Chapter 12
Bacterial Bioreporter Applications in Ecotoxicology: Concepts and Practical Approach

Sulivan Jouanneau, Marie-José Durand, Ali Assaf, Marine Bittel and Gérald Thouand

Abstract Bioreporters are widespread in the ecotoxicological field, and they are used in concert with a range of physico-chemical methods for environmental characterization. These biological methods allow the assessment of other parameters that are not otherwise accessible. Among the broad diversity of available bioreporters, bacterial approaches are particularly interesting for their simple implementation, low cost and timeliness of their response (because of their metabolic kinetics and their growth rate). In this chapter, we are interested in the primary proposed strategies from one initial assumption: the use of one bioreporter to test one parameter. This strategy rapidly reached its limits (for lack of specificity or representativity), thus opening the way for other biological approaches that were more reliable but also more complex (implemented technology and data treatment).

Keywords Bacterial bioreporter · Overall parameters · Specific detection · Environmental monitoring · Ecotoxicology · Approach limits

12.1 Introduction

The physico-chemical approaches, used to monitor the environmental quality, are particularly specific and accurate when the parameters in question are known (for example, the quantification of a specific chemical in river water). In the environment, the diversity and concentration of chemicals are highly variable from one sample to another. Consequently, physico-chemical methods are quickly limited to characterizing the relevant complexity of a sample (Fig. 12.1). Methods based on bioreporters open the vision field to new metrological possibilities in this context.

S. Jouanneau (✉) · M.-J. Durand · A. Assaf · M. Bittel · G. Thouand
University of Nantes, UMR CNRS GEPEA 6144, 18 Bd Gaston Defferre, 85000 La Roche sur Yon, France
e-mail: Sulivan.jouanneau@univ-nantes.fr

M. Bittel
TRONICO Vigicell, 18 Bd Gaston Defferre, 85000 La Roche sur Yon, France

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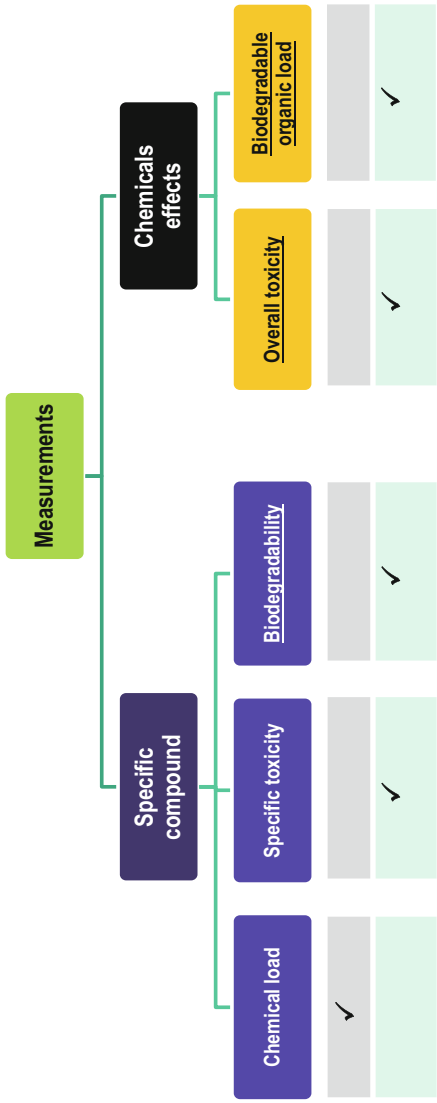


Fig. 12.1 Metrological complementarity of the physico-chemical and biological methods

They allow the improved characterization of the toxic potential of samples via several biological parameters such as the bioavailable concentration and the environmental persistence of specific chemicals or the overall toxicity of the study sample.

Nevertheless, according to Bartell (Bartell 2006; Burger and Gochfeld 2001), a biological indicator must meet certain attributes such as biological relevance (low natural variability, measurable biological signal, and having a response that is attributable to specific stress conditions), methodological relevance (measurement reliability, low cost, and straightforward data interpretation), and societal relevance (social demand).

Among the wide diversity of available natural bioreporters, bacteria fulfill the required specifications to be relevant biological indicators. These microbial reporters have been the subject of a large number of publications over the last decade (Su et al. 2011; Sun et al. 2015; Ponomareva et al. 2011; Dai and Choi 2013). However, the list of potential targets has remained limited (primarily in terms of overall parameters). In addition, to enlarge the detection range of these natural bioreporters, scientists can employ genetic engineering to produce new bacterial strains to detect some specific parameters (Park et al. 2013; Lei et al. 2006; Durand et al. 2003; Ivask et al. 2009; Hakkila et al. 2004; Charrier et al. 2011; Gu and Gil 2001).

In this chapter, the primary strategies proposed in the context of environmental monitoring and their respective limits are detailed point-by-point.

12.2 The Dream of Using "One Cell to Measure One Parameter": The Monoparametric Approach

As shown above, the primary objective was to develop methodological alternatives based on bacterial cells that could be complementary to physico-chemical monitoring methods. The initial ambition was to propose relevant bioreporters in this context (natural ones or those obtained by genetic engineering). For that purpose, the first strategy under consideration (as detailed in the following paragraphs) was to implement bioreporters that could provide a specific response. In other words, one bacterial strain was identified or developed to measure one analyte or one parameter (for measurements of the overall toxicity from the environmental persistence or the bioavailable concentration).

12.2.1 Detection of an Overall Parameter

One of the best-known microbial bioreporters is the natural bioluminescent bacterial strain *Aliivibrio fischeri* (formerly known as *Vibrio fischeri*). This strain is implemented in a bioassay to allow the assessment of water toxicity (for overall

acute toxicity measurement). This strategy was incorporated into an international standard (ISO 11348) and commercialized into kits under the trade denominations Microtox® (Modern Water, UK), ToxAlert® (Merck, Germany), BioTox™ (Aboatox, Finland) or LUMIStox™ (Hach Lange, US) (Jennings et al. 2001; Turner et al. 2010). The underlying principle consists of the exposure, over a short time period, of bioluminescent cells to a test sample. According to the inhibition level of the biological signal, it is possible to deduce the acute toxicity of the sample. The simplicity and robustness of this method have contributed greatly to its democratization. An "online version" of this bioassay for field applications has also been proposed by Microlan (iTox-Toxcontrol).

This assessment strategy (monitoring the inhibition of a biological signal) was enlarged to the other natural bioluminescent strains (Peinado et al. 2002) as well as some constitutive bioreporters (Peinado et al. 2002; Chang et al. 2004) (i.e., the biological signal is not controlled by induction or repression). In this latter case, the biological signal is not always bioluminescent but can be fluorescent or colorimetric.

To assess chronic overall toxicity, the principle is similar to that of an acute bioassay, but the exposure duration is significantly increased. The primary parameter that is monitored in this case is relative to the cellular growth rate; several indicators can be used, such as the cellular density (Radix et al. 2000; Gellert et al. 1999), as described in the ISO 10712:1995 standard (for a bioassay based on the growth inhibition of *Pseudomonas putida*), or the bioluminescence (Gellert 2000; Menz et al. 2013).

Within this global framework of environmental monitoring, another overall parameter was studied, namely the biodegradable organic load. The primary bioassay that was developed to assess this parameter is detailed in ISO 5815 standard (ISO 2003a, b) (the measurement of the biochemical oxygen demand-BOD). The principle underlying this rudimentary method (Great Britain 1908) consists of exposing a water sample (carbon sources) to an unknown environmental inoculum over a 5-day period under controlled conditions (in terms of temperature and a dark room) and with a limited concentration of dissolved oxygen (saturation concentration in water). The monitoring of the oxygen consumption (as a marker of metabolic activity) during this period allows the quantification of biodegradation activity by microorganisms, and consequently, to assess the biodegradable organic load in the sample under analysis. The primary limit of this approach concerns the environmental diversity of the inoculum, which inevitably induces significant variability between assays (Jouanneau et al. 2014).

Many studies have proposed alternative methods to allow this problematic limit, and for that reason, the analytical strategies are based on the implementation of only one biological indicator in most cases (Jouanneau et al. 2014; Yoshida et al. 2001; Chee et al. 2005; Raud et al. 2012). Other markers of metabolic activity were proposed to replace oxygen consumption (the limiting factor of the reference method), such as mediator redox (Yoshida et al. 2001; Pasco et al. 2004; Dudal et al. 2006).

The ease-of-use of these methods, both at the implementation and data analysis levels, has greatly contributed to the deployment of these analytical approaches

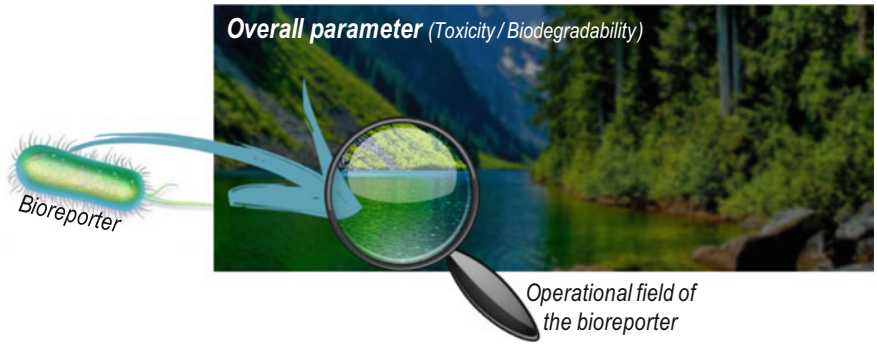


Fig. 12.2 Lack of inherent representativity of a unique bioreporter in a monitoring context of an overall parameter

within the scientific community in addition to physico-chemical methods. Over the two last decades, many publications have been dedicated to this topic (Su et al. 2011; Lei et al. 2006; Ivask et al. 2009; D'Souza 2001).

In both cases (assessment of overall toxicity and environmental persistence), mono-parametric (only one bioreporter) approaches have led to the same metrological limitations, namely, a lack of representativity of the provided biological information. The field of view is relative to the selected biological indicator (biological model) and is consequently too narrow to be easily extrapolated to another organism. As a result, the operational domain is restricted by the intrinsic properties of bioreporters such as the metabolic abilities required to assimilate some organic compounds (environmental persistence) or their natural robustness in resisting environmental stress (Fig. 12.2).

12.2.2 Measurement of Specific Compounds

This strategy was also deployed for the detection and/or quantification of specific targets (e.g., specific toxicity, chemicals). In this case, the metrological strategy was based on the implementation of genetically engineered bioreporters, which were modified to acquire some specific detection properties (Durand et al. 2003; Ivask et al. 2007, 2009; Hakkila et al. 2004; Charrier et al. 2011; Belkin 2003, 2006; Gueune et al. 2009; Roda et al. 2011; King et al. 1990).

Two methodologies could be used to obtain specific bacterial bioreporters, as depicted in Fig. 12.3. First, the bacterial genes involved in the degradation pathway of an organic substance or in the mechanism of resistance against metal compounds are described; in this case, reporter genes (*lux CDABE*, the *luc* gene from fireflies or *gfp* genes) are inserted downstream from a promoter of these known genes. In the second case, the reporter genes are inserted randomly within the chromosome of the

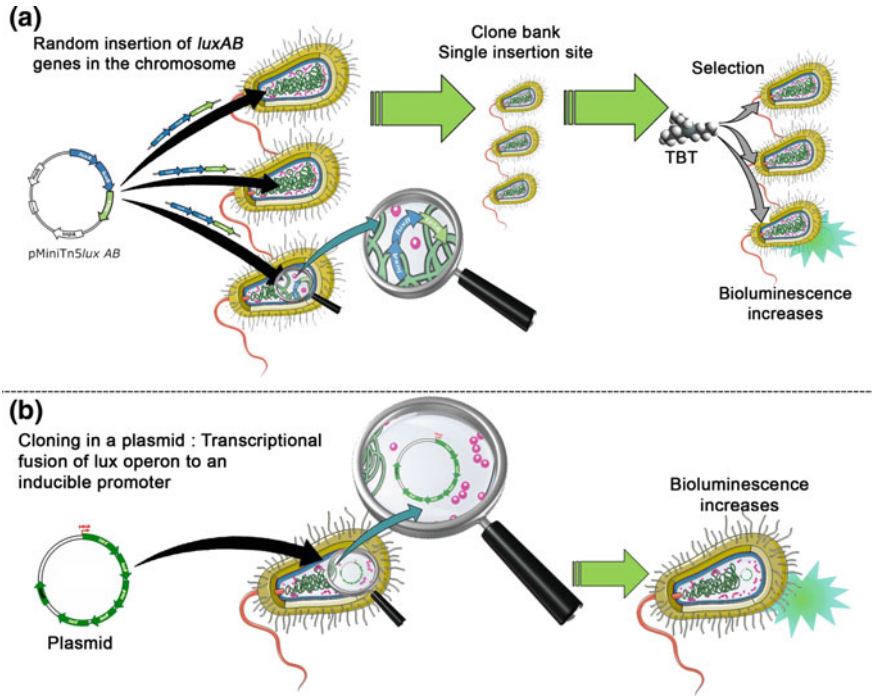


Fig. 12.3 Strategies for designing specific recombinant bacteria. **a** The luciferase genes (*luxAB*) are randomly inserted into the chromosome of *Escherichia coli*. Each clone from the bank is tested against a particular compound (TBT, or tributyltin) and selected when bioluminescence is induced. Decanal (aldehyde) must be added to the medium when *luxAB* genes are used instead of *luxCDABE*. **b** The reporter genes (*lux, luc,* and *gfp*) are inserted downstream of the promoter of a known gene (in *red*). When the bacteria are exposed to the analyte (M), the bioluminescence increases

bacteria (*Escherichia coli*, in most cases) to obtain a clone bank. Each clone is tested against a substance and selected when bioluminescence increases.

12.2.2.1 Example of Specific Strains for Organometallic or Organic Compound Analysis

A recombinant *E. coli* for sensing organometallic compounds has been obtained by using a strategy that was developed by Guzzo and Dubow (Guzzo et al. 1991) for identifying uncharacterized *E. coli* genes. A unique insertion of the *luxAB* genes was made in the *E. coli* chromosome to obtain a library, and the library was screened for bioluminescence in the presence and absence of organotin (Fig. 12.4). One clone called TBT3 was selected, and it displayed augmented luminescence in a dose-dependent manner upon exposure to tributyltin (TBT). Its sensitivity to TBT was 0.08 µM (26 µg/L) and 0.1 nM (0.03 µg/L) for dibutyltin (DBT, one of the

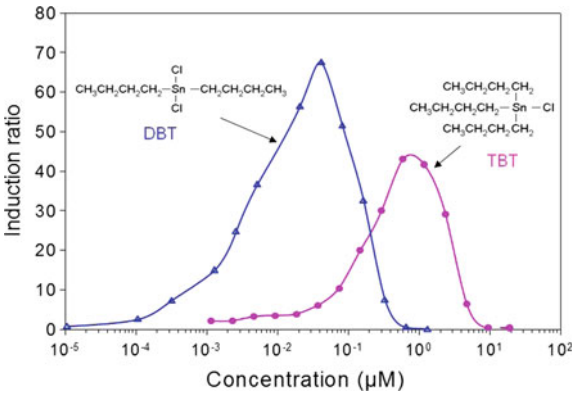


Fig. 12.4 Detection of tributyltin and dibutyltin in sea water with the TBT3 strain (Durand et al. 2003). Induction radio: The bioluminescence of TBT3 was recorded after 1 h of incubation with the compound/background bioluminescence of TBT3

degradation products of TBT), and this strain was not induced by other organotin or other compounds (Durand et al. 2003; Gueune et al. 2008, 2009). A simple bioassay for detecting TBT in paint and wastewater from shipyards has been developed (Gueune et al. 2009).

Organic compounds could be detected with recombinant bacteria by cloning the gene involved in the degradation pathway upstream of the reporter genes. Benzene and atrazine detection will be used as examples.

The TOL plasmid of *Pseudomonas putida* contains the genes of enzymes that are involved in degrading benzene and its derivatives. These genes have been used to build a plasmid with the luciferase gene in fireflies (pTSN316). An *E. coli* H10 that was transformed with this plasmid produces luminescence in the presence of aromatic compounds (Nakazawa et al. 1980). Nevertheless, applications of this bacterium are limited because of the accumulation of toxic derivatives in the cell for benzene concentrations higher than 0.5 ppm (Berno et al. 2004).

The development of recombinant strains for organic pollutant detection requires a good knowledge of the genes that are involved in the degradation pathway. Few of these constructions have been dedicated to field applications for the monitoring of BTEX (Benzene, Toluene, Ethylbenzene, and Xylenes), toluene or naphthalene (Xu et al. 2013; Fernández-Piñas et al. 2014).

The s-triazine family of herbicides has been used intensively in Europe and is still applied in some countries. These herbicides have various toxic effects ranging from aquatic organisms to humans as well as a low degradation rate leading to the accumulation of cyanuric acid compounds, which are common byproducts of s-triazine. Triazine monitoring in the environment is performed by chemical analysis or immunoassay. Nevertheless, Hua et al. (2015) proposed a bacterial bioreporter to monitor atrazine and cyanuric acid. They used the plasmid p-ADP-1, which is borne by *Pseudomonas* sp. and is involved in the atrazine degradation pathway for the genetic construction of recombinant *E. coli*. Atrazine degradation is encoded by *atz* genes, which are divided into two sets of (i) the three constitutive genes *atzA*, *atzB* and *atzC*, which are involved in the degradation of atrazine in cyanuric acid compounds, and (ii) the mineralization of cyanuric acid, which

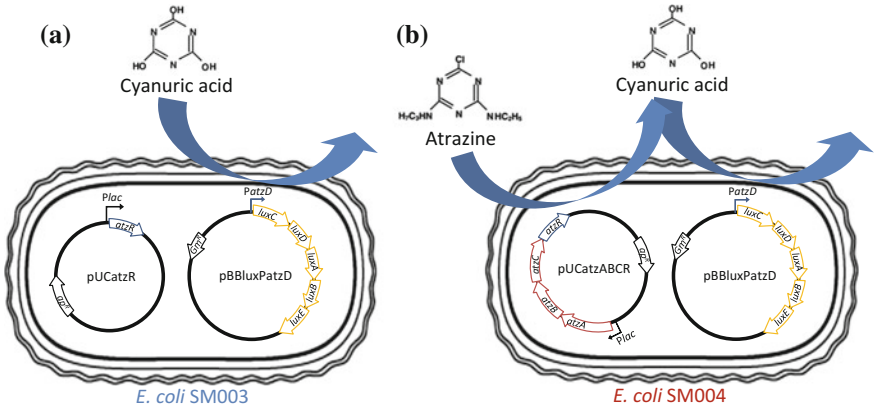


Fig. 12.5 Bioreporter designed to specifically detect cyanuric acid and atrazine (Hua et al. 2015)

requires the inducible *atzDEF* operon, the expression of which is dependent on its regulator *atzR*. Because the first set (i) of genes is constitutively expressed, the promoter cannot be used as a bioreporter. The strategy was to create two complementary bioluminescent strains, with one induced only by cyanuric acid (Fig. 12.5a) and the other for atrazine detection after its degradation in cyanuric acid (Hua et al. 2015) (Fig. 12.5b). The detection range reported for atrazine was from 0.22 to 15 μM and from 7.83 to 2.89 mM for cyanuric acid. Nevertheless, field applications are still in development.

12.2.2.2 Bacteria for the Detection of Inorganic Compounds (Metal Trace Elements, or MTE)

The construction of a metal bioreporter requires knowledge of MTE regulation by the cells. The resistance mechanisms of non-essential MTE efflux pumps are effective for exporting MTE to the outside of the cell, and these transporters could be encoded chromosomally or by mobile genetic elements (Ma et al. 2009). The genes involved in these mechanisms are used to construct recombinant strains for MTE detection.

Approximately thirty bioluminescent bacteria have been described in the literature, and generally, the promoters of genes involved in MTE resistance are cloned upstream of reporter gene(s), in a low copy plasmid inside a host strain (*E. coli*, *Pseudomonas* sp., *Bacillus subtilis*, *Staphylococcus aureus*, and others). Many studies have been performed with standard metal solutions, and the induction of strains was dose-responsive after an incubation period of 60–180 min. Some of these recombinant strains are very sensitive, for example, to construction with the *merR* promoter for which the detection limits reported for Hg^{2+} ranged from 0.03 to 0.003 μg/L (Durand et al. 2015).

The experimental procedure for monitoring MTE detection by recombinant bacteria are usually conducted with batch culture cells or freeze-dried cells, or they are included in a biosensor. Applications of environmental samples with bioluminescent bacteria (bioassay or biosensors) have been intensively reviewed in recent years, and most of them concern wastewater or leachate from polluted soil (Xu et al. 2013; Durand et al. 2015; Eltzov and Marks 2011; Checa et al. 2012). For example, one application of bacterial bioreporters concerns the monitoring of arsenic in water samples. Arsenic is an abundant element in Earth's crust, and its concentration could be enhanced by anthropogenic activities or by hydrogeological conditions, leading to the serious contamination of groundwater in some parts of the world. Because of its high toxicity, the WHO (World Health Organization) has recommended a 10 µg/L limit for inorganic arsenic in drinking water. Nevertheless, despite the use of water treatment systems and the available chemical analysis, the contamination of drinking water in developing countries (Bangladesh, Laos, Cambodia and Vietnam) has remained a problem because of the high cost of these techniques. It could be useful to monitor arsenic alternative methods based on bacterial bioreporters (Kaur et al. 2015; Merulla et al. 2013). More than 20 bacteria that were engineered for arsenic detection with different reporter genes have been created over the last 15 years because their resistance system is well-characterized (Diesel et al. 2009). Resistance against arsenic is provided by the *ars* operon, which is found on the plasmid or in the chromosome (the *arsRDABC* operon for *E. coli*), and the expression of this operon is controlled by ArsR protein. In the absence of arsenic, ArsR is bound to a specific DNA sequence (operator) and represses the transcription of defense genes (*arsD, arsC*, *arsA* and *arsD*). In the presence of arsenic, ArsR loses its affinity for the operator, and *arsDCAB* genes are transcribed. The bioreporter for arsenic detection bears a plasmid with a second copy of the operator-promotor sequence of ArsR that is transcriptionally fused with genes for a reporter protein (luciferase, GFP, β-galactosidase, or others). When arsenic enters bioreporter cells and binds to ArsR, the *arsR* promotor is derepressed, leading to the transcription of reporter genes.

Siegfried et al. (2012). have developed a bioassay for field applications, and validations of this assay have been performed in Bangladesh under realistic conditions; more than 2000 bioreporter analyses have been conducted. A cross analysis with 24 groundwater samples by ICP-MS and the bioassay kit are consistent for arsenic concentrations below 150 µg/l and more than 10 µg/L. Because of its low cost, this bioassay is a useful tool for developing countries to monitor arsenic.

12.2.2.3 Limits of These Strategies for Monitoring a Specific Parameter

One of the primary limits of using bioreporters to monitor one specific compound is their lack of specificity. None of these reporters are specific to one substance, even for organic substances, because the genes involved in the degradation pathway or in MTE resistance could be induced by more than one substance (Fig. 12.6).

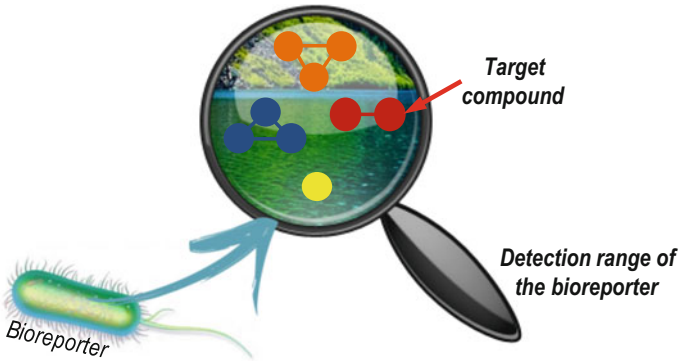


Fig. 12.6 Primary limit (lack of specificity) of approaches that are based on an unique biological indicator

Several tactics were proposed to increase the performance of the MTE molecular manipulation detection (Yagur-Kroll and Belkin 2014). Hynninen et al. (2010) reported an improvement of the detection threshold consecutive to an altering of the metal efflux system of *Pseudomonas putida*-based Cd/Zn/Pb-bioreporters (genetic modification of four metal efflux transporter genes). These metabolic changes cause intracellular accumulation of MTE enhancing, at the same time, cellular sensitivity. Another strategy to enhance sensitivity as developed by Merulla and Van der Meer (2016) was to decrease the background level of the strains. These investigators used the ArsR-operator system after adding a second copy of the operator for ArsR. A decrease in the reporter gene expression is observed in the absence of arsenite, but inducible control in the presence of effectors is preserved.

Improving the specificity of the strain remained difficult, so the identification of MTE in the environmental sample could be performed by cross-detecting a panel of the strain. This strategy has been applied by Jouanneau et al. (2011) (see Sect. 12.3.2). The concept was extended both for the assessment of overall parameters and for the measurement of specific chemicals.

12.3 The Multicellular Approach to Measuring One Parameter

Despite a clear benefit relative to the implementation of these mono-parametric approaches (ease of use and simplified interpretation of biological data), these latter approaches have important bottlenecks (limited representativity and lack of specificity) that significantly restrict their potential development and/or commercialization (except in the case of the bioassay based on bioluminescent bacteria, as in ISO standard 11348).

To circumvent these limitations, a strategic evolution was proposed that consisted in a multiplication of the number of bioreporters used by the parameters. The primary aim was to improve the quality of the selected biological information. This concept was extended for both the assessment of the overall parameters and for the measurement of specific chemicals.

12.3.1 Assessment of Overall Parameters

To make up for the absence of representativity in the mono-parametric strategy as induced by the limited natural properties of the implemented bioreporters, a second-generation strategy was proposed. This latter strategy is based on the implementation of several biological reporters and permits improvements in the metrological mesh (Fig. 12.7).

12.3.1.1 Microbial Assay for Overall Toxicity Assessment

In accordance with this concept, two multi-parametric assays were listed in the literature for applications to the field of toxicity assessment. These bioassays are commercialized by the NCIMB Company (UK) under the trade name MARA and LumiMARA. The MARA assay is based on the growth inhibition (chronic test; 18-h duration) of 11 microbial strains (*Microbacterium* sp., *Brevundimonas diminuta, Citrobacter freundii, Comamonas testosteroni, Enterococcus casseliflavus, Delftia acidovorans, Kurthia gibsonii, Staphylococcus warnerii, Pseudomonas aurantiaca, Serratia rubidaea,* and *Pichia anomala* yeast) (Fai and Grant 2010; Wadhia 2008; Wadhia et al. 2007) (Fig. 12.8).

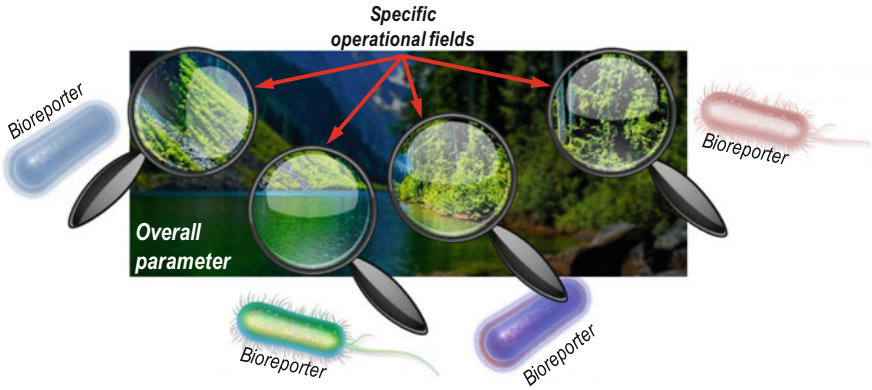


Fig. 12.7 Methodological improvements were implemented to increase the representativity of bioreporter information

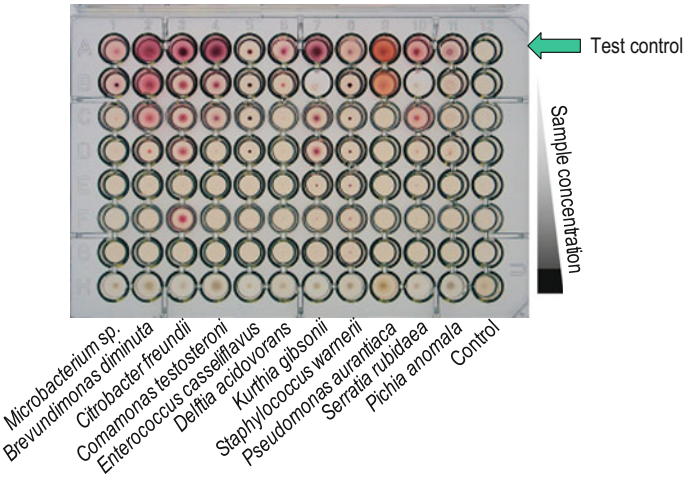


Fig. 12.8 Implementation of a MARA assay using a chemical

Table 12.1 Bioluminescent strains implemented in the LumiMARA assay

LumiMARA strains	Reference
Aliivibrio fischeri	NCIMB 30268
Aliivibrio fischeri	NCIMB 30274
Photobacterium leiognathi	NCIMB 30266
Photobacterium leiognathi	NCIMB 30269
Photobacterium phosphoreum	NCIMB 30267
Photobacterium phosphoreum	NCIMB 30270
Photobacterium phosphoreum	NCIMB 30271
Photorhabdus asymbiotica	NCIMB 30276
Photorhabdus luminescens	NCIMB 30275
Vibrio harveyi	NCIMB 30272
Vibrio harveyi	NCIMB 30273

The toxic effect is highlighted by assessing of the bioluminescence inhibition emitted by cells

The inhibition of the growth of the micro-organisms is measured well-by-well by assessing of the intensity the redox dye (2,3,5-Triphenyl-tetrazolium chloride—red dye—marker of metabolic activity).

The LumiMARA assay resembles the bioluminescence tests described in Sect. 12.2.1, but it is based on a set of 11 bacterial bioreporters (Table 12.1). It is dedicated to the assessment of acute toxicity (for exposure durations of 15–30 min) (Jung et al. 2015).

Conceptually, these strategies allow the enlargement of metrological points of view by comparing them with mono-parametric approaches (for a better representativity of the overall toxicity). Nevertheless, a technological obstacle persists in

relation to interpretations of the biological information. In fact, each bioreporter provides individual data without overall integration, which should result in an easy reading. In other words, these assays look more like a compilation of several biological measurements than an overall approach to toxicity.

The strategy described below proposes to remove this limit by implementing a data analysis algorithm.

12.3.1.2 Biodegradable Organic Matter Detection by Using a Bacterial Set

As explained in Sect. 12.2.1, the most common method (ISO 5815) for assessing biodegradable organic matter is based on the implementation of environmental inocula, which can be highly variable, and at its origin, it can have a significant lack of methodological robustness. The mono-parametric approaches have reached their limits, and thus it was pertinent to consider other methodological paths based on several bioreporters. In addition, to address the lack of representativity in the mono-parametric approaches (cf. Sect. 12.2.1), or, conversely, the strong variability generated by the wide diversity of inocula, the use of several biological reporters was considered. The primary difficulty is to determine the size of the bacterial set.

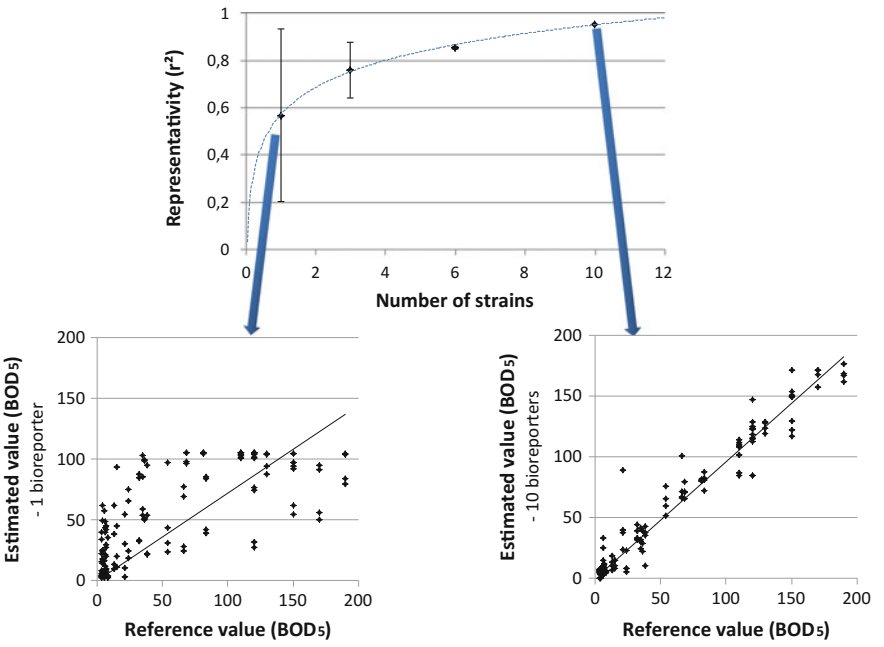


Fig. 12.9 Representativity of biological information (according to the number of implemented strains) in comparison with the reference method (from experimental data from work in progress)

Indeed, an insufficient number of strains risks generating similar limitations as those found with the mono-parametric approach (the lack of representativity), but too many strains can increase the assay costs without a significant gain of the representativity (Fig. 12.9).

Two pathways are described in the literature. First, the different biological indicators are mixed to form an artificial inoculum (Catterall et al. 2003). That approach allows the representativity of the given measurement to increase while ensuring an easy reading of the biological information (the bacterial inoculum provides only one piece of global information). This strategy, which is simple to implement, still poses some limits, such as the control of the cellular proportions (the fraction of each strain in the inoculum) during the assays, which can induce a significant level of variability.

The second strategy consists in using several strains separately (Raud and Kikas 2013) (Jouanneau et al. work in progress). The individual control of each bacterial strain over the entire analysis duration leads to a clear improvement in the reproducibility of results. However, each strain provides specific information; in others words, each biological datum is a component of the global parameter. Consequently, a supplementary step is required to analyze the biological data. To that end, complex statistical tools for data mining are needed (decision trees, neural networks, etc.). These empirical models are designed from an existing database consisting of biological information that is obtained from known samples (and characterized according to the reference method).

12.3.2 Specific Detection of Metals

As widely shown in the past (cf. Sect. 12.2.2.3), the primary limitation of approaches based on only one bioreporter is the lack of specificity. To illustrate this limit, Table 12.2 shows MTE detection by different bioluminescent strains that were transformed with a plasmid containing the bioluminescence genes *luxCDABE* from *Aliivibrio fischeri* under the control of heavy metal-inducible promoters. With the exception of some strains (Durand et al. 2003; Hua et al. 2015), it is not rare when one "specific" strain really detects several chemical targets (Ivask et al. 2009; Jouanneau et al. 2011).

To overcome this problem, a multi-parametric strategy was proposed on the basis of an analysis of crossed data from several bioreporters (Jouanneau et al. 2011; Elad et al. 2008). Contrary to the overall approach, the aim is not to improve the representativity but to increase the specificity of analytical strategies, as shown in Fig. 12.10.

The example that was proposed to illustrate this strategy is based on the works of Jouanneau et al. (2011), which were dedicated to the detection and quantification of four potentially mixed metals in environmental samples. The heart of the strategy is based on five bioluminescent bioreporters (genetic engineering), each of which had a specific plasmid (four inducible plasmids and one constitutive one). The lack of

Table 12.2 Detection of MTE with various strains of recombinant *E. coli*

Metals	Detection limits (μM)					
Ag	*E. coli* K12MG1655 pBarslux[a]	*E. coli* K12MG1655 pBcoplux[a]	*E. coli* K12MG1655 pBmerlux[a]	*E. coli* K12MG1655 pBzntlux[a]	*E. coli* K12MG1655 pBpbrlux[b]	*E. coli* K12MG1655 pBgollux[b]
Ag		2.75				4.27
$As^{(III)}$	0.256		15.6	28.52		
$As^{(V)}$	0.3		12.65	9.32		
Cd	5.9		0.011	0.0045	0.16	
$Cr^{(III)}$						
$Cr^{(VI)}$				597.2		
Co				0.22		
Cu		90.5		16.92		250
Fe			16.1	4.34		
Mn						
Hg			$1.70 \cdot 10^{-7}$	0.01		
Ni				4.4		
Au		10				50
Pb	4.16			2.2	3.8	
Zn				1.7	3.59	
Sn				12.95		

[a]Charrier et al. (2011), Jouanneau et al. (2011); [b]Hua A, unpublished results

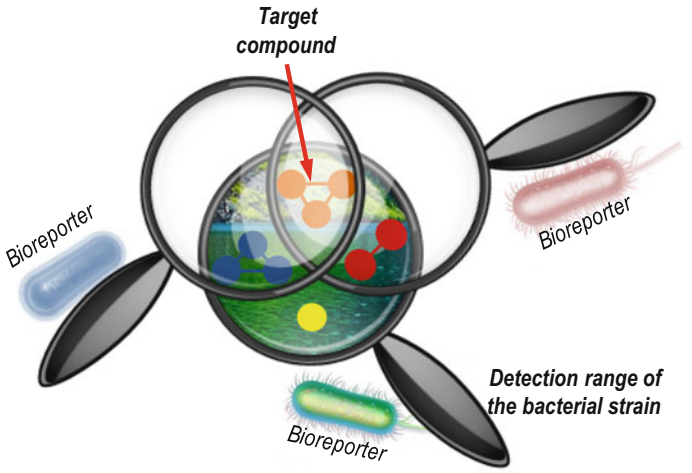
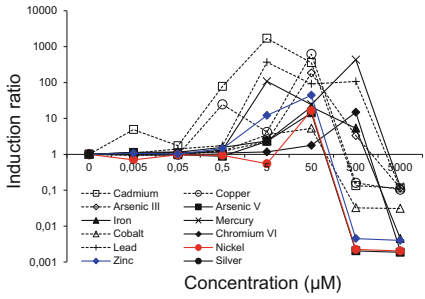
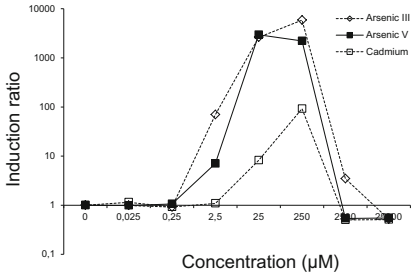


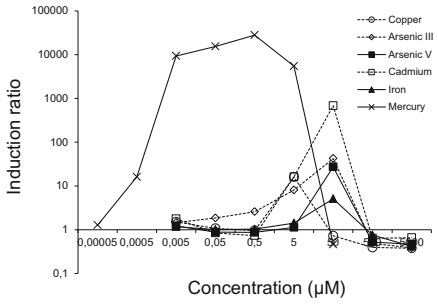
Fig. 12.10 Methodological strategy that was implemented to increase the specificity of approaches based on bacterial reporters



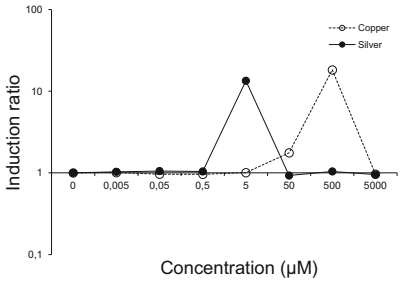
Escherichia coli K12 MG1655 pBZntlux



Escherichia coli K12 MG1655 pBArslux



Escherichia coli K12 MG1655 pBMerlux



Escherichia coli K12 MG1655 pBCoplux

Fig. 12.11 Detection ranges obtained with the inducible strains (Jouanneau et al. 2011)

individual specificity did not allow the reliable detection of the metals in the samples (several metals were detected by the same strain), as shown in Fig. 12.11.

To improve the selectivity of the strains, the authors (Jouanneau et al. 2011) proposed simultaneously mining the different bioreporter data to cross the biological information. For that purpose, data mining algorithms are needed to analyze these complex multi-parametric data and to build biological "fingerprints" of pollution. The selected model was based on decision trees (with a step-by-step classification algorithm) and was designed from an existing database of known metal mixtures. Each bioreporter is involved, if required, in the classification model at different levels according to the investigated chemical (Fig. 12.12) to assess its concentration in study samples. In this example (Fig. 12.12) the first branch separates the data into two groups according to the segmentation value of the variable 'Zntlux' (1.425—bioluminescence value produced by the bioreporter *E. coli* K12 MG1655 pBZntlux). If the bioluminescence value 'Zntlux' is higher than this first threshold, the cadmium concentration is estimated between 250 nM and 50 µM. Otherwise a second classification level is performed until the decision tree allows a determination of the concentration range.

Thanks to this crossing strategy, the reliable identification and quantification of chemicals (in this case, 4 metals) from "specific" bioreporters has been made possible at a reliability superior to 94%.

Although the results are promising, this strategy nevertheless has some drawbacks. As explained below, the design of interpretation algorithms requires an existing database. The size of this database is directly dependent on the number of chemical targets. For example, the addition of one supplementary metal (at three concentration levels) requires a database that will be three times larger than the database used in this study (at 192 tested conditions). Moreover, the development of new models requires the implementation of other "specific" bioreporters. That implementation implies two primary bottlenecks. The first one concerns the multiplication of the strain number, inducing an extra cost of implementation (growth, preparation, storage, etc.). Second, the catalog of available strains is limited, and the development of a new bioreporter is therefore required, which is generally time-consuming, complex and costly.

12.4 Detection of One Analyte by Using Several Parameters in One Cell: The Multi-parametric Approach

As seen before, the first biological assessment methods were intended to follow a single bioreporter (monoparametric). The collected information provides a first analytical dimension, that is, the partial evaluation of the searched parameter (overall or specific parameter). To improve their performances, a second dimension came with the concept of multicellular analysis. The aim was to increase the

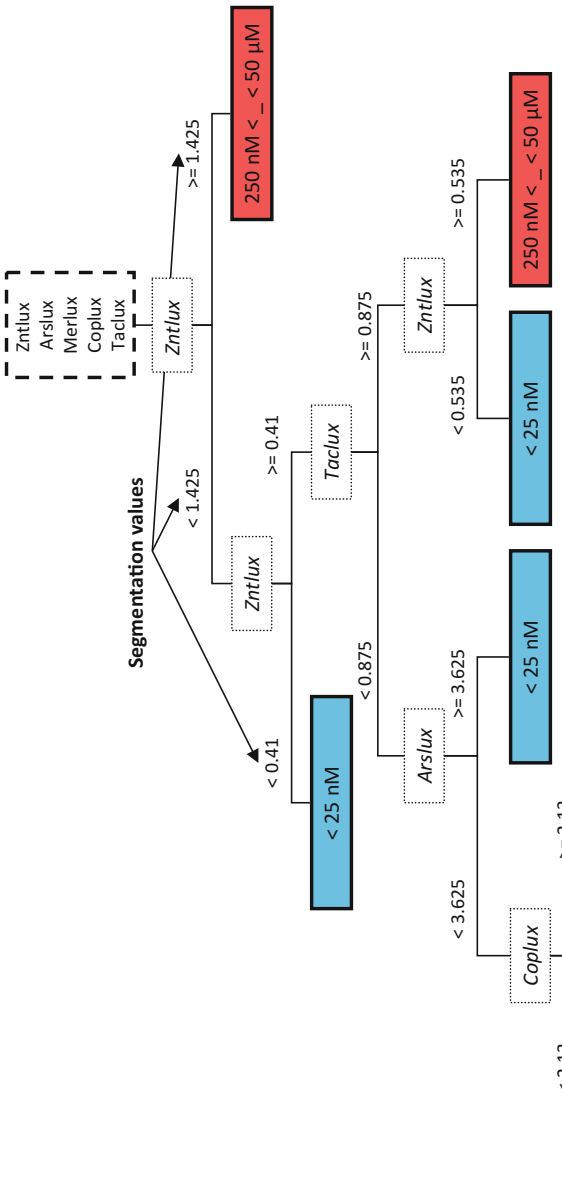


Fig. 12.12 Determination algorithm (decision tree) designed to assess the cadmium concentration (He et al. 2013). *Zntlux*, *Arslux*, *Merlux*, *Coplux* and *Taclux*: variable names relative to the biological data provided by the 5 bioluminescent bioreporters. The segmentation values correspond to the thresholds allowing a classification of biological information

representativity or specificity through the simultaneous observation of several microbial bioreporter responses in the studied sample. However, although the results were promising, these techniques can involve complex genetic manipulations (in the case of specific detection), and they are time-consuming and generally costly.

To circumvent these difficulties, a third generation of approaches was considered to limit the number of biological indicators in use, and, in the same time, to ensure the reliability of the biological information. This last generation was primarily implemented within the framework of specific detection.

12.4.1 Strains Containing Several Genetic Constructions

The first strategy under consideration consists in the construction of bioreporters that can emit different signals depending on the analytes that are in the sample. This strategy was proposed by Roda et al. (2011). To limit the number of necessary bio-indicators, the authors designed a bacterial strain harboring a DNA sequence that could highlight copper (*Photinus pyralis* wild-type luciferase—λ_{max} = 557 nm, inducible promoter: *copA*) and another sequence to assess the overall toxicity (a red-emitting mutant *P. pyralis* with thermostable luciferase—λ_{max} = 618 nm, constitutive regulation) of the analyzed sample (internal viability control) (Fig. 12.13). Thanks to this double genetic construction, it is easy to discriminate between the quantification/detection area and the toxic area.

The constitutive signal allows to monitor the overall toxicity level in the analysed sample. At the same time, the analyte-induced signal allows to detect specifically copper.

This approach nevertheless has similar limits to the strategies based on several bioreporters. Indeed, the strain catalog is extremely restricted, and the complexity of genetic construction makes the design of new strains even more arduous.

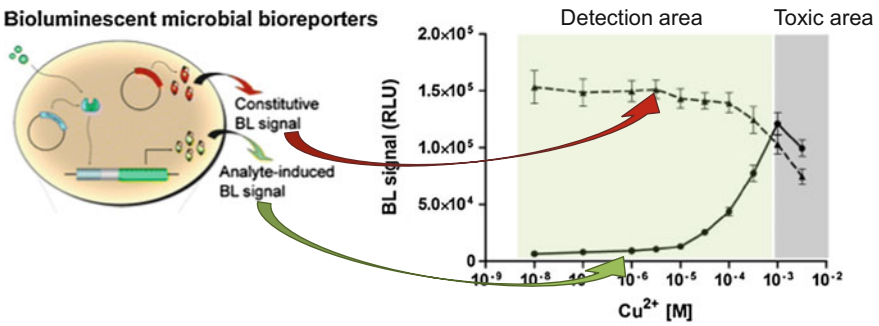


Fig. 12.13 Schematic representation of the designed microbial bioreporter to allow the simultaneous assessment of the copper concentration and overall toxicity (Roda et al. 2011) (with permission of Springer)

12.4.2 Detection by Raman Spectroscopy

Biological indicators interact with an exposed sample as a whole and set up a metabolic response that is adapted to different environmental stimuli. Despite this adaptation, most bioreporters provide only one or two biological data, which are converted into a specific or overall parameter. This strategy, which has been used up until the present, allows researchers to obtain a limited view of the global interactions between the bioreporter and the analyzed sample.

Within this framework, Raman spectroscopy offers a multi-parametric approach that provides an overview of these physiological changes as caused by the toxic agent. This technique is an optical method based on the interaction of light with matter. The difference between laser energy and inelastic scattered photons corresponds to the vibrational energy levels of chemical bonds present in the sample (Pence and Mahadevan-Jansen 2016). Raman spectra can be considered as the molecular fingerprint of the analyzed sample (Fig. 12.14).

The importance of this technique is its status as a non-invasive action and a rapid way to access a large amount of information (it is a multiparametric approach). The resulting Raman spectrum offers an overall view that is very useful for understanding the analyzed sample. One measurement of a few seconds is able to provide the molecular composition of a bacterial cell, allowing, for example, the determination of its physiological state (DNA/RNA bands) and/or its biological molecule content (carbohydrates, proteins, and lipids). However, the richness of the Raman spectroscopy signal is both an advantage and a drawback of the method. The differences in the spectra are difficult to distinguish by simple visual inspection, and the sought-after effects are often hidden among all the information. Consequently,

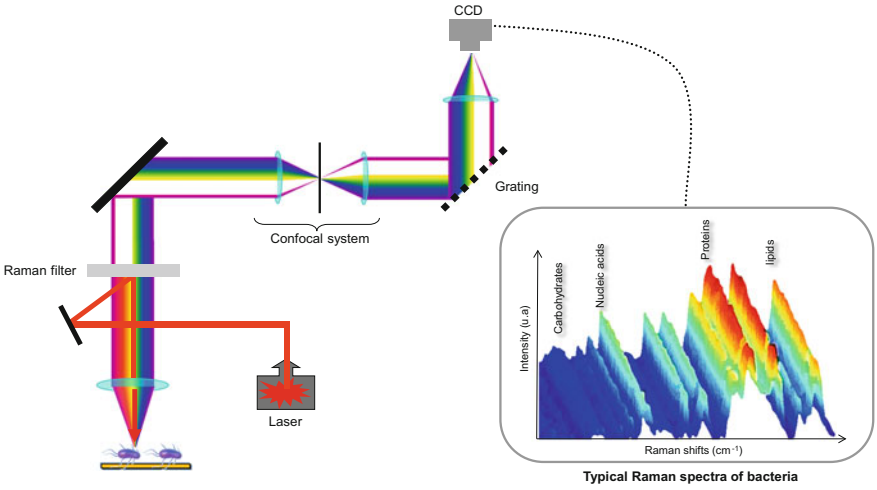


Fig. 12.14 Metrological strategy of Raman spectroscopy, which provides a molecular fingerprint of the bioreporter/sample interaction

statistical tools such as multivariate algorithms are needed (Rae et al. 2014) to highlight the differences between spectra.

Raman applications are numerous and varied, and they extend to all domains of the sciences, from physics and chemistry to the analysis of biological species at the nanoscale (Marshall and Marshall 1922; Fan et al. 2010; Marshall et al. 2010; Vankeirsbilck et al. 2002). The latest progress in Raman spectroscopy (several sources of irradiation and very sensitive detectors) opens new research perspectives. Alongside its use as an alternative optical method for microbial detection, it has also been shown that physiological variations can be observed through Raman spectra. The effects of toxic molecules such as antibiotics can be highlighted, and the observed differences can be matched with physiological events such as the fragmentation of DNA in dying cells or decreasing protein synthesis (Choo-Smith et al. 2001; Huang et al. 2010; Neugebauer et al. 2006; Schuster et al. 2000). By allowing, in one shot, a multiparametric overview of pollutant toxicity targets, this method could allow the rapid identification of toxicity mechanisms towards the better monitoring of pollutant impacts.

This third analytical dimension permits the identification and understanding of toxicity mechanisms, which is necessary to understand their potential effects on the environment and human health. However, this information is currently out of reach without complex and expensive metabolomics methods (Fig. 12.15).

12.4.2.1 Example of Arsenic Toxicity in *E. Coli*

Over the past decade, some researchers have started to investigate these approaches in relation to environmental applications and toxicity assessments. Most of the time, the results have shown significant variations in the observed microorganism spectra following pollutant exposure. However, the primary challenges come from the difficulty in linking significant changes in Raman bands with actual toxicity impacts on cells (Singer et al. 2005; Tian et al. 2012). In fact, the richness of the Raman spectroscopy signal is both an advantage and a drawback to the method, and researched effects are often hidden among all the information. Thus, these data require important analytical work and the implementation of statistical tools (Rae et al. 2014; Jarvis and Goodacre 2005; Lasch 2012). In this context, recent work on the use of Raman spectroscopy for the monitoring of arsenic toxicity shows interesting results (Bittel et al. 2015) (Fig. 12.16).

Spectra for *E. coli* bacteria that were exposed to four arsenic concentrations were collected and analyzed. The first results show the differences between the spectral variations in the molecular footprint of the sample. However, it was difficult, without analytical tools, to evaluate the significance of those differences and to establish clear links with the toxicity. To highlight the specific effects of the metal, special attention has been paid to the statistical data analysis. A spectra "quality-test" based on Independent Component Analysis (Rutledge and Jouan-Rimbaud Bouveresse 2013) has been designed and has allowed a significant

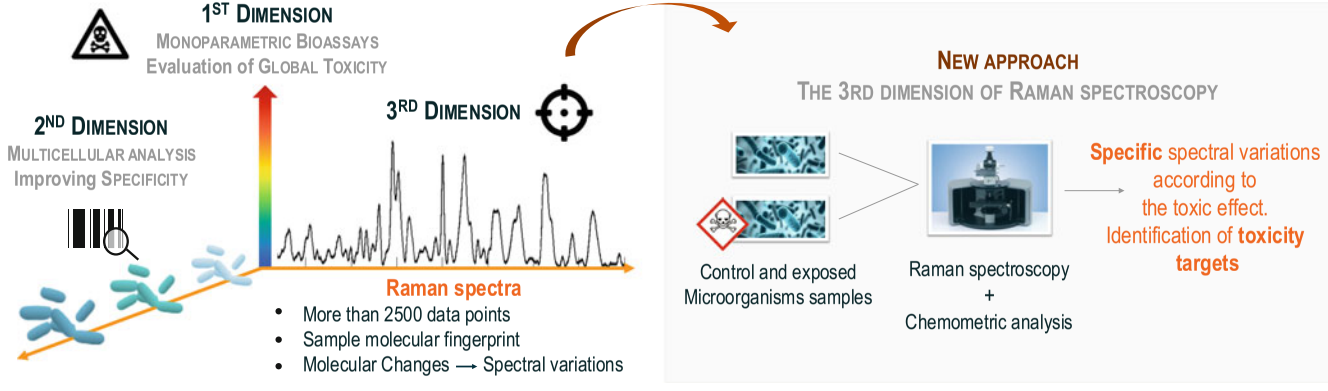


Fig. 12.15 Toxicity assessment, at 3 complimentary dimensions

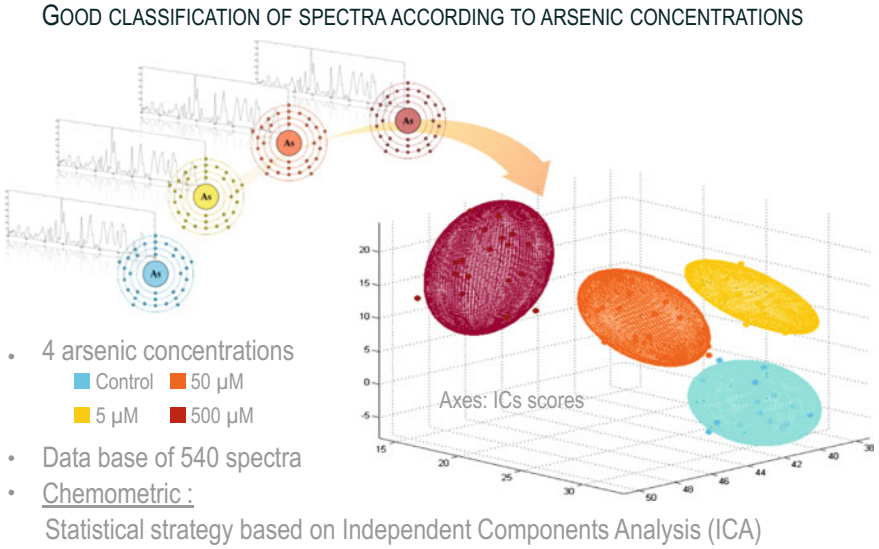


Fig. 12.16 Observation of an arsenic "dose-response" effect on *E. coli* according to Raman spectra[80]

improvement in the correct classification of spectra according to the tested arsenic concentrations. The results were finally consistent with a dose-response effect.

However, the primary advantage of using Raman spectroscopy as an observation method for bioassays and biosensors is its analysis of a multiparametric signal rather than the monoparametric measurements that are traditionally obtained with other techniques. Thus, a chemical interpretation of the results has been performed to link the observed variations to the physiological event that was induced by arsenic exposure. For this purpose, the most significant Raman bands have been identified. They were determined during the classification process, and they illustrate that the toxicity impact is significant on related biomolecules.

For example, some band's variations could be linked to the production of specific membrane transporters for the evacuation of the toxic compounds or the accumulation of arsenic in the bacterial cell wall (465–525 cm^{-1}). Some others can be associated with a denaturation phenomenon or the decrease of protein production accompanying a cell growth slowdown (1200–1400 cm^{-1}). As a last example, variations in a band that correspond to the phosphodioxy groups PO_2^- that are characteristic of nucleic acids (1100 cm^{-1}) were particularly interesting. Indeed, arsenic is known to be a structural analog of phosphate and to damage DNA by breaking phosphate bonds. Thus, the highlighted variations were consistent with known toxicity mechanisms, thus allowing the definition of "spectral signatures" of the metal's effect on the bacteria (Fig. 12.17).

Thus, the multiparametric aspect of Raman spectroscopy permits an overview of toxicity impacts on microorganisms. It helps to identify pollutant toxicity targets

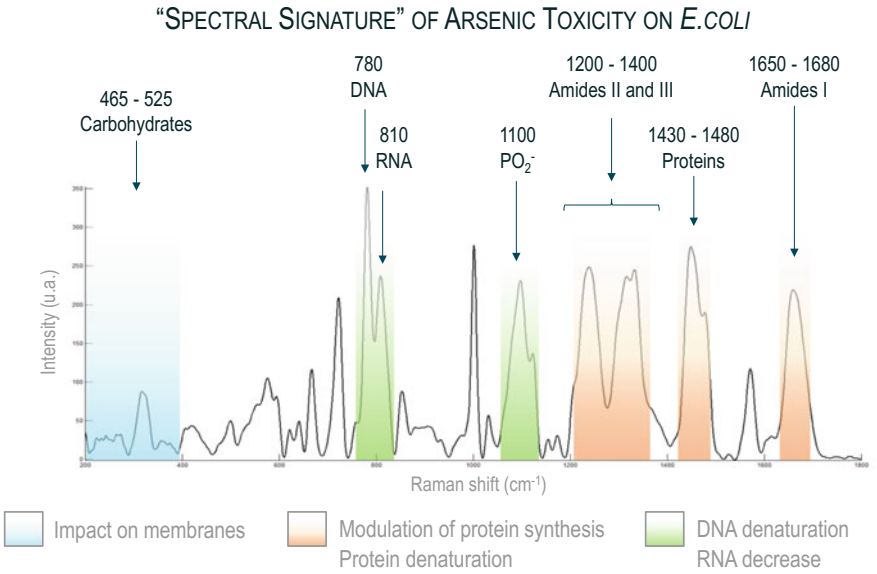


Fig. 12.17 Model spectra from *E. coli*. The highlighted Raman bands are particularly impacted by arsenic toxicity

without the need for complex and expensive metabolomic methods. When coupled with classical monoparametric bioassays involving different types of microorganisms, it could provide a fast and reliable biological alternative method to improve toxicity assessments.

12.5 Conclusions

Driven by both an omnipresent regulatory pressure and a will for environmental preservation, biological approaches were proposed in addition to physico-chemical methods. The primary objective was to focus on a biological characterization of analyzed samples. Some parameters, such as the overall or specific toxicity, the environmental persistence or the bioavailable fraction of chemicals are not measurable via physico-chemical approaches.

Many analytical methods were proposed by using living organisms (bioreporters) from different trophic levels, but among the wide spectra of implemented biological candidates, the bacterial cells were particularly interesting. The notable benefits of these biological indicators (easy to produce, high growth rate, limited biological demands) have widely contributed to their democratization in environmental monitoring and more broadly, to the field of metrological methods.

Initially, these bacterial strategies were based on an ideal vision, namely to use one bioreporter per parameter. However, these first strategies quickly reached their limits for overall or specific parameters. Although they are very easy to put into practice and they are inexpensive, these methods lacked significant robustness in the desired analytical context. From this observation, the proposed strategies have continued to evolve to increase their robustness, becoming more and more complex. The proposed strategies are based on the multiplication of collected biological information by increasing the number of implemented bioreporters or biomarkers. In these two cases, a supplementary data-mining step was required to interpret the multi-parametric information. This latest is generally ensured via statistical approaches such as PCA, neural networks, decision trees, and others.

Thus, through these overall approaches (biological recognition and statistical data mining), the biological methods based on bioreporter(s) appear now as pertinent methodologies in the stringent framework of environmental analysis.

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