially for acidic and basic compounds [19]. In the EN method, the citrate buffer provides a pH of 5–5.5, while in the AOAC method, the acetate buffer provides a more acidic pH of 4.8 [108]. Higher recoveries and bigger number of compounds were extracted using AOAC method [103, 104, 106]. This is referred to the acidic conditions obtained by

citrate buffer, which improves the extraction of some pharmaceuticals such as sulfonamides, macrolides, and ß-lactams. However, satisfactory recoveries were obtained using the original unbuffered method [102, 105] and the EN citrate-buffered method [101] as reported in Table 5.

5 Extraction Procedures for Pharmaceuticals from Plant Tissues

The irrigation with reclaimed wastewater represents a source of xenobiotics as PhACs that are not removed by conventional WWTPs [109–111]. Consequently, agricultural soils contain a wide range of pharmaceuticals that could be uptaken by plants through the roots [40, 112]. Vegetables have different types of more or less branched roots (hairy roots in lettuce, napiform roots of radish, or tuberous roots of potatoes). However, the type or shape of the root does not influence the radical absorption which is similar for all the roots [2]. The PhACs dissolved in water arrive in the rhizosphere, come into contact with the root through the epidermis, and could enter the vascular system with the absorption of the roots [2]. Depending on several PhACs, physico-chemical properties such as polarity, ionizable ease, or lipophilicity could affect the pass through the endodermis and then arriving to the vascular tissue which is responsible for transporting nutrients to the rest of the plant [113]. Therefore, the PhACs capable of entering the vascular tissue can be translocated from the root to the aerial tissues such as leaves and fruits. In fact, the main source of PhACs in the leaves is related to their translocation from the roots to the leaves of the most polar compounds that move with water [114]. Contrary, non-polar (high K_{ow}) PhACs are more susceptible to remain in the root compartment commonly in the cell vacuoles and then bioaccumulating in the root [2]. Moreover, when PhACs are inside the tissues, they could be metabolized, creating new molecules, usually with unknown fates and properties [115]. Therefore, in order to be able to detect and quantify the pharmaceutical products absorbed by plants at low environmental concentrations, accurate and reliable analysis methods are required to be applied to the different tissues of which the plants are made [116]. In fact, each fabric presents a separate challenge to overcome. Plants are characterized by containing large quantities of sugars, waxes, fatty acids, and chlorophyll, which complicates the analysis due to the difficulty of eliminating these substances. These components of the matrix, if not carefully removed, can interfere with instrumental analysis by acting as co-eluents of the target compounds [117, 118]. Taking this into account, the different extraction processes (QuEChERS, ASE, USE, MAE, etc.) are followed by a subsequent cleaning phase to obtain clean extracts for accurate detection and quantification. Ideally, a selective extraction technique allows the recovery of the compounds of interest, while the interferents remain without being extracted into the matrix [45, 119, 120]. Extraction methods that require the use of high temperatures such as ASE or MAE and in some cases due to the USE are more likely to obtain

extracts containing co-extracted elements of the matrix. Nevertheless, to overcome this problem, modern analytical techniques include strategies such as the reduction of the sample quantities, the lower solvent consumption, or the reduction of the extraction time, to favor the reduction of unwanted components of the matrix. The most used clean-up processes are SPE and d-SPE which allow to obtain fairly clear extracts. However, the skip of this step could improve the recoveries of some compounds that are retained by the SPE adsorbents. LC-MS is usually the instrumental analysis performed, thanks to its ability to achieve low limits of quantification and detection, good repeatability, and reproducibility [7, 121]. Indeed, LC-MS/MS have been the instrumental analysis with great confidence to confirm the analytes and its capability to reach very low limits of detection and quantification [122, 123]. All details for each extraction methodology for pharmaceuticals in plants are reported in Table 6.

5.1 Assisted Solvent Extraction (ASE)

In order to perform a good extraction by means of ASE, the selection of an appropriate organic solvent as well as temperature is crucial. In fact, ASE provides the advantage to choose between several solvents allowing to extract compounds of different families in different matrices [124]. ASE uses temperature and pressure in its procedure, which means that modification on the solvent physico-chemical properties occurs facilitating the release of pharmaceuticals. As an example, solvent density decreases allowing a more extensive wet into the sample, whereas solvent boiling point increases due to the effect of the pressure [124]. However, high temperatures could deteriorate the PhACs, especially the thermolabile ones; then, a compromise between enhance on the extraction efficiency and the temperature is established. First, a dispersing agent (sand, hydromatrix, or Florisil) is mixed with the sample in order to avoid sample agglomerates, obtaining a greater surface and, consequently, facilitating the solvent interaction. Moreover, Florisil is the dispersant agent more used before the extraction starts, in order to improve the extraction efficiency through the adsorption of sugar and waxes with the subsequent retention [125, 126]. Samples are placed in a stainless-steel extraction cell and capped with filters at both ends to retain the sample into the cell but letting out the extraction solvent containing the extracted compounds [127]. Once the solvent is introduced into the system (different volumes could be employed), the extraction cell is placed in the oven, and the system starts setting the desired temperature and pressure. Another important parameter to consider consists of the time and cycles used, as the more time the solvent is in contact with the sample, the more extraction efficiency is performed. However, the co-extractive compounds are also released. Then, a compromise between time and extraction efficiency is created and should be optimized [124]. SPE was usually performed as the clean-up step after the extraction, removing interferences and obtaining cleaner extracts [74, 128]. For this reason, it is easier to adjust the pressure than the temperature of the ASE [128, 129]. Similar to

Table 6 Extraction methodologies from plants

Matrix and target molecules	Weight/ volume of sample	Extraction solvent	Salts	Buffer	Clean-up	Analytical method	Reference
Honeys, honeybees, and pollens – pesticides, veterinary drugs, and a synergist	2 g dry weight	8 mL H2O, 10 mL ACN, 3 mL hexane	4 g anhydrous $MgSO_4$, 1 g NaCl	1 g sodium citrate dehydrate, 500 mg disodium citrate sesquihydrate	15 mL solution for d-SPE (900 mg of anhydrous $MgSO_4$, 150 mg PSA bonded silica, 150 mg C18 bonded silica)	HPLC-MS/MS, GC-ToF	[146]
Milk and chicken feed – benzimidazoles and coccidiostats	10 g dry weight	10 mL deionized water, 10 mL MeCN	4 g of $MgSO_4$, 1 g NaCl	None	150 mg $MgSO_4$, 50 mg C18, 50 mg PSA	DART-HRMS	[147]
Animal feed – 13 sulfonamides	5 g dry weight	10 mL H_2O, 10 mL acetic acid 0.1% (v/v) in ACN: MeOH (75:25 v/v)	4 g anhydrous $MgSO_4$	0.5 g sodium acetate	200 mg PSA	HPLC-MS/MS	[148]
Tomato fruit – lufenuron	15 g wet weight	15 mL ACN	6 g anhydrous $MgSO_4$, 1.5 g NaCl	None	100 mg PSA, 600 mg anhydrous $MgSO_4$	HPLC	[36]
White radish, Chinese cabbage, cucumber, string bean, and green pepper – 26 veterinary antimicrobials	10 g wet weight	10 mL MeCN/MeOH (85:15, v/v)	4 g of anhydrous $MgSO_4$ and 1 g of NaCl	1 g of $H_3Cit{\cdot}H_2O$, 0.5 g of $Na_3Cit{\cdot}2H_2O$	70 mg PSA	HPLC-MS/MS	[149]
Herbal dietary supplements – 96 pharmaceuticals, plant toxins, and other plant secondary metabolites	1 g dry weight	10 mL H_2O, 10 mL ACN, ACN (2% formic acid)	4 g anhydrous $MgSO_4$, 1 g NaCl	None	0.22 μm Teflon filter, d-SPE (100 mg C18, 300 mg $MgSO_4$)	UHPLC-HRMS	[143]

(continued)

Table 6 (continued)

Matrix and target molecules	Weight/ volume of sample	Extraction solvent	Salts	Buffer	Clean-up	Analytical method	Reference
Celery and lettuce – 11 pharmaceutical compounds	500 mg dry weight	7 mL ACN/MeOH/H_2O (74.1:0:28.6), (60.7:10.7:28.6), (53.6:17.9:28.6), (35.7:35.7:28.6)	2 g anhydrous Na_2SO_4, 0.5 g NaCl	150 mg L^{-1} Na_2EDTA	d-SPE (12.5 mg C18, 12.5 PSA, 225 mg Na_2SO_4)	HPLC-MS/MS	[119]
Lettuce leaves – carbamazepine, flumequine, and thiabendazole	10 g wet weight	10 mL of ACN	6 g of $MgSO_4$	1.5 g of NaOAc	d-SPE (125 mg PSA, 150 mg C18, 750 mg $MgSO_4$), 0.45 μm PTFE filter	LC-QqLIT-MS/MS	[122]
Sedative functional foods (based on *Salvia miltiorrhiza* and *Schisandra chinensis*) – 11 blockers	2 g wet weight	5 ml of acetic acid/ACN/MeOH (0.1:3:7, v/v/v)	15 ml of acetic acid/ACN/MeOH (0.1:3:7, v/v/v) (second round)	None	d-SPE (50 mg PSA, 500 mg $MgSO_4$)	UPLC-MS/MS	[150]
Baby food (some of vegetal origin) – sulfonamides	5 g dry weight 15 g wet weight	10 mL ultrapure water, 15 mL of 1% acetic acid in ACN 15 mL of 1% acetic acid in ACN	6 g $MgSO_4$	1.5 g NaOAc	150 mg PSA, 150 mg C18, 900 mg $MgSO_4$	UHPLC-HR/MS	[139]
Buckwheat and related products – atropine and scopolamine	5 g dry weight	10 mL H_2O, 10 mL ACN containing 1% (v/v) of formic acid	4 g of anhydrous Na_2SO_4	1 g NH_4Ac	25 mg of PSA, 25 mg of GBC	UHPLC-MS/MS	[35]
Lettuce – 13 wastewater-derived contaminants	0.5 g dry weight	20 mL ACN-MeOH (1:1, v/v), MTBE-MeOH (1:1, v/v), hexane-	4 g $MgSO_4$, 1 g NaCl	1 g sodium citrate	0.2 μm PTFE filters, liquid-liquid extracted with hexane (hexane	HPLC-MS	[7]

		acetone (1:1, v/v), MTBE-ACN (1:1, v/v), EtAc-MeOH (1:1, v/v), 0.5% formic acid in ACN-MeOH (1:1, v/v)			washed), d-SPE (PSA, $MgSO_4$)		
Lettuce, cabbage, and tomato – 28 organic micropollutants	0.5 g dry weight	3 mL H_2O, 10 mL ACN	4 g anhydrous $MgSO_4$, 1 g NaCl	None	150 mg PSA, 900 mg $MgSO_4$, 0.2 μm syringe RC filter	LC-MS/MS	[45]
Plant food supplements – 26 pharmaceutical compounds	0.5 g or 5 mL	10 or 5 mL H_2O (0.1% formic acid), 10 mL ACN	4 g anhydrous $MgSO_4$, 1 g NaCl	None	0.22 μm PTFE filter	UHPLC-MS/MS	[140]
Radish roots and leaves – chlortetracycline, enrofloxacin, sulphathiazole	10 g wet weight	20 mL MeCN with 1% HAc	None	1 g SCTD, 500 mg SCDS, 100 mg EDTA-Na_2	d-SPE (30 mg PSA, 30 mg C18)	LC-MS/MS	[141]
Lettuce, grapes, milk, chicken, pork, beef – 17 pesticides, 16 alkaloids, 18 drugs	1 g wet weight	1 mL H_2O (only in low water content samples), 1 mL ACN	400 mg $MgSO_4$, 100 mg NaCl	1 mL of pH 10 glycine buffer	d-SPE (75 mg, $MgSO_4$:PSA:C18:GCB 3:1:1:1 (w/w/w/w))	GC-MS, LC-MS/MS	[151]
Lettuce shoots and roots – 11 pharmaceutical compounds	0.25 or 0.5 g dry weight	2 mL H_2O, 5 mL ACN/MeOH (65:35, v/v)	2 g Na_2SO_4, 0.5 g NaCl	150 mg/L Na_2EDTA	12.5 mg C18, 12.5 mg PSA, 225 mg Na_2SO_4	HPLC-MS/MS	[32]
Radish and enzyme extracts – 15 pharmaceuticals	0.5 g dry weight	2 mL H_2O, 5 mL ACN/MeOH (65:35, v/v)	2 g Na_2SO_4, 1 g NaCl	150 mg/L Na_2EDTA	250 mg Na_2SO_4 d-SPE (25 mg C18, 25 mg PSA) d-SPE (25 mg C18,	LC-MS/MS	[46]

(continued)

Table 6 (continued)

Matrix and target molecules	Weight/ volume of sample	Extraction solvent	Salts	Buffer	Clean-up	Analytical method	Reference
					25 mg PSA, 10 mg GCB)		
Lettuce, radish, and strawberry – 74 microcontaminants including pharmaceuticals, metabolites, and pesticides	10 g wet weight	10 mL of 1% acetic acid in MeCN and 20 μL of the extraction quality control solution, 10 mL of MeCN	6 g of anhydrous $MgSO_4$ 4 g of anhydrous $MgSO_4$, 1 g of NaCl	1.5 g of NaOAc 1 g trisodium citrate dehydrate and 0.5 g disodium hydrogencitrate sesquihydrate	d-SPE (750 mg of anhydrous $MgSO_4$ and 125 mg of C18) d-SPE (750 mg of anhydrous $MgSO_4$, 125 mg of C18, and 125 mg of PSA)	HPLC-MS/MS	[121]
Cabbage, cucumber, cauliflower, leek, and other commonly consumed vegetables – 49 antibiotics (17 sulfonamides, 16 quinolones, 6 macrolides, 5 β-lactams, 5 tetracyclines)	10 g wet weight	10 mL ACN (0.248% H_3PO_4 in phosphate buffer and 0.1% of formic acid in formic acid buffer)	4 g $MgSO_4$, 1 g NaCl	McIlvaine buffer (0.049, or 0.19, or 0.46 g Na_2HPO_4, 0.5 g citrate) Phosphate buffer (0.5 g KH_2PO_4) Formic acid buffer	d-SPE (10 mg PSA, 25 mg C18, 2.5 mg GCB) Other concentrations were tested with less recovery	UHPLC-MS/MS	[152]
14 different fruits and vegetables – penicillins G and V	1 g wet weight	2 mL of ACN	0.5 g of NaCl	40 mg of EDTA	0.45 μm membrane filter	HPLC-QToF-MS/MS	[123]
Lepidium sativum – diclofenac, ketoprofen, naproxen, and mefenamic acid	1 g wet weight	3 mL ACN/0.1 M HCl (2/1, v/v) 4 mL ACN/0.1 M HCl (1/1, v/v)	0.8 g $MgSO_4$ and 0.2 g NaCl 1.2 g $MgSO_4$, 0.3 g NaCl	None	None	HPLC-MS/MS	[153]
Leafy vegetables – 20 antibiotics	10 g wet weight	10 mL of ACN/MeOH (85:15, v/v)	4 g of anhydrous $MgSO_4$, 1 g of NaCl	1 g of citric acid monohydrate, 0.5 g of trisodium citrate dihydrate	C18 (30, 50, and 70 mg) and GCB (10, 30, and 50 mg)	UHPLC-MS/MS	[154]

Lettuce crops – 24 carbamazepine transformation products	10 g wet weight	10 mL of MeCN at 1% of acetic acid	6 g of $MgSO_4$	1.5 g of NaOAc	d-SPE (125 mg PSA, 125 mg C18, 750 mg anhydrous $MgSO_4$)	LC-QTOF-MS/MS	[155]
Lepidium sativum – atorvastatin, fluvastatin, simvastatin	1 g wet weight	4 mL ACN:H_2O (1:1, v/v)	1.2 g $MgSO_4$, 0.3 g NaCl	None	None	HPLC DTIM QTOF-MS, HPLC QQQ/MS/MS	[156]
Soil and beet root – bisoprolol, metoprolol, sulfamethoxazole, trimethoprim, caffeine	10 g dry weight	10 mL ACN (1% acetic acid)	4 g anhydrous $MgSO_4$, 1 g NaCl	1 g trisodium citrate dehydrate, 0.5 g disodium hydrogen citrate sesquihydrate	150 mg PSA, 900 $MgSO_4$ mg, 150 mg C18	LC-MS/MS	[157]
Lettuce, maize, and radish – atenolol, carbamazepine, and triclosan	1 g dry weight	10 mL ACN	4 g $MgSO_4$ + 1 g NaCl	Bond Elut QuEChERS Dispersive Universal Kit	None	LC-MS/MS QqQ	[158]
Chicory – diclofenac	2 g wet weight	4 mL ACN	2 g $MgSO_4$ + 0,5 g NaCl	0,5 g sodium citrate tribasic dihydrate +0,25 g sodium citrate dibasic sesquihydrate	150 mg $MgSO_4$ + 25 mg PSA + 25 mg C18E	UPLC-MS/MS QToF	[159]
Lettuce – 4-hydroxydiclofenac, 5-methyl-benzotriazole, acesulfame, acetaminophen, acridone, benzotriazole, bezafibrate,	1 g dry weight	10 mL CAN +50 µL formic acid	4 g $MgSO_4$ + 1 g NaCl	None	150 mg PSA, 900 $MgSO_4$ mg, 150 mg C18	HPLC-MS/MS QToF	[160]

(continued)

Table 6 (continued)

Matrix and target molecules	Weight/ volume of sample	Extraction solvent	Salts	Buffer	Clean-up	Analytical method	Reference
bisphenol A, caffeine, carbamazepine, carbamazepine-10,11-epoxide, chloramphenicol, ciprofloxacin, citalopram, climbazole, clofibric acid, diclofenac, diltiazem, fipronil, fipronil-desulfinyl, fipronil sulfone, fluconazole, furosemide, gemfibrozil, hydrochlorothiazide, ibuprofen, indomethacin, irbesartan, ketoprofen, lamotrigine, metoprolol, metronidazole, N-acetylsulfamethoxazole, oxcarbamazepine, propranolol, sucralose, sulfamethazine, sulfamethoxazole, triclosan, valsartan, valsartan acid, verapamil							
Cucumber, tomato, and lettuce – tetracycline, oxytetracycline, chlortetracycline, sulfamethazine, sulfamethoxazole, sulfadimethoxine	500 mg dry weight	8 mL MeOH:HCl (95:5, v/v)	None (sonication method)	None	SPE clean-up (Oasis HLB 60 mg cartridge)	LC-MS/MS (ion trap mass spectrometer)	[112]

Soil and radish leaf – chlordiazepoxide, clonazepam, diazepam, flurazepam, nordiazepam, oxazepam, temazepam, triazolam	1 g soil 1 g wet weight plant	Soil: 1 g sand +2*5 mL MeOH +1*5 mL acetone Plant: 1 g sand +3*5 mL ACN:H_2O (70:30, v/v) (15 mL total)	None (sonication method)	None	SPE clean-up (Oasis HLB 6 mL 200 mg)	LC-MS/MS	[31]
Radish and ryegrass – carbamazepine, diclofenac, fluoxetine, propranolol, sulfamethazine, triclosan	1 g wet weight of plant (leaf and root)	1 g sand +3*5 mL ACN:H_2O (70:30, v/v)	None (sonication method)	None	SPE clean-up (Oasis HLB 6 mL 200 mg)	LC-MS/MS (Thermo Finnigan TSQ Quantum Discovery Max)	[133]
Chinese white cabbage, water spinach, Chinese radish, corn, and rice – tetracycline, sulfamethazine, norfloxacin, erythromycin, chloramphenicol	1 g weight crop	30 mL acidified ACN:acetone (1:1, v/v, pH = 3)	None (ultrasonication)	None	SPE clean-up HLB cartridges	LC-MS/MS QqQ	[136]
Tomato – diclofenac, sulfamethoxazole, and trimethoprim	10 g wet plant	TMP and DCF: 10 mL MeOH + $NaHCO_3$ (pH 6–7) SMX: 10 mL MeOH (pH 3–4)	None (sonication method)	None	None	UPLC-MS/MS QqQ	[44]
Pisum sativum L. var. *axiphium* – carbamazepine, atenolol, sulfamethoxazole, and metabolites	0,1 g dry weight	1 mL ACN:H_2O (1:1, v/v, 0,1% formic acid)	None (sonication method)	None	None	LC-MS/MS QqQ	[43]

(continued)

Table 6 (continued)

Matrix and target molecules	Weight/ volume of sample	Extraction solvent	Salts	Buffer	Clean-up	Analytical method	Reference
Chickpea – metformin	0,1 g dry weight	2 mL dichloromethane	None (sonication method)	1,9 mL (ammonium acetate 450 mmol L^{-1} formic acid 50 mmol L^{-1})	SPE (Bond Elut-LMS cartridges 1 mL, capacity 25 mg)	LC-ToF-MS	[42]
Celery, lettuce, cabbage, spinach, carrot, cucumber, bell pepper, tomato – 16 PhACs and 3 PCP	0,2 g dry weight	20 mL tert-butyl ether	None (USE method)	None	SPE clean-up (HLB cartridge 150 mg)	UPLC-MS/MS	[137]
Lettuce, tomato, cauliflower, and broad bean seeds – sulfathiazole, sulfamethizole, sulfadiazine, sulfamethazine, sulfadimethoxine, sulfamethoxazole, ofloxacin, enrofloxacin, clindamycin, and trimethoprim	1 g wet weight	10 mL MeOH	None (USE method)	None	SPE clean-up (Strata-X cartridge, 100 mg/6 mL)	LC-MS/MS (Waters TQ detector)	[134]
Lettuce, spinach, arugula, and radish – carbamazepine, atenolol, sulfamethoxazole	0,1 g dry weight	4 mL ACN:H_2O (1:1, v/v, acidified 0,1% formic acid)	None (USE method)	None	None	LC-MS/MS QqQ	[41]
Alfalfa – diclofenac, sulfamethoxazole, trimethoprim	5 g wet weight	10 mL MeOH:H_2O (1:1, v/v)	None (USE method)	None	SPE clean-up (Oasis HLB)	UPLC-MS/MS TQD	[161]
Cucumber – 17 PPCs	0,2 g dry weight	20 mL tert-butyl ether	None (USE method)	None	SPE clean-up (Oasis HLB 150 mg)	UPLC-MS/MS QqQ	[37]
Lettuce – 19 PPCs	0,2 g dry weight	20 mL tert-butyl ether +20 mL ACN	None (USE method)	None	SPE clean-up (Oasis HLB	UPLC-MS/MS	[120]

Typha latifolia and *Berula erecta* – iopamidol, iohexol, iomeprol, iopromide, propranolol, carbamazepine, naproxen, ibuprofen, diclofenac	0,2 wet weight	10 mL MeOH:acetone (95:5, v/v)	None (ultrasonic solvent extraction)	None	SPE clean-up (reversed phase Phenomenex Strata-X 200 mg/ 6 mL + normal phase Supelclean LC-Florisil 1 g/ 6 mL)	HPLC-DAD	[162]
Typha angustifolia, *Arundo donax*, and *Lemna minor* – 31 CECs	1 g wet weight	8 mL EtAc 3% NH_4OH + 2*5 mL ACN 4% formic acid	4 g Florisil +2 g $MgSO_4$ (ultrasound-assisted)	None	Glass-column clean-up (1 g $MgSO_4$ + 1 g C18)	GC-MS	[135]
Cress – sertraline, clomipramine, and trazodone	1 g wet weight	2 mL ACN	None (sonication + centrifugation)	None	None	LC-MS/MS	[163]
Pea – trimethoprim, sulfamonomethoxine, sulfamethoxazole, sulfadimethoxine, crotamiton, gliclazide, carbamazepine, losartan, cyclophosphamide, acetaminophen, ketoprofen, diclofenac, indomethacin	Wet weight	ACN – MeOH 0,5% formic acid	None (sonication + centrifugation)	None	SPE (Oasis HLB)	LC-MS/MS	[164]
Cucumber – carbamazepine, carbamazepine epoxide, 10,11-dihydroxycarbamazepine, 2-hydroxycarbamazepine, 3-hydroxycarbamazepine, lamotrigine	1 g wet plant	MeOH +1 g Florisil	None (ASE extraction)	None	None	LC-MS/MS QqQ	[126]

(continued)

Table 6 (continued)

Matrix and target molecules	Weight/ volume of sample	Extraction solvent	Salts	Buffer	Clean-up	Analytical method	Reference
Cabbage – salbutamol, trimethoprim, sulfamethoxazole, carbamazepine, sertraline, triclosan	8 g wet plant	50 mL ACN:H_2O (55:45, v/v)	None (pressurized liquid extraction, PLE)	None	None	LC-MS	[40]
Carrot, potato – bezafibrate, carbamazepine, diclofenac, gemfibrozil, ibuprofen, clofibric acid, sulfapyridine, ketoprofen, sulfamethoxazole, 10,11-epoxycarbamazepine	1 g dry weight	MeOH +1 g Florisil	None (ASE method)	None	None	LC-MS	[125]
Lettuce and carrot – tetracycline and amoxicillin	0,1 g dry weight	100% Milli-Q water (final extract 30 mL)	None (PLE method)	None	SPE clean-up (Oasis HLB cartridges hydrophilic-lipophilic balance, 200 mg/6 mL)	LC-MS/MS QTrap Sciex	[39]
Grass – oxytetracycline, sulfamethoxazole, ketoconazole	1 g wet weight	MeOH:citric acid (0,2 M, 50:50, v/v, pH = 4,5 with NaOH)	None (PLE method)	Na_2EDTA	SPE clean-up (Strata-X 200 mg/ 6 mL, reverse phase)	LC-MS Orbitrap Thermo Fisher	[165]
Soybean and wheat – ketoprofen, naproxen, diclofenac, ibuprofen	0,5 g dry weight	0,01 M NaOH	None (pressurized hot water extraction – ASE)	None	Hollow fiber liquid-phase microextraction clean-up	LC-MS	[131]

Wheat – sulfamethoxazole, trimethoprim, ofloxacin, carbamazepine	1 g dry weight	MeOH 70 mL	None (ASE method)	None	SPE clean-up (Oasis HLB)	LC-MS/MS	[129]
Cowpea, turnip, chard, collards, basil, lettuce, and cilantro – atenolol, ofloxacin, and diclofenac	– Cowpea, turnip: 3 g dry weight – Chard, collards: 1,5 g dry weight – Basil, lettuce cilantro: 1 g dry weight	70–75 mL methylene chloride:MeOH (75:25, v/v)	None (ASE method)	None	Turnip and Chard: SPE clean-up (Oasis HLB 3 mL, 540 mg)	LC-MS	[128]
Lettuce, spinach, carrot – azithromycin, roxithromycin, clarithromycin, clindamycin, methamphetamine, MDMA, pseudoephedrine, DMPEA	1 g dry weight	MTBE:MeOH (90:10, v/v) 5 mL final extract	None (ASE method)	None	TurboVap clean with hexane	LC-MS/MS ion trap	[130]
Phragmites australis – 81 micropollutants	0,5 g dry weight	25 mL MeOH:H_2O (25:75, v/v)	None (microwave-assisted extraction)	None	SPE clean-up (Oasis HLB)	UHPLC-MS/MS QqQ	[145]
Cereals (wheat, barley, oat, and rice) – 4 fluoroquinolones, 3 tetracyclines, 7 sulfonamides,	2 g wet weight	MAE: 6 mL ACN: MeOH:phosphoric acid 5% (7:1:2, v/v) USE: 7 mL ACN:	None (MAE, USE method)	None	d-SPE clean-up (250 mg PSA)	LC-MS/MS QqQ	[33]

(continued)

Table 6 (continued)

Matrix and target molecules	Weight/ volume of sample	Extraction solvent	Salts	Buffer	Clean-up	Analytical method	Reference
4 macrolides, and lincomycin		MeOH:phosphoric acid 5% (7:1:2, v/v)					
Lettuce – lincomycin, roxithromycin, doxycycline hyclate, demeclocycline, sulfamethoxazole, oxytetracycline	2 g wet weight	10 mL (80% isopropyl alcohol, 20% 0,04 M citric acid solution)	None (freeze-thaw extraction)	None	SPE clean-up (200 mg Oasis HLB)	LC-MS/MS	[166]
Brassica juncea – acetaminophen	0,5 g wet weight (root and leaf)	1 mL HCl 0,1 M	None (solid-liquid extraction)	None	SPE method, Phenomenex Strata-X 300 mg	LC-MS	[28]
Cucumber, eggplant, long bean, and wheat – sulfamonomethoxine, sulfathiazole, acetylsulfamethoxazole, sulfisoxazole, sulfachlorpyridazine, sulfamethazine, sulfamerazine, trimethoprim, chloramphenicol, ibuprofen, sulfamethoxazole, and triclosan	5 g wet grain crop 2 g wet fruit and vegetables	220 mL ACN	None (Soxhlet extraction)	None	None	LC-MS/MS QqQ	[34]

soil, MeOH, ACN, and water are the most used solvents for the extraction procedure. Different mixtures have been reported in order to achieve the optimum polarity conditions to extract the targeted compounds and avoiding interferences from the matrix plant [40, 74, 130]. Additionally, buffer mixtures such as citric acid and NaOH are included providing the properly pH into the mixture and enhancing the extraction efficiency. The most used extraction solvent, MeOH, provided good results in carrot and potato, coming to detect ten PhACs in a single analysis [40] but also in wheat [129] and in cucumber [126]. Using water as extractant solvent helped Azanu et al. to extract tetracycline and amoxicillin from lettuce and carrot [39]. On the other hand, Cortés et al. managed to extract four pharmaceuticals from soybean and wheat using buffered water (0.01 M NaOH) [131]. It is worth to mention that the greenest method consists of using water as a unique extraction solvent.

Up to 15 different plant matrices were studied under ASE methodology in only 9 reports (Table 6), indicating the versatility of ASE. Moreover, lettuce and carrot were the matrices most studied (three reports for each matrix). Reports showed the capability to develop one method for the detection of PhACs in several matrices [39, 130].

5.2 Ultrasound Solvent Extraction (USE)

The USE methodology is certainly the most widely performed extraction method in the environmental field. The base of the USE is centered in the formation of small cavities inside the sample by means of ultrasound, for a subsequent penetration of the solvent causing a greater contact surface between the sample and the extraction solvent, enhancing PhACs extraction [132]. A further comminution of the samples could occur due to the increase in collisions between the particles due to the vibration of ultrasound and to the disturbances created for cavitation [132]. Dispersant agents such as sand are commonly employed to help the formation of cavities into the samples by impact of sand particles into the sample [133]. USE is known to be an easy extraction method but also for the use of low quantities of solvent. However, the heat that is usually generated by particle collisions on the one hand could facilitate the release of some classes of target compounds; in the case of PhACs, it could lead to negative effects due to a possible thermal degradation. The sonication time and the number of cycles are parameters that must be optimized to ensure good extraction performance. Normally, the sonication time varied from 10 to 30 min, and 1 to 4 cycles are performed [112]. The organic solvents most used in the extraction of PhACs from plants are mainly the same as previously seen as MeOH, ACN, and water, used individually or in different mixtures [41, 134]. In particular, the ACN/water mixture has been used successfully for the extraction of pharmaceutical residues in radish, ryegrass, *Pisum sativum*, lettuce, spinach, and rocket with satisfactory results [31, 41, 43, 133]. Buffered mixtures containing $NaHCO_3$, formic acid, or NH_4OH were also used providing an adequate pH for a better extraction

performance especially for the acidic compounds which are more sensitive to the pH [44, 112, 135].

Up to 16 studies have been reported to employ USE in order to extract PhACs from plant matrices (Table 6). The versatility and applicability of USE have been widely demonstrated on about 20 different vegetable matrices with good results. Lettuce, radish, and tomato were the most studied matrices [31, 41, 44, 112, 120, 133, 134, 136, 137].

The most interesting compounds studied were antibiotics, benzodiazepines, and nonsteroidal anti-inflammatory drugs (NSAIDs), such as carbamazepine, diazepam, sulfamethoxazole, sulfamethazine, trimethoprim, etc.

5.3 QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe)

The QuEChERS method is the most used extraction technique for pharmaceutical residues. In fact, more than 25 studies using QuEChERS for drugs in plant tissues are reported in Table 6. The extraction of QuEChERS mainly depends on the selection of three phases: the solvent, the salts required, and the cleaning phase. Many of the reported studies are based on more or less important modifications of the original procedure developed by Anastassiades et al. for the determination of pesticides in food [19]. In the original method, ACN was the solvent selected for its ability to extract non-polar and relatively polar compounds and has the ability to leave lipophilic substances without being extracted [138]. In addition, ACN mixes easily with water allowing the penetration of the organic solvent into the matrix [138]. Reports have shown that ACN is by far the most widely used solvent, sometimes buffered with acidifying compounds to improve extraction efficiency. Different solvents such as H_2O or MeOH have also been used less frequently but also providing good extraction recoveries. The original method requires a sample weight of 10 g of fresh material with a water content greater than 80%. Sometimes, for dry, dehydrated, or fat-rich samples, water is added up to the minimum quantity required for the extraction [35, 139, 140]. Acidification of the organic solution, majorly with acetic acid or formic acid, has shown to improve the recoveries of compounds susceptible to pH [121, 141]. The main difference with the other extraction methods consists in the use of salts which force the analytes to pass from the aqueous phase to the organic phase, known as the salting out process [142]. Taking as a reference the original method, $MgSO_4$ with NaCl were the salts employed [19]. $MgSO_4$ is used for several reasons. In fact, it helps in the separation between phases (ACN/water) by saturation and is also used for its drying capacity. Finally, the drying process involves an exothermic process with consequent release of heat which leads to a better extraction of the non-polar compounds [138]. On the other hand, the main purpose of NaCl consists of the reduction of polar interferences and co-extractives coming from the plant [138]. Three main types of salts are used in

plants, the original non-buffered salts ($MgSO_4$ + NaCl), the EN-buffered salts consisting of the original salts and the addition of citrate buffers (MgSO4 + $C_6H_5Na_3O_7$•$2H_2O$ + $Na_2C_6H_6O_7$•$1.5H_2O$), and the AOAC method with acetate buffer ($MgSO_4$ + CH_3COONa). The pH is extremely important in this extraction procedure. The EN and AOAC extraction salt kits consist of buffered salts which result in a pH between 5 and 5.5 for the EN method and a pH < 5 for the AOAC. In contrast, the final pH of the original method mainly depends on the nature of the initial matrix [142]. However, current studies suggest that the original salt kit is the most used salt mix to extract pharmaceutical residues from plant tissues, with excellent results [45, 140, 143]. On the other hand, AOAC salts and EN salts were widely used providing more stability to the target compounds [7, 121]. Aside from the extraction phase, Anastassiades and colleagues also developed a new clean-up methodology to remove any interferents based on a solid-phase extraction in the dispersive phase (d-SPE) [19]. Further considerations are shown in the clean-up section. The matrices studied so far are mainly vegetables such as lettuce, radish, and cabbage (Table 6). In particular, plants consisting of a branched root are the most studied samples, but fruits such as cucumber, tomato, and strawberry are also being studied. QuEChERS has become the most used extraction method with exponential growth in recent years, and everything seems to indicate that thanks to its versatility and its possible future automation, this growth seems even more pronounced in the near future.

5.4 Other Extraction Methods (MAE, Soxhlet, Solid-Liquid Extraction)

The microwave-assisted extraction method (MAE) is a technique widely used in soils but has not had the same diffusion with plants, at least for now. The MAE consists in the use of a correctly selected organic solvent for the extraction of target drugs which is heated by microwaves, combining sonication and temperature properties [144]. This technique is widely used for the analysis of metals and heavy metals in plant material. However, it is also used in the field of PhACs, although the quantity of solvent is reduced and the obligation to operate at high temperatures could adversely affect the extraction of the target compounds. The organic solvents used are the same as previously described for other methodologies. Basically, ACN, MeOH, and water, including their mixtures, are the solvents selected for vegetables. Only two studies with plants have been reported, one with *Phragmites australis* and the other with cereals [33, 145].

Recent works have demonstrated the ability of the classic Soxhlet extraction method to detect pharmaceutical products in plants and to detect 12 pharmaceutical products in different vegetables [34]. However, Soxhlet extraction is not usually used because, due to the high working temperatures required by Soxhlet and also the long exposure times, this method could alter the properties of the target compounds,

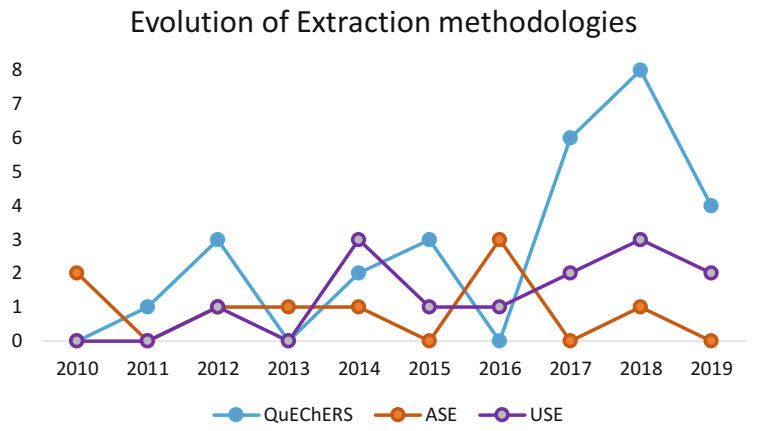

Fig. 1 Comparison of the use of the three main extraction methods (USE, ASE, and QuEChERS) for pharmaceutical residues in plant tissues reported in the last 10 years

in particular thermolabile ones. In addition, a high amount of solvent is required, making the procedure more expensive and polluting than modern methods.

Finally, solid-liquid extraction (SLE) is a simple but effective extraction method used when a liquid matrix contains the target compounds. It is a technique widely used for water analysis. Although the new methodologies (USE, ASE, QuEChERS, etc.) are able to provide more selectivity and also great results, SLE can be used in combination with these techniques as an extract clean-up phase. Despite this, Bartha et al. managed to analyze the paracetamol from the root and leaves of *Brassica juncea* [28].

Figure 1 shows the evolution of the three main extraction techniques for plants over the past 10 years. The use of these techniques between 2010 and 2016 was quite similar, but since 2017 QuEChERS has been the main methodology followed by the authors to extract PhACs from plant tissues, thus demonstrating its versatility in this area.

6 Clean-Up Procedures

Environmental and food samples have very complex matrices, and part of its constituents are involuntarily co-extracted during the usual extraction procedures. Furthermore, although the high temperature used in ASE and MAE and the strong sonication waves used in USE are very effective in the extraction of the compounds of interest, as a counterpart, they facilitate the migration of matrix interference in the final extracts [56]. Consequently, as a main effect, these elements can mask the detection of analytes during chromatography. Therefore, cleaning the extracts is a crucial step. A large diversity of sorbents is today available and successfully

employed for extract clean-up from matrices interfering components. These include, for example, SPEs in the reverse phase (HLB, C8, C18, etc.), normal phase SPEs (alumina, diol, Florisil, silica, etc.), and the ion exchange mode SAX (strong anion exchange) or MCX (mixed-mode cation exchange). The various sorbents mentioned are used in the form of discs, columns, dispersive phase or cartridges. However, the latest format is the most widely used. In this section, we will discuss the commonly used sorbents for soil and crop extract clean-up, as well as the newly introduced sorbents.

6.1 Commonly Used Sorbents

Solid-phase extraction (SPE) in cartridge format is one of the most clean-up techniques used over the last years. The great selectivity of SPE in retaining the targeted compounds and simultaneously eluting the matrix interferences, or vice versa, has made this technique gain wide popularity. Moreover, they allow for a large volume of extract to be purified and concentrated at the same time. As previously mentioned, a large diversity of sorbents is today available; however, for soil and crop extracts, SPEs using hydrophilic and lipophilic balance (HLB) sorbent cartridges solely [72, 82] or in combination with the strong anion exchange sorbent (SAX) [70, 75], which is more expensive, are the purification techniques predominantly employed. HLB cartridges are able to bind non-polar and polar pharmaceuticals at the same time if they are in their neutral form in the extract, whereas, when HLB is employed in tandem with SAX on top of it, SAX cartridges are able to retain matrix interferences such as the negatively charged humic and fulvic acids present in soil extract or the waxes, fatty acids, chlorophyll, and pigments present in plant extracts, while pharmaceuticals are retained on HLB cartridges, resulting in further purified extracts [58].

Those two dominant SPE sorbents are in general used packed in cartridges, and they are mostly employed after a main extraction with ASE, MAE, and USE. However, Malvar and coworkers [69] have recently employed an in-cell clean-up with PLE (or ASE), also known as selective PLE (SPLE), where they added C18 and PSA to the extraction cell with the sample in dispersive form, which allowed to avoid the need of further treatment of sample extract. To our knowledge, this is the only study in which SPLE was employed.

Besides SPE cartridges, dispersive SPE (d-SPE) is nowadays very popular. The concept of d-SPE was first introduced by the fathers of QuEChERS [19], and from that time, it was always employed for cleaning up the extracts of diverse kinds of matrices obtained from QuEChERS extraction. SPE cartridges and d-SPE have similar purpose; the main difference between them remains on the usage mode. For the SPE cartridges, sample extract is loaded into the cartridges, whereas for the d-SPE, sorbents are in powder form (sorbent salts) and are added on the extract, where they absorb matrix interferences resulting in a free analyte extract (Fig. 2). By comparing both SPE techniques, d-SPE is easier to use and saves time.

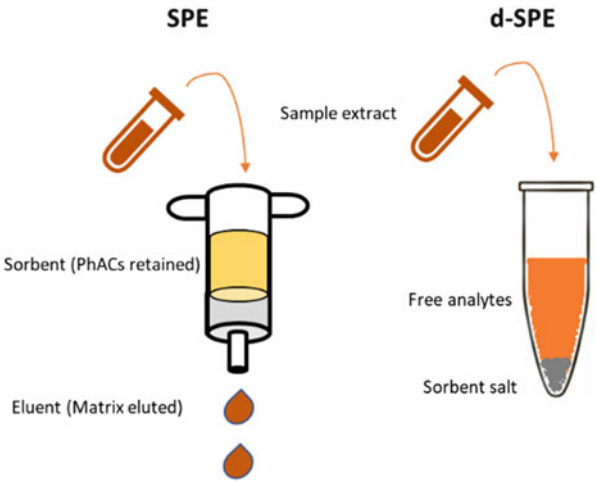

Fig. 2 Employment differences between SPE and d-SPE

Since QuEChERS requires no external forces, less matrix components are extracted. However, most of the reported studies employed the easy and rapid clean-up step using d-SPE, in order to reduce matrix effect and improve limits of detection and quantification. C18, primary secondary amine (PSA), and magnesium sulfate ($MgSO_4$) are the most known and commonly used d-SPE sorbents for extract clean-up. $MgSO_4$ is used as dehydrating agent to remove water excess in organic solvents; C18 eliminates non-polar matrix interferences such as fats, lipids, and some minerals; and PSA is commonly used to retain polar organic acids such as fatty acids and pigments by strong hydrogen bonds. Since only a few studies (there are exactly six studies) employed QuEChERS for soil extraction, we will discuss them in details from here on. The C18 is nearly always used in all the reported studies, either solely or in combination with $MgSO_4$ and PSA. Lee and coworkers used C18 sorbent solely for extract clean-up, and satisfactory recoveries were obtained ranging between 60.2 and 120.3% [103]. The addition of magnesium sulfate and primary secondary amines was excluded in their study, because the first liberates energy that might influence on the stability of the tested compounds whereas the second have the ability to chelate/bind with compounds such as sulfonamides. De Carlo and coworkers [102] tested C18 + $MgSO_4$ and PSA + $MgSO_4$ separately, and the best results were obtained with C18 + $MgSO_4$. The clean-up performed adding C18 + $MgSO_4$ allowed to recover all target analytes, whereas with PSA + $MgSO_4$, only two out of the five target analytes were recovered. This can be explained by the adsorption of analytes on the PSA by strong hydrogen bonds established between the hydrogen-donor hydroxyl group of the studied analyte and the hydrogen-acceptor nitrogen of PSA, together with other weaker hydrogen-bonding interactions. Same observation was obtained with Martínez-Piernas and coworkers [104], where higher recoveries were obtained using the mixture C18 + $MgSO_4$ while the presence of PSA reduced the extraction efficiency. This is also referred to the ability of PSA to act as a chelating agent with acidic compounds such as clofibric acid, furosemide,

indomethacin, ketoprofen, ketorolac, mefenamic acid, and methylprednisolone. Finally, similar recovery yields were obtained by those two studies (88–113% [102] and 70–120% [104]). On the contrary, this PSA effect was not observed with Meng and coworkers [105] when it was added in a reasonable amount (25 mg), and better recoveries (61.4–118.9%) and matrix effect (−40 to 54%) were obtained using the combination 25 mg of PSA, 10 mg of C18, and 100 mg of $MgSO_4$. On the other hand, when the clean-up step was omitted, chromatograms with less quality were obtained, due to the presence of a noisy baseline and interfering peaks derived from co-extracted components [102]. However, no clean-up was employed by Bragança and coworkers (recoveries >80%) [101], and no significant matrix effect was observed in their results. Finally, Salvia and coworkers [106] tested the d-SPE using several sorbents: PSA, PSA + C18, Florisil, silica, aluminum oxide, and SAX. However, great matrix effect was always obtained (> 80%), whereas satisfactory results were obtained using SPE cartridges (SAX and Strata-X cartridges) which was adopted in their final method.

Finally, despite the advantages provided from the clean-up, some authors believed that SPE step causes loss of analytes, so they omitted the clean-up step in both soil and crop matrices [140, 153].

6.2 *New Sorbents*

Several sorbents have been introduced recently for a better clean-up step, among them we list: Z-Sep, Z-Sep+, Z-Sep/C18, ChloroFiltr, CarbonX, Cleanert NANO, Oasis PRiME, and finally EMR-Lipid.

Z-Sep and Z-Sep+ sorbents are based on zirconium dioxide (ZrO_2) and can replace the use of PSA and C18. They were used to clean up extracts with high amounts of fat for the analysis of pesticide residues [167, 168]. Z-Sep is recommended for the clean-up of samples with hydrophobic analytes. Z-Sep+ is used for samples containing greater than 15% fat, and finally Z-Sep/C18 is used for samples containing less than 15% fat [167, 169]. Z-Sep/C18 was used for pharmaceutical analysis from fish samples [170].

ChloroFiltr, CarbonX, and Cleanert NANO are used to remove co-extracted chlorophyll from plant matrices as for GCB (graphitized carbon black) [171–173]. ChloroFiltr is a polymeric-based sorbent; it was used in combination with $MgSO_4$ and PSA without scarifying the recovery of planar analytes [171, 172]. CarbonX in a non-friable form of GCB, and similar to GCB, it reduces the recoveries of planar analytes. It was effectively used in combination with $MgSO_4$, PSA, C18, and Z-Sep for the clean-up of pesticides and environmental contaminants in shrimps [37]. However, CarbonX retain pesticides less strongly than GCB, and it is easier to work with since it is available as filter-vial d-SPE [174] or SPE minicartridges [173, 175]. Cleanert NANO is used to remove colorant and fatty acids. It is composed of functionalized MWCNTs (multiwalled carbon nanotubes),

with deactivated surface to ensure the recovery of pesticides with benzene ring [138].

Oasis PRiME HLB (acronym for Process, Robustness, Improvements, Matrix effects, Ease of use) is the next-generation SPE device. It is a simpler, faster, and cleaner SPE cartridge. It is simple because it does not require any conditioning and equilibration steps; also no SPE expertise is required. Equally important, it can be employed by reversed phase "catch-and-release" SPE or "pass-through" SPE. The three-step catch-and-release protocol consists of loading of the pretreated sample, washing the cartridge, and finally eluting the caught analytes with the proper solvent suitable for target analytes, whereas in the two-step pass-through protocol, the sample is loaded and collected at same time, because in this case matrix interferences are retained while analytes are passing through. It is faster because it provides faster flows with less plugging. Finally, it provides cleaner extracts since it removes more than 95% of common matrix interferences, such as salts, proteins, and phospholipids, with the generic three-step protocol (load, wash, elute) and at least 90% more phospholipids than the generic protocol with Oasis HLB. It is effectively used for pesticide analysis in spices and fruits [176, 177].

Finally, EMR-Lipid sorbent is introduced by Agilent Technologies in 2015 for the removal of phospholipids and proteins [178]. Water should be added to the extract before EMR-Lipid clean-up, adding an additional step to the method. However, it does not function as solid d-SPE; conversely, it dissolves to saturation in the extracts; and the extraction mechanism is based on size exclusion and hydrophobic interactions. It was effectively used for the analysis of multiresidue pesticides and environmental contaminants in kale, salmon, avocado, and pork [179].

7 Analysis of Pharmaceuticals in Earthworms

Invertebrates, living in crop fields, play an important role in terms of accumulation of PhACs but also acting as soil purifiers [180]. Specifically, earthworms are recognized for their use as sentinel organisms belonging to the soil microfauna, in order to evaluate soil contamination by organic contaminants. In addition, they are the main soil-dwelling organism that has the ability to absorb pharmaceutical products from soils since earthworms represent the vast majority of the biomass of total soil life [180, 181].

Numerous studies have shown their ability to absorb pharmaceutical products from soil [182, 183] through consumption or direct contact with the soil, as well as other contaminants such as heavy metals [184, 185] and polychlorinated benzene [186]. Furthermore, unlike plants, earthworms move constantly in the soil favoring contact with large quantities of PhAC that remain in the soil [187].

Therefore, the analysis of worms could assess the global contamination of pollutants in a cultivated field, thanks to its bioaccumulation capacity. On the other hand, terrestrial worms are soil invertebrates widely used for the assessment of the ecotoxicological risk of soil contamination by pharmaceutical products [188]

and pesticides [189]. Therefore, they are a key organism in the terrestrial environment, and their presence reflects a healthy soil environment.

Furthermore, earthworms constitute 60–80% of the soil biomass [190], allowing them to be the preferred organisms for identifying the level of contamination in the soil and in the agro-ecosystem, therefore the contamination of the food chain. In addition, they maintain an integral position being the basis of the food chain. Moreover, wide ranges of PhACs are susceptible to be absorbed by earthworms. Lipophilic and hydrophilic compounds could enter into the earthworm system due to absorption on its skin and diffusion by means of the mucous skin membrane, respectively [183, 191, 192].

Furthermore, earthworms are incredible metabolic machines given the presence of numerous enzymatic processes that take place in their tissues. Recently, vermicomposting, a biotechnological composting process, has shown that some terrestrial worms are capable of transforming organic compounds into a less toxic final product [187].

The quantification of the pharmaceutical products taken and accumulated in earthworms is important not only for assessing the direct risk on earthworms themselves, but it is also the first step to estimate the transfer of contamination through the food chain to the best predators such as birds. For example, Spurgeon and colleagues studied the potential risk of secondary poisoning of metals transferred to earthworms' predators, such as birds, through food chain [193].

However, very few studies have been directed toward the extraction, detection, and quantification of pharmaceutical products in earthworms (Table 7), which may represent an important limitation for the assessment of environmental, ecotoxicological, and human health risk. Furthermore, these reported studies were addressed for risk assessment rather than for method development and validation for analytical purposes. Again, this type of complex biological matrix requires selective and clean extraction to be analyzed with precision. According to our literature survey, only two research groups have undertaken a study dedicated to the multiresidual extraction of pharmaceutical products in earthworm tissues [20, 181], although in the next paragraph we will discuss the different steps and conditions for the preparation, extraction, cleaning, and analysis of the samples reported in the literature.

7.1 Sampling, Sample Preparation, and Extraction

For earthworm sampling from soil field, the International Organization for Standardization (ISO) has standardized a protocol for soil invertebrate (earthworms) sampling (ISO, 2006). Briefly, the field should be distributed to several subplots, then two holes (dimension of one hole: 25 × 50 cm and 20 cm deep) are hand-sorted in each subplot, and earthworms are forced out by pouring in the holes diluted solution (0.5%) of formaldehyde. Collected earthworms are then transferred alive on a moist tissue to the laboratory for analysis. This protocol was followed by [192]. On the other hand, Kinney and coworkers [183] followed another sampling protocol,

Table 7 Extraction methodologies in earthworms

Pharmaceuticals	Extraction method	Sample mass	Extraction solvent	Clean-up	Recoveries (%)	RSD (%)	LOD	Instrument	References
Carbamazepine, diclofenac, fluoxetine, orlistat	USE		Methanol, ethyl acetate, acetonitrile/water (70:30 v/v), and acetonitrile	No clean-up	86.3–100.9%			Liquid scintillation counting (LSC)	[133]
Caffeine, carbamazepine, thiabendazole, trimethoprim, diphenhydramine, d-limonene, galaxolide (HHCB), tonalide (AHTN), acetophenone, indole, isoborneol, camphor, isoquinoline, menthol, 4-tert-octylphenol, para-nonylphenol-total, nonylphenol monoethoxy-total, nonylphenol, diethoxy-total, 4-cumylphenol, octylphenol, monoethoxy octylphenol, diethoxy, phenol, triclosan, 3-beta-coprostanol, cholesterol, beta-sitosterol, stigmastanol, naphthalene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]pyrene, 2-methylnapthalene,	ASE	3–5 g	70:30 ACN/water		27–117%			HPLC-MS and GC-MS	[183]

1-methylnapthalene, 2,6-dimethyl-napthalene, bisphenol A, tributyl phosphate, diethylhexyl phthalate, para-cresol, diazinon, metolachlor, skatol, benzophenone, isophorone									
Triclosan, triclocarban, methyl triclosan	USE	1 g						LC-MS/MS and GC-MS	[192]
Androstenedione, testosterone, progesterone, norethindrone, gestodene, levonorgestrel, estriol, estrone, 17β- and 17-α-estradiol, 17-α-ethinylestradiol, sulfanilamide, sulfadiazine, sulfathiazole, sulfametoxydiazine, trimethoprim, sulfadimerazine, sulfabenzamide, sulfadimethoxine, erythromycin, tylosin roxithromycin, penicillin G, dicyclanil, florfenicol, paracetamol, sulfamethoxazole, fluvoxamine, carbamazepine, ibuprofen, bisphenol A	QuEChERS	250 mg	Acetonitrile/water	d-SPE (MgSO4, PSA, and C18)	45.2–105%		<14 ng/g	LC-MS/MS	[20]

(continued)

Table 7 (continued)

Pharmaceuticals	Extraction method	Sample mass	Extraction solvent	Clean-up	Recoveries (%)	RSD (%)	LOD	Instrument	References
Fluconazole, climbazole, valsartan, valsartan acid, irbesartan, ciprofloxacin, citalopram, metronidazole, sulfamethazine, sulfamethoxazole, 4-nitro-sulfamthoxazole, N-acetyl-sulfamethoxazole, sulfanilic acid, sulfanilamide, diclofenac, 4-hydoxydiclofenac, benzotriazole, 5-methyl-benzotriazole, carbamazepine, carbamazepine-11,12-epoxide, oxcarbamazepine, metoprolol, diltiazem, propranolol, caffeine, verapamil, acridone, fenofibrate, sucralose, clarithromycin, lamotrigine, lamotrigine N2-oxide, N2-methyl-lamotrigine, 5-des-5-oxo-lamotrigine, acesulfame, hydrochlorothiazide, acetaminophen, ibuprofen, gemfibrozil, bisphenol A, chloramphenicol, furosemide, indomethacin, bezafibrate, clofibric acid, fipronil, fipronil sulfone, fipronil-desulfinyl, and cocaine	QuEChERS	0,5 g	Acetonitrile	SPE (Oasis PRiME HLB)	70–99%	1–54%	0.01 ng g^{-1}	LC-QToF-MS	[181]

which consists of removing a soil circle of 40 cm diameter and 25 cm of depth using a cleaned metal-blade spade, and then undamaged earthworms were extracted from the collected soil. It is an older protocol used by Salagovic and coworkers in 1996 [194] in order to assess the genotoxicity of polluted soil on earthworm.

Usually, the very first step before earthworm's tissues extraction is to let them empty their guts. For this depuration step, earthworms are left on wet filter paper for 24 h, and then they are washed with deionized water, dried with a towel, and frozen and/or lyophilized. Only one study reported the use of freeze-drying the earthworms [181]. After lyophilization, earthworms are crushed, homogenized, and stored at −20°C until analysis. Other studies used homogenized fresh earthworm tissue for the analysis [20, 183].

The weights of the samples vary according to the extraction method followed, since for QuEChERS weights of 0.25 and 0.5 g were employed, respectively [20, 181]; when ASE was performed, weights between 3 and 5 g were needed [183].

We have been able to identify at least four different methods applied to the analysis of drugs in earthworms (Table 7). Kinney et al. managed to detect 77 anthropogenic organic waste indicators means of the ASE methodology, although 20 were detected in real samples. Approximately, 50 mL of a 70:30 ACN/water solvent mixture were employed during five static cycles at a temperature of 130°C and 10,300 kPa. Recoveries from 27 to 117% were observed, but overall good recoveries and accuracy results were reported [183].

One of the first studies used a previous extraction method used for the soil but also applicable to earthworms. The method consists of an ultrasonic extraction for the determination of four pharmaceutical drugs (carbamazepine, diclofenac, fluoxetine, and orlistat). Different extraction solvents have been used depending on the compound. For carbamazepine, 20 mL of MeOH was used while for fluoxetine and orlistat 20 mL of ACN/water (7:3, v/v). Finally, 20 mL of ethyl acetate was used for diclofenac. After centrifugation, no cleaning process was performed to avoid reducing recovery results [182].

The use of the most versatile QuEChERS method has been reported by two different studies. In the most recent work by Montemurro et al., an analytical method was developed for the determination of over 50 pollutants present in earthworm wastewater using a rapid extraction method based on QuEChERS with an innovative cleaning step with SPE using Oasis PRiME HLB. The method shows good recovery results from 70 to 99% for most of the studied compounds. By applying this method, the authors managed to detect and quantify 19 PhACs in earthworms grown under controlled conditions, whereas 8 analytes were detected in earthworm samples collected from a cultivated field irrigated with treated wastewater [181].

Bergé and colleagues also used the QuEChERS method as an extraction method to detect 11 steroids, 14 veterinary antibiotics, and 6 human pharmaceutical products. In this case, the AOAC-buffered salt kit was used. For the hydration phase, an emulsion of 9 mL of water/hexane (67:33, v/v) was used, while acetonitrile was the extraction solvent. The cleaning procedure was performed using PSA and C18 (950 mg of $MgSO_4$, 150 mg of PSA, and 150 mg of C18). Recoveries between 45.2 and 105% have been observed with good results of linearity and precision [20].

Finally, all the reports employed the analysis by means of LC-MS/MS. Concretely, Fourier transform (LC-FTMS), single quadrupole ion trap (QTrap), triple quadrupole (QqQ), and time of flight (LC-QToF-MS) mass spectrometers were employed for the detection of analytes of interest (Table 7).

8 Separation and Detection

It is widely known nowadays that pharmaceuticals are present in solid environmental and food matrices, such as soil and crops, at very low concentrations (few μg/kg up to g/kg). Advances in chromatography techniques and mass spectrometry instruments have facilitated the separation and the detection of pharmaceuticals extracted from any kind of environmental and food matrices even at trace levels. Despite this, these samples are analytically very difficult to analyze because of their complexity and their numerous components that interfere with the detection of pharmaceutical products.

Liquid chromatography (LC) and gas chromatography (GC) are both used for pharmaceutical analysis, depending on their polarity and/or volatility. Chromatography techniques used for soil and crops are reported in Tables 2, 3, 4, 5, and 6. However, liquid chromatography (LC) is most widely used since most pharmaceutical products have high polarity and low volatility. Furthermore, whenever GC is used, the extracted pharmaceutical products need an additional derivatization procedure and/or the replacement with a GC-compatible organic solvent before their injection. BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) + TMS (trimethylsilyl) are the derivatization reagents commonly used [54, 80, 81, 85, 92]. Aside from the fact that most pharmaceutical products are polar and non-volatile, some are also thermolabile, such as tetracycline [31, 195], making the derivatization step an essential phase for their detection. In general, organic reactions, such as methylation, silylation, and acetylation, undergo the derivatization of the hydroxyl and carboxyl groups of pharmaceutical products. However, in this way, a further step is added to the analytical protocol, which can affect the efficiency of the method due to the loss of analytes, incomplete reactions, or the introduction of unwanted contaminants [195]. Therefore, LC has an advantage over GC since no derivatization step is necessary. However, it should be remembered that GC is convenient, suitable for routine analysis, and less subject to matrix effects [54, 196].

LC and GC are basically coupled to mass spectrometers (MS). In addition, several studies have used LC coupled with ultraviolet (UV), fluorescence (FLD), or diode array (DAD) detectors for the detection of a few numbers of pharmaceutical products [68, 78, 162]. However, lower detection and quantification limits were obtained compared to LC coupled to MS. Furthermore, the high-resolution mass spectrometry (HRMS) allows to perform a non-target analysis of pharmaceutical, as well as their environmental transformation products, or even the products of the metabolism without compromising the sensitivity of the analysis [196].

The availability of different columns played an important role in multiresidue separation in one single injection. However, the reversed phase (RP) C18 and C8 columns are dominantly used for pharmaceutical separation for LC [72, 197], while fused silica capillary and DB5 columns are commonly used for GC [80, 92].

For GC-MS, helium is always used as carrier gas, and 1–2 μL of samples are injected in split/splitless mode, while the column temperature is programmed from 50 to 300°C, EI ionization temperature is between 200 and 250°C, and finally the standard ionization energy of 70 eV is always used. The traditional ionization mode, electronic impact (EI), was always used as ionization source coupled with GC.

LC chromatography techniques are available as high-performance LC (HPLC) and ultrahigh-performance LC (UHPLC). The latter has a higher sensitivity with 2–3 orders of magnitude compared to HPLC, since it uses columns with particles of smaller dimensions (<2 μm), with consequent better chromatographic separation, better resolution, narrow peak shapes, and a reduced chromatographic run. However, UHPLC is used in only a few numbers of studies [53, 77, 95, 154, 159]. The composition of the mobile phase is an equally important factor for obtaining good ionization and separation efficiencies, reproducible retention times, and peak shapes [198]. Generally, the methanol/water or acetonitrile/water mixtures, at different pH values, are commonly used for the separation of pharmaceutical products under gradient elution. For better ionization and separation of pharmaceutical products, some modifiers are added to the mobile phases. Formic acid, acetic acid, ammonium acetate, and ammonium formate are the modifiers commonly used in the positive/negative ionization modes [58, 74].

When the mass spectrometer is coupled to LC, electrospray ionization (ESI) is always the most used ionization source for PhACs. It is a soft atmospheric ionization technique, easily coupled to LC. The analytes are dissolved in an organic solvent and introduced into the ionization chamber through a fine needle in the form of a spray. A high electrical potential is applied to the needle, resulting in the formation of charged droplets. The droplets are then vaporized by introducing neutral gas (generally nitrogen). Under these conditions, the charged droplets decrease in size as they move inside the source, the droplet of the solvent evaporates, and the charged analytes pass through the ionization chamber toward the analyzer. Therefore, ESI is the atmospheric pressure ionization technique mostly preferred and used since it is excellent with polar and non-polar compounds and for compounds with low thermal stability [199].

Advances and developments in mass spectrometry allow the detection of pharmaceutical by target analysis (using reference standards) with consequent quantification at the trace and ultra-trace levels or by providing the possibility to perform suspect or non-target screening. Additionally, the use of tandem MS/MS offers higher specificity, provided with MS^2 of compounds, thus reducing co-elution problems of matrix interferences. Therefore, it is preferred in the analysis of solid complex matrices. Mass spectrometers with single (MS) or multiple analyzers (MS/MS tandem) are both highly used. Single quadrupole (Q) [54], Orbitrap [74], and ion trap (IT) [59] are used as single MS, while triple quadrupole (QqQ) [58], quadrupole-time of flight (QTOF) [123, 156], and triple quadrupole-linear ion trap

(QTRAP or QLIT) [61] are used as tandem MS/MS for pharmaceutical analysis from soil and crops. However, MS/MS systems offer high sensitivity, selectivity, and precision and lower limits of detection and quantification, particularly in the analysis of complex solid matrices. In other words, hybrid mass spectrometers are built by combining two different analyzers in a single instrument and provide more information on the sample in shorter analysis times, therefore an easy differentiation between target analytes and interfering components of the matrix. Jacobsen and coworker [58] used QqQ for the analysis of chlortetracycline, oxytetracycline, sulfadiazine, erythromycin, and tylosin (TYL) and its degradation products, TYL A, B, C, and D, from soil, obtaining low detection and quantification limits (from 0.6 to 5.6 g/kg and from 25.7 to 73.9 g/kg, respectively). Martínez-Piernas and coworkers [155] used QTOF for the analysis of carbamazepine transformation products from lettuce obtaining detection limits less than 3 ng/g in dry weight samples.

In addition, IT analyzers have the ability to perform multiple compound fragmentation steps (MS^n) and to trap fragment ions resulting in full-scan spectra with high sensitivity. Barron and coworkers [55] used LC-ESI-ion trap-MS in single and tandem MS modes, and they obtained detection limits below 20 ng/g for 20 pharmaceuticals out of 27 from soil, reporting poorer sensitivity for paracetamol, salbutamol, caffeine, pravastatin, indomethacin, and clotrimazole. However, they assumed that this poor sensitivity might be due to their low % recovery. The combination of IT with quadrupole analyzers (QTRAP or QLIT) offers the robustness of a QqQ with the full scan and high sensitivity of IT. Low method detection limits were obtained with García-Galán and coworkers [61] using this instrument, ranging from 0.03 to 2.23 ng/g, for sulfonamide extraction from soil. However, for a better selectivity, QTOF and Orbitrap are the chosen instrument. They provide high-resolution and accurate masses for parent and fragment ions in full-scan spectra. Accordingly, they remove the interfering signals, making it easier to identify the non-target compounds in complex environmental and food matrices [155, 200]. Orbitrap was used by Chitescu and coworkers [74], for the extraction of oxytetracycline, sulfamethoxazole, and ketoconazole from grass, obtaining detection limits lower than 10 μg/kg.

The methodologies managed in full-scan mode or single ion monitoring (SIM) with a single quadrupole showed the need for a highly efficient cleaning phase. On the other hand, the use of multiple reaction monitoring (MRM) mode with MS/MS systems offers maximum selectivity with a reduced matrix effect. It allows identification by monitoring the target compound and usually the most abundant transitions (product ions, generally two) [197].

Finally, to deal with the evaluation of the matrix effects, which can cause an enhancement or reduction of the analyte response, most of the studies used isotopically labeled compounds. However, not all deuterated compounds associated with each analyte are available for purchase or are sometimes very expensive.

9 Conclusions and Future Perspectives

The reuse of urban wastewater has opened up new possibilities for the use and availability of water. Indeed, in order to reduce the human impact on the environment, wastewater can be used to irrigate agricultural fields in areas of the world affected by drought or where availability is normally limited. Furthermore, the reuse of wastewater certainly has a positive impact on the soil as a mean for the development of plants and on the farmers themselves because of the economic advantage they could obtain by using wastewater as a fertilizer and a source of water for crops.

However, it is known that wastewater treatment plants are unable to remove small organic compounds such as PhACs, which remain in the soil and can subsequently enter the plant system where they are absorbed by the roots. Once inside these plants, these compounds can be consumed by grazing animals or even by humans or remain in the environment. For this reason, the study of these compounds in soil and plants is at the center of interest of many scientists.

Determining the presence and concentration of contaminants in soil and plant tissues requires significant effort. Hence, from the research papers reviewed, it is clear that several successful analytical methods have been developed in the last decade to extract, detect, and quantify most of the pharmaceutical products commonly used in various matrices such as vegetable and soil. However, in most cases, these methods are time-consuming, are expensive, and require the use of specialized reagents and personnel. Various research teams have made significant efforts to overcome the obstacles associated with these methods, but in some cases further development is needed. It is therefore necessary to continue the research developing even simpler and more robust analytical techniques that are at the same time environmentally friendly.

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Analytical Approaches for the Determination and Identification of Drug Metabolites in Plants After Uptake

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Contents

Abstract The present chapter gives an overview of analytical methodologies employed for the identification and quantitation of metabolites formed in plants or plant cell cultures from drugs and personal care products after uptake from water or soil. Important aspects like experimental approaches for plant growing, extraction of the investigated analytes from plants, preconcentration strategies, and final analytical techniques allowing the proposal of (at least tentative) structures for drug-related metabolites are discussed. Special emphasis is also set on the elucidation of translocation processes by analyzing different plant parts. In one table, a comprehensive

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overview of the current state of knowledge available from the literature is given, with respect to the topics listed above.

Keywords Drug metabolites, Emerging contaminants, Environmental analysis, Environmental metabolomics, Phytouptake

Abbreviations

17α-E2	17α-Estradiol
17β-E2	17β-Estradiol
ALB	Albendazole
ATE	Atenolol
ATR	Atorvastatin
AVO	Avobenzone
BHT	Butylated hydroxytoluene
BMS	Betamethasone dipropionate
BPA	Bisphenol A
BZP	Benzophenone
CAF	Caffeine
CBZ	Carbamazepine
CDP	Chlordiazepoxide
CIP	Ciprofloxacin
CLA	Clarithromycin
CLI	Clindamycin
CLP	Clomipramine
CP	Clonazepam
DCF	Diclofenac
DP	Diazepam
E1	Estrone
EE2	Ethinylestradiol
ENR	Enrofloxacin
ESI	Electrospray ionization
FAD	Furaltadone
FAZ	Furazolidone
FBZ	Fenbendazole
FL	Fluvastatin
FLU	Flubendazole
FP	Flurazepam
GEM	Gemfibrozil
HPLC	High-performance liquid chromatography
HR-MS	High-resolution mass spectrometry

IBU	Ibuprofen
IPR	Iopromide
KPF	Ketoprofen
LTG	Lamotrigine
MeBT	5-Methyl-1H-benzotriazole
MePB	Methylparaben
MET	Metformin
MFA	Mefenamic acid
NDP	Nordiazepam
NFA	Nitrofurantoin
NFZ	Nitrofurazone
NPX	Naproxen
NSAID	Non-steroidal anti-inflammatory drug
OBZ	Oxybenzone
OC	Octocrylene
OFL	Ofloxacin
OP	Oxazepam
OPL	4-Octylphenol
OS	Octisalate
PAR	Paracetamol
PZE	Phenazone
QqQ	Triple quadrupole
QuEChERS	Quick, easy, cheap, effective, rugged, and safe
SDM	Sulfadimethoxine
SDZ	Sulfadiazine
SER	Sertraline
SIM	Simvastatin
SMR	Sulfamerazine
SMT	Sulfamethizole
SMX	Sulfamethoxazole
SMZ	Sulfamethazine
SPD	Sulfapyridine
STZ	Sulfathiazole
TA	Temazepam
TCP	Tris(2-chloroethyl)phosphate
TCS	Triclosan
TEL	Telmisartan
TM	Triazolam
TMP	Trimethoprim
TRZ	Trazodone

1 Introduction

In recent years, the occurrence of residues of pharmaceuticals and personal care products (PPCPs) in the environment has attracted increased attention in the scientific community. Nowadays it is a well-known fact that effluents from wastewater treatment plants (WWTPs) are an important source for the release of these xenobiotics into surface waters. The increasing availability and use of PPCPs worldwide have made the presence of such contaminants a global phenomenon [1]. As a matter of fact, PPCPs are continuously introduced into the environment so that they are also referred to as pseudopersistent pollutants. The tremendous advances in procedures for environmental analytical chemistry [2, 3] have resulted in a significantly increased number of PPCPs that can be monitored routinely in water samples and have provided extended data sets to be used for a better understanding of their fate in the environment. Although effort has been made to improve the removal rates for PPCPs in WWTPs [4–6], the release of such xenobiotics may still be an issue worldwide.

Typical concentrations of PPCPs in effluents of WWTPs may be up to the low μg L^{-1} range. This means that concentrations in receiving waters can be expected to be in the ng L^{-1} range. Even such low concentrations may result in negative effects on the environment. This is well known in case of the continuous presence of residues of antibiotics, which can lead to the development of antibiotic resistance [7]. Unfortunately, for many other classes of PPCPs, ecotoxicological effects are still poorly understood [8]. Even less knowledge is generally available when it comes to the presence and effects of metabolites of PPCPs possibly generated in the environment.

The presence of PPCPs in effluents of WWTPs has also raised some concerns in the context of food safety. In arid regions, reclaimed water is nowadays frequently employed for irrigation in agriculture as a consequence of increased water scarcity. For example, in 2016 Israel used reclaimed wastewater for approximately 50% of the total irrigation water [9]. Also Mediterranean countries of the European Union depend more and more on reclaimed water in agriculture [10]. This means that agricultural plants can be expected to get into contact with pharmaceuticals not fully removed by WWTPs, which subsequently may result in an uptake of these xenobiotics by the plants. Considerable efforts are made to establish regulations regarding the quality of reclaimed water, but so far comprehensive final standards have not yet been set [11]. Another source for pharmaceuticals potentially taken up by agricultural plants is the use of biosolids [12]. Manure used as a natural fertilizer may contain residues of veterinary drugs which can contaminate agricultural plants via the soil [13, 14].

Various papers and reviews dealing with the uptake of intact PPCPs by plants from the environment have been published within the last few years [15–24]. However, PPCPs taken up by plants may also be subjected to bio-transformation/metabolization processes [25, 26]. A full risk assessment of PPCPs in the environment will require thorough investigations dealing with such transformation pathways because metabolites may show (eco)toxicities even higher than the parent

compound or different modes of action regarding their biological activity. Last, but not least, metabolites of PPCPs formed eventually during the passage through WWTPs may get transformed back to the parent substance when taken up by plants.

In general, the metabolization of xenobiotics in plants follows the pathways known from mammalian organisms. During phase I, xenobiotics are transformed into more polar compounds (that also exhibit a higher water solubility) by reactions like hydroxylation, which are catalyzed by enzymes such as cytochrome P450 oxidases. Phase II involves the conjugation of phase I metabolites with small molecules like hexoses (whereas in mammalian organisms glucuronic acid is commonly attached), amino acids, sulfate, or glutathione. Various transferases are involved in this step, such as glutathione S-transferases (GSTs) with structures close to that in mammalian organisms except for a catalytic serine in place of a tyrosine residue [27]. During phase III the metabolites may be further transformed (an example is the reaction of glutathione conjugates to acetylcysteine conjugates), and in case of mammalian organisms, the metabolites are excreted. In case of plants, metabolites undergo compartmentalization and binding to cell walls. This fraction is not readily available for extraction and analysis, but its extent can be estimated by using ^{14}C-labeled parent drugs and measuring the activity in the plant cells, as recently shown for investigations of the metabolism of naproxen and ibuprofen in the *Arabidopsis* plant [28].

The investigation of the uptake and metabolization of a certain PPCP by a plant may be done in the form of two different approaches, namely, either by a targeted analysis or by an untargeted analysis. In case of targeted analysis, a limited number of metabolites are defined for quantitation prior to the analysis itself. These potentially present metabolites may be selected according to existing knowledge about metabolization pathways in plants or on the basis of studies done on mammalian or microbial organisms. One can also use in silico prediction approaches and tools available as commercial software packages or freeware for selecting the target metabolites (for a review dealing with such tools, see, e.g., [29]). Such a targeted analysis makes an effective optimization of the sample preparation and analyte preconcentration methods for the selected analytes possible, so that quantitation limits can be achieved that may be low enough for investigating plants irrigated with real reclaimed water.

Contrary to targeted analysis, the untargeted approach aims at a comprehensive detection of known and unknown metabolites formed from PPCPs taken up by the plant. For that purpose techniques are available that are nowadays commonly used in metabolomics of mammalian systems and rely on high-performance liquid chromatography (HPLC) hyphenated with high-resolution mass spectrometry (HPLC-HR-MS) [30–32]. When unknown metabolites are the focus, the sample pretreatment including preconcentration steps can hardly be fully optimized which may compromise quantitation limits. Therefore, untargeted analysis of metabolites in plants is often done by treating model plants (or plant cell cultures) with relatively high concentrations of PPCPs. In this way, the detection of new metabolites and their subsequent identification may become possible, and in a second step, a targeted approach can be added for real samples.

This review intends to summarize in a comprehensive and critical way the current knowledge about the fate and metabolization of PPCPs in plants and to give an up-to-date overview of the development of analytical approaches employed in this field.

2 Experimental Approaches for Investigating the Uptake of Drugs by Plants

2.1 Strategies for the Growing of Plants and Their Treatment with Drugs

Since more than a decade, studies investigating not only the uptake but also the subsequent metabolization of drugs by plants were published [12, 33]. To achieve this goal, different approaches were followed. In the majority of reports, plantlets were grown hydroponically in drug-containing media [17, 34] or in a support medium (like agar or perlite) that was wetted with drug-containing aqueous solutions. A smaller number of studies reported the uptake of drugs by plants grown in soil [18, 19]. Here drugs were mostly added by irrigation using either lab-made drug-containing aqueous solutions or even actual reclaimed waters. What the majority of these studies (hydroponic and soil-based ones) have in common is that to facilitate the detection of the parent drugs but even more importantly their metabolites, rather high concentrations of drugs were used. Nevertheless there are a few examples where drug concentrations employed were close to those expected in actual reclaimed waters (e.g., a study where garden cress was treated with NSAIDs at a concentration level as low as 0.001 mg L^{-1} [35]), whereas on the other hand *Armoracia rusticana* cell culture was incubated with paracetamol (PAR) at a concentration level of >160 mg L^{-1} [36].

Although, as just discussed, treatment with somewhat higher concentrations of a drug (or drug cocktail – if the fate of more than one drug is investigated in a single plant experiment) increases the probability to detect also the less abundant drug-related metabolites, two aspects have to be considered. First, drug concentrations should be below a level hampering the organic growth of the plant, and second, plant metabolism should not be affected too much by concentration effects to keep the study meaningful also with respect to actual environmental conditions. When considering the treatment of a single plant with a drug cocktail (to reduce the experimental effort when investigating the fate of several drugs), unwanted interactions between the drugs should be thoroughly observed, unless the investigation of synergistic effects (as might be occurring when using real reclaimed waters) is the actual aim of the study. A further factor that should be kept in mind is that the solvent employed for preparing the drug standard solution (in many cases methanol) used for adding the drug to the growing medium or irrigation water may also exhibit negative effects on plant growing. Here the use of ethanol instead of methanol has proven beneficial.

A range of model plants was used for these tests. Thereby due to their relevance in the production of food and feed, particular emphasis was set on edible plants. Examples therefore are vegetables such as tomato [37–40], cucumber [41–43], bean [39], lettuce [37, 39, 40, 44–49], pea [40], cabbage [50], carrot [51], onion [40, 52], garden cress [35, 53–55], and radish [36, 47, 56–59] treated with NSAIDs, antibiotics, antidepressants, PAR, carbamazepine, or personal care products as well as maize [40], wheat [37], barley [58], sorghum [40], and millet [40] grown in diclofenac-containing medium.

Another approach for investigating the interaction of plants with xenobiotics is the use of cell cultures [28, 36, 42, 51, 56–58, 60–63]. Major advantage of this experimental setup is the fact that cultivating and also incubating cell cultures is affected with less effort than growing whole plants (or at least plantlets). On the other hand, cell cultures do not allow to study transformation phenomena such as a (time-dependent) monitoring of parent drug and metabolite concentrations in different plant parts.

2.2 Harvesting and Extraction

Depending on the study layout (but of course also on the plant model used), the period between the start of a growing experiment and the final harvesting of the plants ranged between a few days (for an example, see [35, 53, 54]) and several months (for an example, see [38, 45, 52]). Whereas in the case of plant cell experiments there is no other choice than using the whole cells for analysis, the situation is different when growing full plants. In this case, depending on the size of the harvested plantlet, it could be divided into several distinct plant parts (commonly roots, stem, leaves, bulbs, or fruit) which were subsequently extracted and analyzed separately. This allowed judging the translocation of the parent drug and the metabolites into different plant compartments. In a series of works, the latter was also studied as a function of time, harvesting plants at several time points and studying the time-resolved distribution of parent drug(s) as well as metabolites within the plant parts investigated.

In most cases, parent drugs and/or metabolites were recovered from plant material and cell cultures by simple and straightforward extraction with appropriate solvents. Plant material was either directly homogenized (e.g., using an Ultra-Turrax – for exemplary papers, see [53–55]) or freeze-dried and grinded to form a fine powder with subsequent solvent extraction (e.g., using an ultrasound bath – for exemplary papers, see [38, 41] or accelerated solvent extraction [37, 43]). For a cleanup and a further preconcentration step, extracts were subjected to a solid phase extraction (SPE) procedure. For the parent drugs, SPE provided acceptable preconcentration factors. For some of the more polar substances such as the sugar-containing metabolites formed from diclofenac (DCF), naproxen (NPX), ketoprofen (KPF), and mefenamic acid in cress, only a very limited preconcentration effect could be

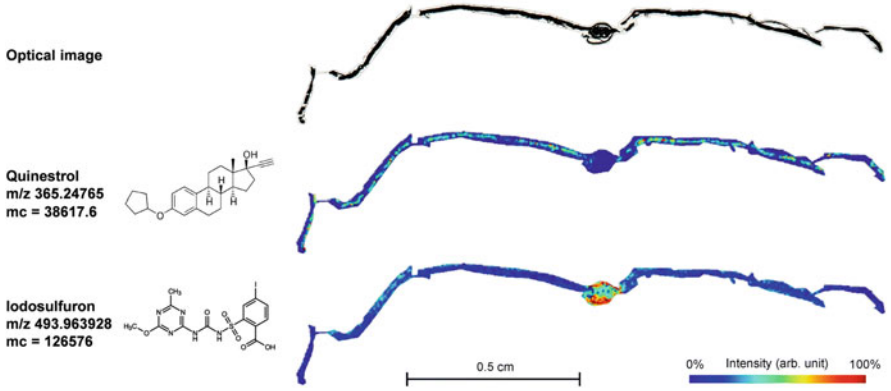

Fig. 1 Tissue distribution of quinestrol and iodosulfuron in *Salix alba* leaves. The mass spectrometry imaging analysis of leaf sections from *Salix alba* revealed different distribution patterns of xenobiotics in the leaf tissue. Quinestrol was mostly sequestered in internal tissue, and iodosulfuron was clearly located in the vascular tissues; intensity given as an arbitrary unit shown as color gradient; mc: maximum count. From [67] with permission

achieved due to insufficient interaction with the SPE sorbent [53]. This observation might be extrapolated also to sugar conjugates from other drugs.

As an alternative pretreatment, a modified QuEChERS approach for extracting parent drugs and their metabolites from plants was used, allowing skipping the SPE step and still providing good limits of detection for both parent drugs and metabolites [35, 45, 54, 64]. One way to improve extraction by an increased cell-rupturing effect, setting free also drug and drug-metabolite molecules within the plant cells, is the use of methods involving matrix solid phase dispersion (MSPD) [65] or sea sand disruption [65]. MSPD is based on the blending of a sample with an abrasive solid material [66]. It permits simultaneous disruption of the sample architecture and extraction of complex biological samples, thereby improving extraction efficiency.

The workflow discussed above, although most widely used, involving either extraction of the whole plant or dissection of plant parts with subsequent analysis of the compounds of interest does only allow a rough estimation on the location of parent drugs and their metabolites within the plant. An alternative approach, enabling direct investigations on the spatial distribution of the drug and its metabolites within plant parts, is mass spectrometry imaging. Hereby the sample of interest (plant part) is examined, after appropriate sample preparation, by techniques such as desorption electrospray ionization (DESI) MS or matrix-assisted laser desorption ionization (MALDI) MS. An example for the use of MALDI MS, for the in situ localization of a series of xenobiotics in *Salix alba* leaves after cryo-sectioning, was published by Vilette et al. [67]. The analysis of cross sections of *Salix alba* leaves by the methodology mentioned above is depicted in Fig. 1.

3 Approaches for Metabolite Detection

In a series of research papers, the main focus was set on the detection and identification of new drug-related metabolites formed within plants after uptake of the parent drug from water or soil. Thereby different strategies have been applied for the structural identification of these metabolites. According to Schymanski et al. [68], there are five levels of structure identification, ranging from level 1 (confirmed structure), where comparison with a reference standard is obligatory, to level 5 where only the accurate mass of the potential metabolite is known. The possibility to characterize drug-related metabolites according to level one is primarily restricted by the commercial availability of appropriate standards or the possibility to synthesize these compounds in-house. An excellent example for a study where a high percentage of the detected metabolites could be assigned to structures according to level 1 following the Schymanski scheme has been published by Riemenschneider et al. [38]. Here out of 17 carbamazepine-derived metabolites found in tomato plants treated with a total of 7.5 mg carbamazepine per plant over a growing period of 89 days, 10 could be identified unambiguously by comparison of their high-resolution mass spectra with those from commercially available standards. Nevertheless, it should be taken into account that (with a very few exceptions) the availability of standards is limited to phase I metabolites. In most cases, only tentative or probable structures (according to levels 2 and 3 of the Schymanski scheme) could be presented for drug-related metabolites formed in the plant. These are mainly based on measuring accurate mass, employing either (quadrupole) time-of-flight mass spectrometers ((Q)TOF/MS) or Orbitrap MS (mostly after chromatographic separation), together with the generation of MS/MS spectra allowing the interpretation of molecule fragmentation. Frequently these data are combined with information from the literature such as published pathways for the formation of metabolites in mammals and/or published MS spectra for analogous compounds. A few older papers propose structures on the basis of low-resolution MS, fragmentation spectra, and data from additional chemical experiments such as enzymatic digestion or alkaline hydrolysis [36, 58, 69]. However, it should be stressed that despite the possibility to measure accurate mass, probable structure for drug-related transformation products in plants cannot always be proposed (level 5).

Appropriate standard substances are even more required, when, in addition to the proposal of structures, quantitative information is desired. The large majority of studies discussed in this chapter involve the use of MS detection employing an electrospray source for ionization. Here the response factor for different substances can vary substantially, allowing proper quantification only if standard substances are available for calibration. One approach showing considerable potential to overcome this obstacle is the methodology proposed by the Kruve group [70–72], showing a way toward standard-free quantification. Thereby they studied the efficiency of negative ion formation in the ESI source via deprotonation of substituted phenols and benzoic acids [70]. The observed correlations between the ionization efficiencies achieved and different molecular properties allowed the construction of a linear

model describing the ionization efficiency of both phenols and benzoic acids. In a further paper, this approach was applied to investigating the influence of different mobile phases on the signal intensities in ESI, allowing to compare results obtained under different chromatographic conditions [71].

A promising approach for investigating the fate of drugs after uptake by a plant was the following. First experiments with rather high concentrations of the drug were performed, and extracts from either the whole plant (or the whole cells in the case of plant cell experiments) or the plant parts of interest were examined using HPLC coupled to HR-$MS^{(n)}$. Thereby accurate masses could be determined (under ideal circumstances allowing the proposal of an unambiguous sum formula), and information on the molecular structure could be obtained from MS^n experiments. Stable isotope labeling with HR-MS (i.e., spiking the nutrient solution with a mixture of deuterated and non-deuterated drug) can be seen as an interesting approach to further improve the workflow in the detection of drug-derived metabolites within the plant [63]. Combining the findings from HR-MS with knowledge from plant biology or drug metabolism in mammals at best allowed proposing a metabolic pathway for the investigated drug within the plant organism. Subsequently, to improve the applicability of this experimental approach also for actual environmental samples (here the concentrations of both the parent drug and the drug-related metabolites will be much lower), information from previous MS^n experiments was employed for setting up a multiple reaction monitoring (MRM) method on a highly sensitive QqQ MS, thereby allowing the investigation of plants treated with environmentally relevant concentrations of the drug. Examples for the successful application of this scheme can be found in the literature [35, 53].

As common for metabolomics research, two different approaches, namely, a targeted and a non-targeted one, can be distinguished. Most of the studies published so far investigating the uptake, translocation, and metabolization of drugs and personal care products (PCPs) in plants clearly use a targeted approach. Based on knowledge about regular plant metabolism, comparison with metabolic studies in mammals and humans, and selective search for potential transformation products, plant extracts are searched for drug-related metabolites. Only a few papers employing a more untargeted approach can be found in the relevant literature (for a good example, see [67]). Vilette et al. analyzed surface water and superficial sludge (at the entrance of a tertiary treatment wetland) with respect to the presence of micropollutants (HR-MS spectra were processed with common metabolomics software; sum formulas were proposed and entered into open-access data bases) and compared these data with those from *Salix alba* growing on the edge of the wetland. A total of 1,027 compounds were tentatively identified (level 3 according to Schymanski) whereby 96 could be found in all 3 matrices (see also Fig. 2) [67].

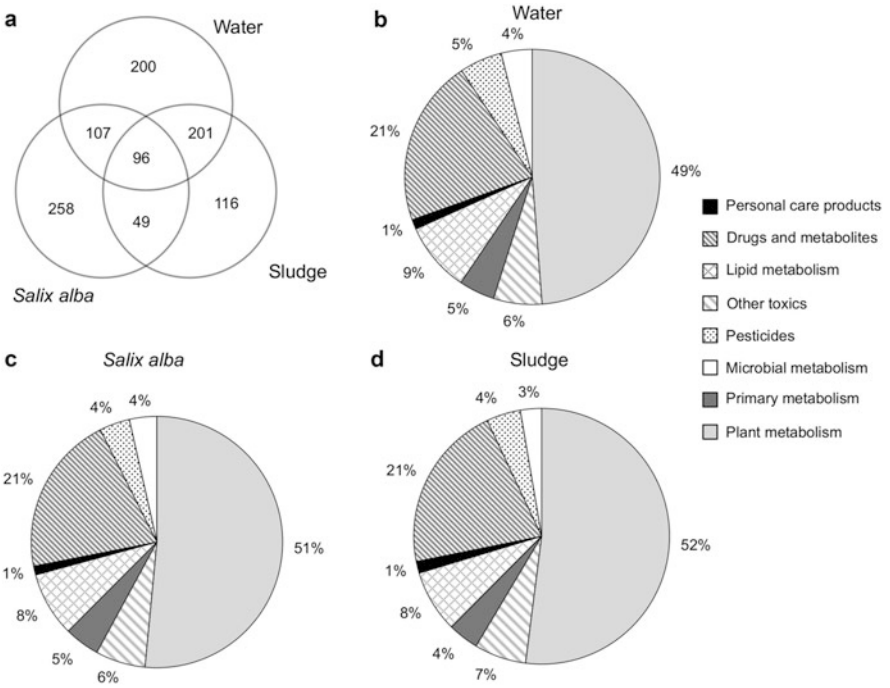

Fig. 2 Non-targeted metabolome analysis of water, sludge, and plant leaf extracts. Surface water (**b**), leaves from *Salix alba* (**c**), and superficial sludge (**d**) were analyzed by liquid chromatography coupled to mass spectrometry to obtain their metabolic profile. A total of 1,027 compounds were tentatively identified (level 3 of Schymanski classification). Only some of the compounds were common to the three compartments (**a**), but the same classes of molecules were found with a similar distribution in all three compartments of the environment. Water and sludge were sampled in triplicate, eight leaf samples of each age (young, intermediate, old) were extracted individually, and the results were pooled after acquisition. From [67] with permission

4 More Detailed Discussion of Papers Published Within This Field

A comprehensive overview of the current state of literature within the field discussed in this book chapter is provided in Table 1. Thereby information about drugs and personal care products investigated, plant or plant cell models employed, metabolites detected (phase I + II), as well as strategies used for metabolite detection and identification is given.

Table 1 Overview of studies on the analysis of metabolites from drugs and personal care products in plants after uptake

Parent drug(s)	Metabolites		Plant material	Growing conditions	Extraction/cleanup/ enrichment	Analysis of extracts	Ref.
	Phase I	Phase II					
ALB	x	x	Ribwort plantain – plant, cell suspension	Seeds germinated on Murashige and Skoog basal medium Cell culture was initiated from the leaves of regenerants Incubated with 10 μM ALB	Liquid-liquid extraction of homogenized frozen samples	UPLC-QqQ-MS^2	[61]
ALB, FBZ, FLU	x	x	Harebell – cell suspension	Suspensions of harebell cells in Murashige and Skoog culture medium Incubated with 10 μM ALB, FBZ, FLU	Liquid-liquid extraction of homogenized cell suspensions	LC-QTOF-MS^2	[86]
ATE, CBZ, SMX	x	–	Lamb's lettuce, spinach, arugula, radish – plant	Greenhouse conditions watered with 1 mg L^{-1} drug solutions	Liquid extraction of homogenized frozen samples	LC-QqQ-MS^2	[47]
ATR, FL, SIM	x	x	Cress – plant	Hydroponic (drug concentrations 0.001–1 mg L^{-1})	Ultra-Turrax extraction QuEChERS cleanup	LC-QqQ-MS^2 LC-DTIM-QTOF-MS	[54]
AVO, OC, OS	x	x	Duckweed, *Cyperus alternifolius* – plant	Hydroponic in nutrition solution (drug concentrations 9.6–40.6 mg L^{-1})	Ultra-Turrax extraction	LC-QTOF-MS LC-DTIM-QTOF-MS	[90]
BMS, TEL	x	x	*Salix alba* – leaves	Growing on the bank close to the water outlet of a two-stage vertical flow constructed wetland	Frozen leaves were ground in liquid nitrogen and extracted with liquid extraction MALDI with HCCA	LC-QTOF-MS MALDI-MSI FT-ICR-MS	[67]

BPA, BHT, BZP, CAF, CBZ, MePB, MeBT, OPL, PZE, TCP, TCS	–	x	Lettuce – plant	In pots under greenhouse conditions watered with 0.05–50 μg L^{-1} drug solutions	Extraction using sonication SPE with Strata-X Derivatization prior to injection	GC-MS^2	[44]
CAF	x	–	Lettuce – plant	Hydroponic in nutrition solution (CAF concentrations 0.575 mg L^{-1})	Liquid extraction of freeze-dried samples SPE with Oasis HLB	LC-QTrap-MS^2	[46]
CBZ	x	–	Tomato, wheat, lettuce – plant	Grown in lysimeters containing arable soil irrigated with treated wastewater	Freeze-dried plant material was ground and extracted using an accelerated solvent extractor	LC-QqQ-MS^2	[37]
CBZ	x	–	*Typha* spp. – plant	Hydroponic in nutrition solution (drug concentrations 0.5–2 mg L^{-1})	Extraction using matrix solid phase dispersion or sea sand disruption method	LC-QqQ-MS^2	[65]
CBZ	x	x	Tomato – plant	Hydroponic in nutrition solution (drug concentrations 0.5 mg L^{-1})	Ultrasonic extraction of freeze-dried samples	LC-QqQ-MS^2 UPLC-QTOF-MS	[38]
CBZ	x	–	Lettuce – plant	In pots under greenhouse conditions irrigated with 1 mg L^{-1} CBZ	QuEChERS extraction	LC-QTOF-MS	[45]
CBZ	x	x	Horseradish – hairy root culture	Grown in Murashige and Skoog medium (10–250 μM CBZ)	Frozen samples were ground and extracted with sonication	LC-QTOF-MS	[57]
CBZ	x	–	White-rot fungus – plant	Cultures in Petri dishes (10.8 mg L^{-1} CBZ) and solid-state fermentation (0.025 mg g^{-1} CBZ)	Ultrasonic extraction	LC-QqQ-MS^2 LC-Q-TOF-MS	[73]
CBZ (LTG)	x	–	Cucumber – plant	Hydroponic in nutrition solution (drug concentrations 1.8 μM)	Plant material was ground and extracted using an accelerated solvent extractor	LC-QqQ-MS^2	[43]

(continued)

Table 1 (continued)

Parent drug(s)	Metabolites		Plant material	Growing conditions	Extraction/cleanup/ enrichment	Analysis of extracts	Ref.
	Phase I	Phase II					
CDP, CP, DP, FP, NDP, OP, TA, TM	x	–	Radish, silverbeet – plant	Grown on soil (drug concentration 0.5 mg kg^{-1})	Ultrasonication extraction of freeze-dried samples SPE with Oasis HLB	LC-QqQ-MS^2	[59]
CIP	x	–	Chinese flowering cabbage – plant	Hydroponic in nutrition solution (1 mg L^{-1} CIP)	Ultrasonication extraction of freeze-dried samples SPE with Oasis HLB	UPLC-QqQ-MS^2	[50]
CLA, SDZ	x	–	Lettuce – plant	Hydroponic in nutrition solution and clean perlite for plant fixation (drug concentrations 1 mg L^{-1})	Extraction using sonication SPE with Oasis Prime HLB	UPLC-QqQ-MS^2 UPLC-QTOF-MS^2	[48]
CLI, ENR, OFL, SDM, SDZ, SMT, SMX, SMZ, STZ, TMP	x	x	Lettuce, tomato, cauliflower, broad beans – plant	Farm plot irrigated with reclaimed water	Ultrasonic extraction of homogenized vegetable sample SPE with Strata-X	LC-QqQ-MS^2	[39]
CLP, SER, TRZ	x	x	Cress – plant	Hydroponic (drug concentrations 10 mg L^{-1})	Ultra-Turrax extraction	LC-QTOF-MS LC-Orbitrap-MS	[55]
DCF	x	x	*Arabidopsis thaliana* – cell suspension	Cells grown in medium (DCF 3.59 mg L^{-1})	Freeze-dried cell samples extracted with sonication SPE with Oasis HLB	UPLC-QqQ-MS^2 LC-TOF-MS	[60]
DCF	x	x	Barley – plant, horse radish – hairy root culture	Hydroponic in Murashige and Skoog medium (10 μM DCF)	Liquid extraction of homogenized frozen samples SPE with Strata-X	LC-IT-MS^2	[58]

DCF	x	–	Poplar – plant	Plantlets in pots filled with perlite in a growth chamber (DCF 1 mg L^{-1})	Plant material ground in liquid nitrogen and extracted with sonication SPE with Strata-X	LC-IT-MS	[82]
DCF	x	x	Cattail – plant	Hydroponic in nutrition solution (DCF 1 mg L^{-1})	Samples ground in liquid nitrogen and extracted with liquid extraction SPE with Strata-X	LC-IT-MS	[75]
DCF	x	x	Tomato, salad, onion, rice, amaranth, millet, maize, pea, sorghum – plant	Hydroponic (DCF 20 mg L^{-1})	Ultra-Turrax extraction	LC-DTIM-QTOF-MS^2	[40]
DCF, KPF, MFA, NPX	x	x	Cress – plant	Hydroponic (drug concentrations 0.01–1 mg L^{-1})	Ultra-Turrax extraction SPE with Strata-X	LC-QqQ-MS^2 LC-QTOF-MS^2 LC-Orbitrap-MS	[53]
DCF, KPF, MFA, NPX	x	x	Cress – plant	Hydroponic (drug concentrations 0.001–0.1 mg L^{-1})	Ultra-Turrax extraction SPE with Strata-X and QuEChERS cleanup	LC-QqQ-MS^2	[35]
EE2, 17β-E2	x	–	Lettuce – plant	Hydroponic in nutrition solution (drug concentrations 0.1–10,000 μg L^{-1})	Extraction using sonication SPE with Oasis HLB and CARB cartridges Derivatization prior to injection	GC-MS	[49]
FAD, FAZ, NFA, NFZ	x	–	Spring onion – plant	In soil (drug concentrations 4 mg kg^{-1}) Incubation of the homogenate of spring onion leaf/root bulb (drug concentrations 1 mg L^{-1})	Protein-bound metabolites were liberated and derivatized Extraction with ethyl acetate	LC-QTrap-MS^2	[52]

(continued)

Table 1 (continued)

Parent drug(s)	Metabolites		Plant material	Growing conditions	Extraction/cleanup/ enrichment	Analysis of extracts	Ref.
	Phase I	Phase II					
FBZ, FLU	x	x	Ribwort plantain – plant, cell suspension	Cultivated with Murashige and Skoog medium (drug concentrations 1 mg L^{-1})	Liquid extraction of homogenized frozen samples	UPLC-QqQ-MS^2	[87]
GEM	x	x	*Arabidopsis thaliana* – plant, cell suspension	Hydroponic in nutrient solution (GEM 1 mg L^{-1}) and cell culture (GEM 2.5 mg L^{-1})	Liquid extraction of freeze-dried samples SPE with Oasis HLB	LC-QqQ-MS^2 LC-TOF-MS	[63]
IBU	x	x	Duckweed – plant	Grown in Murashige and Skoog medium (drug concentrations 1 mg L^{-1})	Liquid extraction of freeze-dried samples with sonication SPE with Oasis HLB	LC-Orbitrap-MS	[77]
IBU	x	x	*Phragmites australis* – plant	Pots filled with clean perlite and culture medium (IBU 60 µg L^{-1})	Liquid extraction of frozen samples SPE with Oasis HLB	LC-Orbitrap-MS	[69]
IBU	x	x	*Arabidopsis thaliana* – cell suspension	Grown on medium (200 µM IBU)	Liquid extraction of homogenized samples SPE with Oasis HLB	LC-QTOF-MS^2 LC-Orbitrap-MS	[79]
IBU	x	–	*Typha angustifolia* – plant	Horizontal subsurface flow constructed wetland system (IBU 0.1 mg L^{-1})	Sea sand disruption method	LC-QqQ-MS^2	[70]
IBU	x	–	Duckweed – plant	Grown in Murashige and Skoog medium (IBU 0.02–1 mg L^{-1})	Plant material ground in liquid nitrogen and extracted with sonication SPE with Oasis HLB	LC-FLD	[81]

IBU, NPX	x	x	*Arabidopsis thaliana* – plant, cell suspension	Cells grown in medium (drug concentrations 2 mg L^{-1})	Liquid extraction of freeze-dried samples SPE with Oasis HLB	UPLC-QqQ-MS^2 LC-TOF-MS	[28]
IPR	x	–	*Typha latifolia* – plant	Pots filled with perlite and nutrition solution (20 μM IPR)	Ultrasonication extraction of (under liquid nitrogen) ground samples SPE with Oasis HLB	LC-IT-MS^2	[85]
MET	x	–	*Typha latifolia* – plant	Pots filled with perlite and nutrition solution (MET 6.5–32.3 mg L^{-1})	Ultrasonic liquid extraction SPE with Bond Elut LMS	LC-IT-MS	[84]
OBZ	x	x	*Cyperus alternifolius* – plant	Hydroponic in nutrient solution (5–50 μM OBZ)	Liquid extraction of ground samples SPE with Oasis HLB	LC-IT-MS	[89]
OBZ	–	x	Horseradish – hairy root culture	Grown in Murashige and Skoog medium (100 μM OBZ)	Ultrasonication extraction of (under liquid nitrogen) ground samples SPE with Oasis HLB	LC-IT-MS^2 LC-TOF-MS	[62]
PAR	–	x	Cucumber, common bean, tomato, alfalfa, wheat – plant	Hydroponic in nutrient solution (PAR 0.06–5 mg L^{-1})	Liquid extraction of freeze-dried samples SPE with Oasis HLB	UPLC-QqQ-MS^2	[41]
PAR	–	x	Horseradish – hairy root culture	Grown in Murashige and Skoog media (1 mM PAR)	Samples ground in liquid nitrogen and extracted with liquid extraction SPE with Strata-X	LC-IT-MS^2	[36]
SDZ, SMR, SMX, SMZ, SPD	x	x	*Canna indica*, *Iris pseudacorus* – plant	Hydroponic in nutrition solution (drug concentrations 0.04–2.4 μM)	Liquid extraction and cleanup with graphitized carbon black and octadecylsilane-silica	LC-Qtrap-MS^2 UPLC-QTOF-MS	[83]

(continued)

Table 1 (continued)

Parent drug(s)	Metabolites		Plant material	Growing conditions	Extraction/cleanup/ enrichment	Analysis of extracts	Ref.
	Phase I	Phase II					
SMX	x	x	Cucumber – plant, *Arabidopsis thaliana* – cell suspension	Cells grown in medium (SMX 1 mg L^{-1}) Hydroponic (SMX 1 mg L^{-1})	Liquid extraction of freeze-dried samples SPE with Oasis HLB	UPLC-$QqQ\text{-}MS^2$ LC-TOF-MS	[42]
TCS	x	x	Freshwater algae – plant	Grown in culture medium	Liquid extraction with sonication	UPLC-$QqQ\text{-}MS^2$	[88]
TCS	x	x	Horseradish – hairy root culture	Grown in Murashige and Skoog culture medium (TCS 0.6 mg L^{-1})	Samples ground in liquid nitrogen and extracted with liquid extraction	UPLC-QTOF-MS	[56]
TCS	x	x	Carrot – plant, cell suspension	Grown in culture medium (TCS 1 mg L^{-1}) Carrot grown on soil (TCS 3 mg kg^{-1})	Liquid extraction of cells QuEChERS for carrots	GC-MS LC-QTrap-MS LC-$QTOF\text{-}MS^2$	[51]

17α-E2 17α-estradiol, *17β-E2* 17β-estradiol, *ALB* albendazole, *ATE* atenolol, *ATR* atorvastatin, *AVO* avobenzone, *BHT* butylated hydroxytoluene, *BMS* betamethasone dipropionate, *BPA* bisphenol A, *BZP* benzophenone, *CAF* caffeine, *CBZ* carbamazepine, *CDP* chlordiazepoxide, *CIP* ciprofloxacin, *CLA* clarithromycin, *CLI* clindamycin, *CLP* clomipramine, *CP* clonazepam, *DCF* diclofenac, *DP* diazepam, *E1* estrone, *EE2* ethinylestradiol, *ENR* enrofloxacin, *FAD* furaltadone, *FAZ* furazolidone, *FBZ* fenbendazole, *FL* fluvastatin, *FLD* fluorescence detector, *FLU* flubendazole, *FP* flurazepam, *FT-ICR* Fourier transform ion cyclotron resonance, *GC* gas chromatography, *GEM* gemfibrozil, *HCCA* α-cyano-4-hydroxycinnamic acid, *IBU* ibuprofen, *IPR* iopromide, *IT* ion trap, *KPF* ketoprofen, *LTG* lamotrigine, *MeBT* 5-methyl-1H-benzotriazole, *MePB* methylparaben, *MET* metformin, *MFA* mefenamic acid, *NDP* nordiazepam, *NFA* nitrofurantoin, *NFZ* nitrofurazone, *NPX* naproxen, *OBZ* oxybenzone, *OC* octocrylene, *OFL* ofloxacin, *OP* oxazepam, *OPL* 4-octylphenol, *OS* octisalate, *PAR* paracetamol, *PZE* phenazone, *SDM* sulfadimethoxine, *SDZ* sulfadiazine, *SER* sertraline, *SIM* simvastatin, *SMR* sulfamerazine, *SMT* sulfamethizole, *SMX* sulfamethoxazole, *SMZ* sulfamethazine, *SPD* sulfapyridine, *STZ* sulfathiazole, *TA* temazepam, *TCP* tris(2-chloroethyl)phosphate, *TCS* triclosan, *TEL* telmisartan, *TM* triazolam, *TMP* trimethoprim, *TRZ* trazodone

4.1 Carbamazepine

Carbamazepine (CBZ), a drug used for treatment of epilepsy and neuropathic pain, is one of the most thoroughly investigated substances regarding its uptake and metabolization in plants [25]. The studies published so far were focusing on plants with different intended usage. Those comprised edible plants such as cucumber [43], tomato [37, 38], lettuce [37, 45, 47], spinach [47], arugula [47], radish [47], and wheat [37], plants seen as potential candidates for phytotreatment (*Typha* spp.) [65], a fungus (*Pleurotus ostreatus*) [73], and cell cultures from horseradish [57]. Thereby, upon treatment with CBZ, apart from phase I metabolites formed by dehydration, hydroxylation, and subsequent epoxidation, also several metabolites belonging to phase II derived from conjugation of hydroxylated CBZ with amino acids and hexoses were found. From the manifold studies on the interaction of plants with CBZ, three stand out. One investigated synergistic effects when besides CBZ also lamotrigine was taken up by the plant. In this case, a reduced formation of dihydroxy-CBZ from the corresponding epoxide was observed due to inhibition of epoxide hydrolase, a finding corresponding with results from human pharmacokinetic studies [43]. Due to the increased experimental effort, not too many studies use plants cultivated in soil. Kodesova et al. investigated the uptake of CBZ (next to two other common drugs) from eleven topsoils and two sub-soils (whereby drug stability in the soil was also considered) [47].

The third study to be highlighted in this context investigated CBZ transformation products in *Armoracia rusticana* cell culture. Thereby the effect of endophytic rhizobacteria (*Rhizobium radiobacter* and *Diaphorobacter nitroreducens*) on the formation of these metabolites was examined [57]. The results from this work suggested that the reduction in CBZ concentrations could potentially be modulated by the endophytic community. In a more fundamental way, this issue was already researched in a study exploring the general potential of endophytic bacteria in phytoremediation [74].

4.2 Non-steroidal Anti-inflammatory Drugs

With sales revenues exceeding 13 billion dollars alone in the United States, non-steroidal anti-inflammatory drugs (NSAIDs) can be regarded as one of the most prevalent drug classes for use in humans. As a negative side effect, traces of NSAIDs are ubiquitous in the WWTP effluents and hence in the aquatic environment. This is also reflected in a particular scientific interest in the interaction of this group of pharmaceuticals with plants. Thereby the majority of studies involved the use of either diclofenac (DCF) [35, 40, 53, 58, 60, 75, 76] or ibuprofen (IBU) [28, 69, 77–81], the two most prominent representatives within the group of NSAIDs. To a lesser content, also naproxen [28, 35, 53], mefenamic acid [35, 53], and KPF [35, 53] were investigated with respect to their interaction with plants such

as *Arabidopsis thaliana* [28, 60, 79], *Lemna gibba* [77, 81], *Phragmites australis* [69], *Typha angustifolia/latifolia* [75], poplar [82] (the latter three plant types are commonly used in the realization of constructed wetlands), and the edible plants barley [58] and garden cress [35, 40, 53] upon uptake from water. In the following, a few selected papers on this topic will be discussed in detail.

The first report on the metabolization of DCF in plants was by Huber et al. [58]. In an experiment with barley, they could prove the formation of a phase I metabolite (hydroxylated DCF) in the plant that was subsequently transformed into a phase II metabolite by conjugation with glucose. This analytical work was based on HPLC with low-resolution MS (ion trap) whereby the formation of the glucose conjugate was further bolstered by enzymatic cleavage. In 2017 Fu et al. proposed a degradation/transformation pathway for DCF in *Arabidopsis* cells. The strategy employed to detect and further identify DCF-related metabolites was the use of data from HPLC-HR-MS, whereby the characteristic isotopic pattern resulting from the two chlorine atoms in DCF was employed as a first hint, and subsequently comparison with standard substances. This approach resulted in an unambiguous identification of a number of phase I + II metabolites [60]. A special asset of this work and a second one published by the same group [28] (focusing on IBU and naproxen) was that additional experiments with ^{14}C-labeled drugs allowed a judgment on the non-extractable phase III metabolites integrated in cell walls (a portion that is not considered in the majority of works within this field).

Mlynek et al. presented an interesting workflow with respect to the use of drift-tube ion-mobility (DT-IM) coupled to HR-MS for the detection of DCF-related metabolites in hydroponically grown garden cress (see Fig. 3) [40]. In this approach, drift times were recorded for the peaks eluting from HPLC, and, as a QTOF/MS instrument was used, all analytes were subsequently fragmented with low collision energies to yield significant fragments. This allowed to assign these signals to DCF-related drug metabolites (all in all 30 DCF metabolites formed in the plant could be identified).

As already stated in the previous section, to facilitate the detection and (at least tentative) identification of the drug metabolites formed within the plant, most studies employ relatively high concentrations of the parent drug (commonly in mg L^{-1} to high μg L^{-1} range). Nonetheless, in several papers the authors tried to approximate the drug concentrations used to those expected in real environmental waters. Examples for such a practice have been published by Emhofer et al. (employing DCF, NPX, KPF, and mefenamic acid at concentrations of 1 μg L^{-1} each) [35] or Di Baccio et al. [81] and He et al. [69] with IBU concentrations of 20 μg L^{-1} and 60 μg L^{-1}, respectively.

4.3 Antibiotics

Many antibiotics are applied for the treatment of humans as well as animals. For this reason they can enter the environment either via the sewage system or via the use of

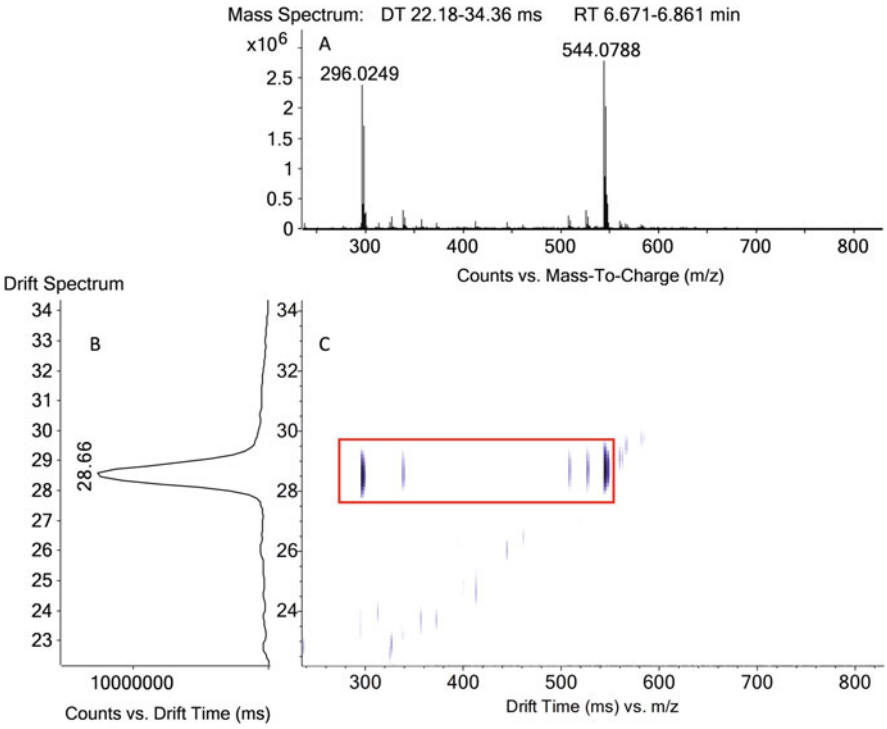

Fig. 3 Mass spectrum and drift-tube ion-mobility data for a peak observed in the chromatogram at 6.8 min in an onion sample. (**a**) shows the mass spectrum from 6.67 to 6.86 min, (**b**) the drift spectrum for the selected range of 22.2 to 34.4 ms, and (**c**) the corresponding drift time versus m/z plot. The peak at 6.8 min was identified as DCF-glucose-malonic acid. From [40] with permission

manure of treated animals as a fertilizer. Sulfonamides [42, 48, 83], fluoroquinolones [39, 50], clarithromycin [48], and clindamycin [39] were investigated with respect to their uptake and metabolization by plants. Studies published so far were looking for metabolites from ofloxacin and enrofloxacin as well as clindamycin and several sulfonamides in field-grown vegetables (in this case reclaimed water was used for irrigation) [39]. Thereby, although only a smaller number of metabolites could be detected, the authors achieved quantification in real vegetable samples. Further studies described the detection of a series of clarithromycin and sulfadiazine metabolites in lettuce [48], the investigations on the intraspecies variability of cabbage varieties with respect to accumulation and metabolization of ciprofloxacin [50], and metabolism of sulfonamides in cucumber [42] and wetland plants [83]. One specific outcome of the latter research was the proposal of metabolic pathways for several sulfonamides.

4.4 Other Drugs for Use in Humans

One of the more early examples for research focused on the formation of drug metabolites in plants after treatment with the parent drug (here PAR) is the detection of PAR-glutathione, PAR-cysteine, and PAR-glucose in *Armoracia rusticana* cell culture reported by Huber et al. [36]. In this work, the cell culture was incubated with an exceptionally high concentration of PAR (~160 mg L^{-1}), and extracts from the harvested cells were subsequently analyzed by HPLC coupled to a low-resolution mass spectrometer (ion trap). MS^2 experiments were used to acquire knowledge on the metabolite structures. To further support data from HPLC-MS analysis, enzymatic hydrolysis was performed revealing the cleavage of glucose from the PAR-glucoside. A further study on PAR metabolism by cucumber was published 10 years later by Sun et al. [41].

A major problem encountered in many of the studies devoted to identifying the metabolites formed in plants upon uptake of PPCPs is the lack of suitable standards, particularly when it comes to phase II metabolites. For this reason as much information as possible about the compounds detected should be gathered. Investigating the interaction of garden cress with three statins, Emhofer et al. introduced DT-IM QTOF/MS offering a further parameter (collision cross sections) for compound characterization [54]. Stable isotope labeling-assisted metabolite probing for the detection of metabolites formed within the plant was tested on the example of *Arabidopsis thaliana* treated with gemfibrozil [63]. The unique diagnostic pattern due to the use of 1:3 mixture of deuterated and non-deuterated gemfibrozil facilitated the spotting of 11 novel phase II drug-conjugates in plant extracts [63]. The workflow employed in that study is depicted in Fig. 4.

MS imaging (MSI) may be used to illustrate the tissue distribution of drugs as well as their metabolites with a plant. Vilette et al. presented an MSI approach for elucidating the spatial distribution of telmisartan and its metabolites (among other drugs) in *Salix alba* leaves [67]. A multi-analyte approach was chosen by Hurtado et al. when analyzing lettuce grown in the presence of a series of different contaminants [44]. Their approach involved the use of ß-glucosidase to cleave off glucose moieties from phase II metabolites derived from a variety of parent drugs and PCPs. Comparing the results from extracts treated with ß-glucosidase and untreated ones allowed to judge the degree of metabolization. Information about further studies on interactions of plants with caffeine [46], benzodiazepines [59], antidepressants [55], steroid estrogens [49], and metformin [84] may be extracted from Table 1.

The X-ray contrast agent iopromide was tested with *Typha latifolia* whereby a large number of mainly phase I metabolites were detected [85].

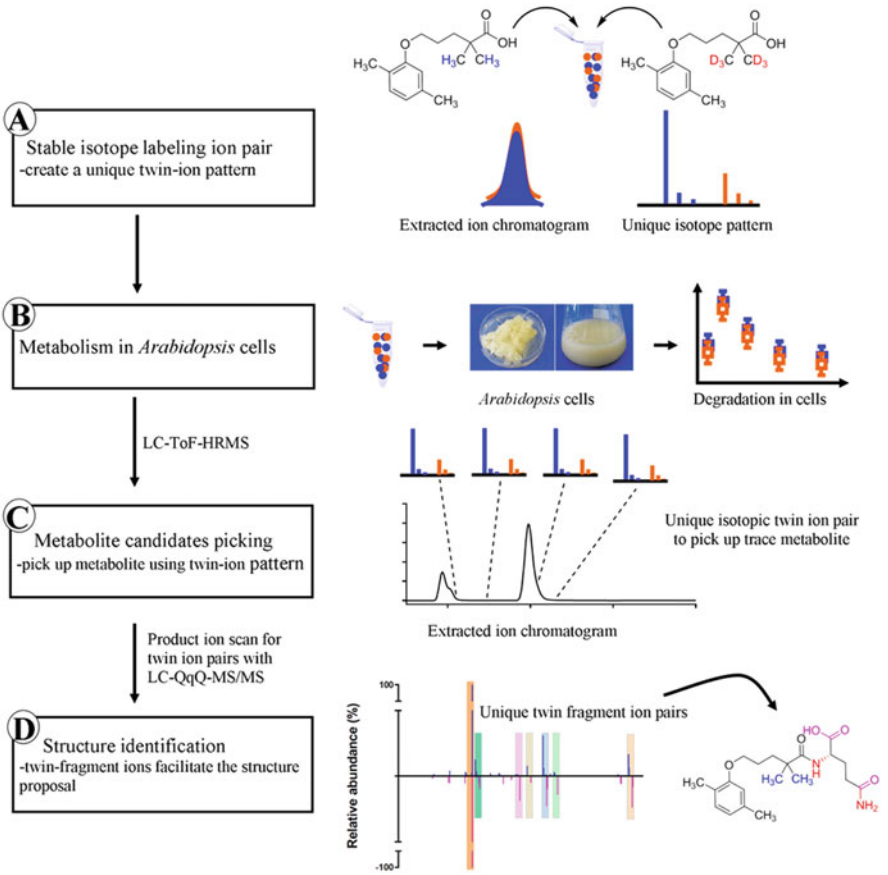

Fig. 4 Schematics of identifying unknown metabolites of gemfibrozil in *Arabidopsis* cells using stable isotope labeling coupled to high-resolution mass spectrometry. Main steps include (**a**) create a mixture of labeled and unlabeled gemfibrozil, (**b**) expose the gemfibrozil mixture to *Arabidopsis* cells, (**c**) identify metabolite candidates, and (**d**) elucidate structure of metabolites. Reprinted with permission from [63]. Copyright (2018) American Chemical Society

4.5 Drugs for Veterinary Use Only

Benzimidazole anthelmintics are frequently employed in the treatment of animals against their infestation with parasitic worms with the unwanted side effect that these substances are released to the environment and subsequently can interact with the agricultural system. Uptake and metabolization of anthelmintics have been investigated in *Campanula rotundifolia* [86] and *Plantago lanceolata* [61, 87] whereby experiments with whole plants allowed to propose schemes for metabolization of albendazole, fenbendazole, and flubendazole. As an example of the metabolic pathway of drugs in plants, the transformation of albendazole in *Plantago*

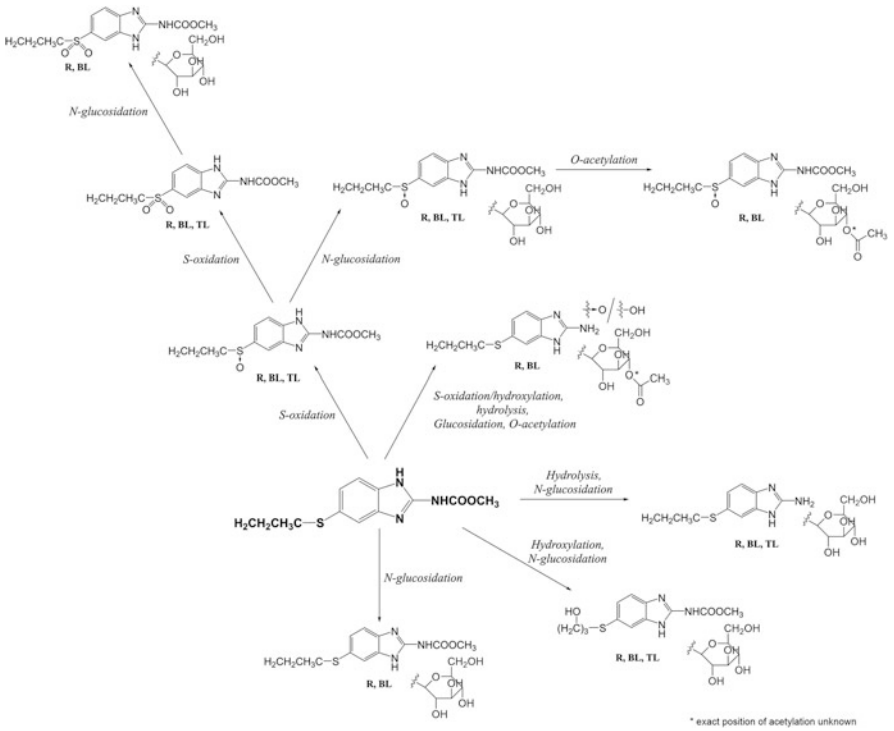

Fig. 5 The metabolic pathway of ABZ in roots (R), basal parts of leaves (BL), and tops of leaves (TL) from *Plantago* regenerants. For several metabolites more than one isomer was detected. Reproduced with modifications from [61] with permission

regenerants is depicted in Fig. 5. Unfortunately even substances banned in many developed countries such as nitrofuran can still represent a hazard as it is still used (on a legal or illegal basis) for animal production. Wang et al. investigated uptake and metabolism of nitrofuran in spring onion by in vitro (incubation of homogenized plant material) and in vivo (spring onion grown in soil) experiments [52].

4.6 Personal Care Products

From the quite substantial group of PCPs, several sub-groups have been studied with respect to their interaction with plants. In this chapter we included two groups of substances, the bacteriostatic agent triclosan in combination with freshwater algae [88], horseradish [56], and carrot [51] and a series of UV filters commonly employed in sunscreen agents and by this way introduced into the aquatic system. The latter include oxybenzone (investigated in combination with horseradish [62] and *Cyperus alternifolius* [89]) as well as avobenzone, octocrylene, and octisalate where uptake

and metabolism by *Cyperus alternifolius* and *Lemna gibba* [90] were investigated. As already discussed shortly, a targeted approach for metabolite detection is always somewhat biased, as unexpected conjugates might be overlooked. Macherius et al. provided a sophisticated workflow for the identification of triclosan-related plant metabolites in horseradish [56]. They recorded HR-MS spectra for both treated and untreated horseradish hairy root tissue, whereby abundant metabolites were simply detected by comparing base peak chromatograms from both series. For less abundant conjugates, they subjected the data to data filtering and multivariate statistics (principal component analysis). Another work worth mentioning employing novel instrumentation for metabolite detection and (at least tentative) identification was published by Seyer et al. [90]. Here the interaction of several UV filters commonly employed in sunscreen agents with two water-borne plants (*Lemna gibba* and *Cyperus alternifolius*) was investigated. The use of DTIM-HR-MS provided an increased amount of data available for metabolite structure proposal.

5 Conclusions and Perspectives

In particular within the last 10 years, interest in the uptake and metabolization of pharmaceuticals by plants has faced increasing interest, leading to a series of publications in this field. There are several driving forces for conducting such studies. The first category are basic studies, mainly devoted to the identification of metabolites originating from drugs or personal care products formed in plants, ideally allowing to propose a (tentative) metabolic pathway. Second, when investigating edible plants (where uptake of xenobiotics via the use of reclaimed waters for irrigation is of particular interest), drugs and their metabolites might affect their use for food and feed. Third, there is research focusing on the potential of certain plants (such as *Typha* spp. [65], *Typha angustifolia/latifolia* [80], *Phragmites australis* [69], or *Lemna gibba* [77]) for phytoremediation of contaminated land.

Regarding current and future trends related to this scientific field, there are a number of promising developments that either can already be seen or imagined for the years coming. Climate change and with it the need for employing reclaimed waters in agriculture are an increasingly important factor within many countries. As a consequence, plants used for production of food and feed might be affected by xenobiotics (and their metabolites) taken up from irrigation water. For this reason, there is a high probability that research in this field will face growing importance. Whereas the determination of the parent drugs in food and feed at the required trace level was already accomplished [16], for the corresponding metabolites, methods for their detection and even more their quantitation in edible plants definitely need to be substantially improved [25].

To achieve these improvements, advances need to be made in all analytical steps. These include new approaches for extraction and preconcentration of the metabolites of interest and finally optimization of HPLC-$MS^{(n)}$ analysis. The final goal of these efforts should be to achieve comparable standards as already accomplished in the

analysis of other environmentally relevant analytes such as pesticide residues. Although a range of metabolites formed in plants upon uptake of xenobiotics from the environment has already been identified according to the highest standards (level 1 of the scheme presented by Schymanski [68]), more standard substances should also be available for phase II metabolites, thereby allowing to move from tentative formulas and structures to unambiguously identified ones. Standards are also required for quantitative analysis, when following traditional workflows in HPLC-ESI-MS. Here new approaches allowing the standard-free quantification in HPLC-ESI-MS might be an alternative worth considering [70]. For a more comprehensive understanding of metabolism pathways in plants, improved knowledge about the exact location of metabolites within the plant is of utmost concern. Besides a move toward more miniaturized methods for sampling (i.e., analyzing smaller and smaller plant parts), the use of imaging techniques as demonstrated by Villette et al. might be the way to go [67].

Finally despite the fact that a range of pharmaceuticals has already been investigated with respect to their metabolization in plants, there is still a lot of work left for the future with important classes of pharmaceuticals still missing completely.

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Conclusions and Future Perspectives

Sandra Pérez, Serge Chiron, Damià Barceló, Nicola Montemurro, and Peter Eichhorn

Contents

Abstract During the course of this book, we have witnessed how wastewater represents an important agronomic resource especially in areas of the world affected by drought or where availability is usually limited. However, this practice raises many fears not only in the environmental field but especially in the case of food safety. In fact, wastewater represents the main source of diffusion of pharmaceutical residues (including degradation products and metabolites) in the aquatic and terrestrial environment. For assessing the occurrence of drugs and related substances, sensitive analytical methods have recently become available.

Keywords Agrosystems, Ecotoxicity, Human health, Metabolism, Vermiremediation

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1 Introduction

Pharmaceuticals constitute a structurally diverse class of mostly synthetic organic compounds that have been designed and optimized for their efficient and selective interaction with a specific macromolecular target whose modulation has been demonstrated to result in the desired pharmacological response in the treated organism. In case of drugs for the diagnosis, prevention, and treatment of medical conditions in humans, the ultimate goal of all research and development efforts in industrial settings is to deliver medicines with proven efficacy and safety for a given indication. Therefore, drugs receiving marketing authorization from the regulatory agencies have undergone extensive testing in clinical trials involving large number of patients to fully satisfy the two aforementioned essential requirements. As desirable as it may appear from an environmental perspective, pharmaceutical compounds are designed in the first place to improve the health of human beings according to the treatment plan with a given route of administration. In many cases this means that high metabolic stability in the human body is a desirable feature; only when the rate of biotransformation is low enough can systemic exposure at an acceptable dose be achieved. If drug metabolism is extensively studied at the drug discovery stage, including the elucidation of metabolic pathways and the identification of the enzymes responsible for each of the reactions, then these investigations aim at gaining a broad understanding of the clearance mechanisms as part of the overall characterization of the elimination routes and at assessing the potential of drug-drug interactions in clinical settings. Whether a drug is extensively metabolized or rather excreted in unaltered form from the human body is indeed an important characteristic of the compound, but directing its design toward one pathway or the other is not a goal per se. In other words, whatever the excretion profile looks like, drug safety in the narrow sense essentially ends once all drug-related material has been eliminated from the human body. At this very moment, though, it may become an environmental problem.

After more than two decades of extensive studies on the environmental occurrence, distribution and fate of drugs, and the generation of a huge body of literature on these topics, it is an undeniable matter of fact that the large majority of sewage-borne drugs are incompletely removed from the waste stream ultimately finding their way into the aquatic environment, or, if the treated wastewater is reused in agriculture for irrigation purposes, they can become soil contaminants.

2 Presence of Drugs in Soils and Their Remediation

Soil is an environmental compartment, which is exposed to PhACs residues through TWW reuse and acts as a sink facilitating their secondary transfer to others compartments (e.g., soil-living organisms and plants). More research is needed to quantify the current risk of PhACs in wastewater reuse systems, in particular

where multiple receptors are considered, such as exposure to wildlife and the soil microbial community. This new knowledge will enable the development of thresholds for safe reuse of wastewater. Earthworms are key organisms to evaluate soil quality and represent ideal sentinel organisms for assessing soil contamination, as they are in contact with soil and soil solution, tend to migrate over only short distances, and are widely distributed in soils around the globe. The possible role of *Lumbricus terrestris* involvement on reducing pesticides and pharmaceuticals toxicity has been demonstrated.

There are two general strategies for mitigation of PhACs in soil or their availability to crops. One is simply to decrease the concentrations of drugs before the reclaimed water is irrigated into agricultural soil. Since conventional WWTPs cannot remove most of the PhACs from domestic effluents, some advanced treatment technologies (e.g., ozone and adsorption on activated carbon) have been strongly recommended. However, these systems often require a high energy input and/or can produce undesirable by-products. The development of additional effective and low-cost treatments are needed to improve water quality. Constructed wetlands (CWs), as an eco-sustainable wastewater treatment system, have been attracted more attention especially in rural regions. However, the conventional CWs are insufficient as they are unable to remove PhACs efficiently. There is therefore an urgent need to develop more cost-effective CW systems to enhance the removal efficiency of different PhACs classes. Promising technologies are the microelectrolysis CWs, in which the electron transfer, microbial growth and metabolic enzyme activity, and biodegradation ability of microorganism can be promoted or bioaugmented CWs with endophytic microorganisms which can also help in the degradation of PhACs. The other strategy is to increase the degradation of PhACs in soil by involving a broad range of agricultural practices that have the potential to lower the amount of pharmaceuticals and/or their bioavailability but also by including possibility of on-site remediation. Amendment with fungi (e.g., *Trichoderma* spp.) or with adapted soil microorganisms to pharmaceutical biodegradation might be a wise choice for minimizing the level of PhACs in agricultural soil. The use of earthworms to clean biosolids and manure (ex situ vermiremediation) and to reduce pharmaceutical bioavailability to plants (in situ vermiremediation) has also been suggested. The impact of earthworms on soil physicochemical and biological properties together with the tolerance of these organisms to PhACs makes these bioremediation strategies viable in soils receiving pharmaceutical-contaminated amendments and water.

3 Presence, Uptake, and Metabolism of Drugs in Crops

From a quantitative perspective, it can be anticipated that the drug loads reaching wastewater treatment plants will increase in the near future owing to the aging population, demographic growth, expanding lifestyle diseases particularly in Western countries, and those adopting their bad habits while at the same time climate

change increases the pressure on freshwater resources in many regions where precipitations are scarce and subject to large seasonal and annual fluctuations. Concentrations of human pharmaceuticals and their metabolites are detected continuously in wastewaters. Since the use of treated wastewater to irrigate crops is getting widespread in regions where freshwater is limited, drugs are released in the agrosystems by wastewater irrigation. Then soils are exposed to drugs, and crops can take up and translocate them becoming this fact a food safety issue. Many of the reported plant uptake studies have been performed in hydroponic settings; the information on the fate of these compounds in soil-plant system is not always available. Only through field experiments can the actual potential uptake of drugs by crops be fully assessed and integrated into a database for risk assessment. The chemical uptake by plant roots in soil-root-leaves system depends largely on sorption and desorption of contaminants in soils and their physicochemical properties.

In general, organic chemicals with logP >4 are expected to have a high potential for root retention and low translocation capacity. Moderately hydrophobic compounds are most likely to be translocated by plants, as observed in previous research with the most translocatable compounds exhibiting a logP between 1 and 4. The molecular mass histogram demonstrates that translocatable compounds generally have a molecular mass of <350 Da, below Lipinski's cutoff of 500 Da. Hydrogen bond donor and acceptor histograms appear to have cutoffs around 4 and 7, respectively. Carbamazepine as a neutral compound is expected to show high mobility in plants because of its low molecular weight (236 Da), a moderate logP (2.8), and low hydrogen bonding capacity (1 donor and 1 acceptor). Other compounds that can potentially be translocated are expected to be identified by considering the balance between water solubility, passive membrane permeability, and size. Once they are translocated the compounds can covalently bound to vacuoles. There are a few studies evaluating the formation of non-extractable residues of drugs, the most informative experimental approach being the use of radiolabeled ^{14}C compounds. Recently, in a study using ^{14}C-labeled diclofenac and naproxen, a large fraction of these compounds was observed to be converted into not extractable material in plant tissues. There is the need to conduct more studies with radiolabeled drugs to facilitate quantitative assessment of covalent binding, in order to evaluate which compounds are most prone to be bound. At the last step prior to human consumption are studies to determine the relevance of the release of bound-residues upon cooking or other forms of food processing.

4 Toxicity in the Environment and Humans

Few studies have evaluated the exposure of drugs through the intake of vegetables. Some studies have demonstrated that health threat due to the consumption of vegetables irrigated with treated wastewater is low. However, it has been detected that in root crops for two compounds (lamotrigine and 10,11-epoxycarbamazepine) detected in carrot roots, carrot leaves, and sweet potato leaves, a health risk was

suggested. The level of toxicity of these compounds and other potential toxic drugs should be determined, after which regulation of maximum permissible levels in treated wastewater for irrigation could be established.

In the environment, the toxicity of drugs has been evaluated in soil biota. *Lumbricus terrestris* has appeared as a good alternative to the *Eisenia* spp. models in acute toxicity testing and as a good bioindicator in soil toxicity assessment. Research has primarily centered on evaluating the effects of veterinary pharmaceuticals (antibiotics) with the most common end-points considering survival, reproduction, and alterations in behavior. More research, characterizing the risk using a wider variety of pharmaceuticals under environmentally relevant exposure scenarios is urgently needed by incorporating "omic" disciplines as effective and reliable biomarkers, as well as recent technical advances in metabolite identification into toxicity evaluation. As far as phytotoxicity of PhACs is concerned, it is important to highlight that the majority of studies that have elucidated their effects on plants have used high exposure concentrations into a growth medium, which do not resemble in vivo situations occurring in the natural soil environment. More recently, research has started to explore the potential for sublethal effects or changes in key plant parameters at lower, environmentally relevant concentrations. One promising approach is the measurement of the alteration of plant hormones concentrations because these play an integral role in plant growth processes as well as in biotic and abiotic stress responses and because hormone-triggered changes may be the basis for more long-term visual phytotoxic responses. The assessment of the ecotoxicity of PhACs on soil microorganisms is lagging behind the risk assessment procedures for terrestrial macroorganisms, despite their well-documented pivotal role in ecosystem functioning. We are still missing several pieces of the puzzle including (1) a meaningful tiered approach, (2) well-defined experimental protocols, and (3) functional microbial groups, which can act as bioindicators. For pesticides regulation, key functional microbial groups were identified as potential microbial indicators such as arbuscular mycorrhizal fungi (AMF) due to their key role in plant nutrition and ammonia-oxidizing microorganisms (AOM) due to their involvement in the cycling of nitrogen. However, similar work has not been conducted for PhACs yet.

5 Methods for the Analysis of PhACs in Soil-Crops Systems

In the past, research has focused on the analysis and determination of PhACs in particular in waste or surface water. Only recently, due to the growing demand for water in the agricultural sector, the attention of the scientific community has started to shift to the effects of emerging contaminants in soil and plants. Therefore, the big challenge is to identify chemicals that could potentially have harmful effects on human health, terrestrial and aquatic organisms, and ecosystems.

The main problem concerns the search for more efficient and more accurate analytical methods, as well as the need to obtain reliable analytical information on a greater number of known chemical compounds that may be present in the tested

samples. Indeed, determining the presence and quantification of pharmaceutical residues in soil and plant tissues requires significant effort. Therefore, rapid progress and improvements in instrumentation over the last decade have led to the development of new analytical procedures and control measurement tools that allow the detection, identification, and quantification of an ever wider range of analytes at an ever lower content level in samples characterized by a complex and variable matrix such as soil or plant tissues. In these procedures, improvements have been made in the detection, separation, identification, and quantification steps of the widest possible spectrum of analytes for a careful assessment of the state of the environment and the life of aquatic and terrestrial organisms, as well as the impact on human health.

These improvements, which can be used for the detection and quantification of analytes, can include, for example, ecological extraction procedures thanks to the use of alternative and more environmentally friendly solvents. New solutions can also be represented by the introduction of simplified analytical protocols for the preparation of samples and extraction processes, or by the application of miniaturized techniques that allow you to work on small quantities of sample, or by the introduction of new sorbents for the cleanup of the matrix.

High-resolution mass spectrometry (HR-MS) also has many unexplored potentials for studying the effects of drugs in soil or plants. In fact, its use together with mathematical and chemometric models allows the development of methodologies for the evaluation of the presence of thousands of unknown compounds or for the study of metabolism through the retrospective analysis of previously acquired data.

Finally, in the near future, it will be possible to use direct analytical techniques which allow to eliminate the treatment of the sample and which can be used for the direct detection and/or determination of analytes, limiting the loss of the analytes of interest due to the strong processes: extraction. This approach is particularly interesting because no sample preparation is necessary for the analysis of the tested material.

A last wish would be to soon establish limits of xenobiotic contaminants in the water reused in agriculture, as well as we should think of standardized analytical methods as in the case of pesticides.

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