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New challenges in environmental analytical chemistry: Identification of toxic compounds in complex mixtures

Marie-Hélène Dévier [a], Patrick Mazellier [a], Sélim Aït-Aïssa [b], Hélène Budzinski [a,*]

[a] Environnements et Paléoenvironnements Océaniques et Continentaux (EPOC, UMR 5805, CNRS), Équipe Laboratoire de Physico- et Toxico-Chimie de l'Environnement (LPTC), CNRS, université Bordeaux 1, 351, cours de la Libération, 33405 Talence cedex, France
[b] Unité Écotoxicologie in vitro et in vivo, Institut National de l'Environnement Industriel et des Risques (INERIS), Parc ALATA, BP2, 60550 Verneuil-en-Halatte, France

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ABSTRACT

Toxic effects evidenced in the environment are most often caused by mixtures of known and unknown pollutants. One of the key challenges in environmental chemistry and ecotoxicology is to characterize and identify those toxicants in relation with the effect. However, many of the current bottlenecks in the assessment of organic contaminants in our environment are related to the difficulty of evaluating various chemical classes and biological effects within complex mixtures and more precisely to link both approaches. To tackle these analytical challenges, the bioanalytical concept has emerged during the last decade. In this article, we describe through some outstanding examples the current limitations in the chemical-driven approach such as problems encountered for a correct evaluation of water quality when the continuous introduction of new chemicals has to be taken into account in monitoring for correct evaluation of this quality and could led to tremendous analytical costs or some of the integrated bioanalytical approaches as promising powerful tools to improve environmental risk assessment by taking into account the link presence/effect.

1. Introduction

In Europe, increasingly restrictive regulations take into account the problem of release of chemicals and especially of organic chemicals in the environment: the European Water Framework Directive (WFD – 2000/60/EC), the European Marine Strategy Directive (MSD – 2008/56/EC), the European Soil Framework Directive (SFD – 2004/35/EC), the Biocides Directive (Council Directive 98/8/EC) and more recently, the European Regulation for Registration, Evaluation, Authorization and Restriction of Chemicals (REACH). However, to date, about 16 million compounds

Abbreviations: AhR, aryl hydrocarbon receptor; AR, androgen receptor; DBP, disinfection by-product; DGT, Diffusive Gradient in Thin film; DLC, dioxin-like compound; EDA, effect-directed analysis; EDC, endocrine-disrupting compound; ER, estrogen receptor; fmol, fentomole; FT-ICR, Fourier transform ion cyclotron resonance mass analyzer; GC, gas chromatography; GC/MS, gas chromatography - mass spectrometry; GR, glucocorticoid receptor; IT, ion trap mass analyzer; LC, liquid chromatography; LC/MS, liquid chromatography - mass spectrometry; LDPE, low density polyethylene; LIT, linear ion trap mass analyzer; LTQ Orbitrap, hybrid linear ion trap - Orbitrap mass analyzer; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MSD, European Marine Strategy Directive; NR, nuclear receptor; PAH, polycyclic aromatic hydrocarbon; PBDE, polybromodiethylether; PCB, polychlorobiphenyl; POCIS, Polar Organic Contaminant Integrative Sampler; ppb, part per billion; PPCP, pharmaceuticals and personal-care product; ppt, part per trillion; PXR, pregnane X receptor; Q, quadrupole mass analyzer; QqLIT, hybrid quadrupole - linear ion trap mass analyzer; QqQ, triple quadrupole mass analyzer; QqToF, hybrid quadrupole - time-of-flight mass analyzer; QSAR, quantitative structure-activity relationship; REACH, European Regulation for Registration, Evaluation, Authorization and Restriction of Chemicals; SFD, European Soil Framework Directive; SPMD, Semi-Permeable Membrane Device; SPME, solid-phase microextraction; TIE, Toxicity Identification Evaluation; ToF, time-of-flight mass analyzer; TP, transformation product; TWA, time-weighted average; US EPA, United States Environmental Protection Agency; WFD, European Water Framework Directive; WWTP, wastewater treatment plant.

* Corresponding author.
E-mail address: h.budzinski@epoc.u-bordeaux1.fr (H. Budzinski).

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are known and registered in the Chemical Abstracts Service (CAS) and of course all these compounds are not subjected to specific regulations. For example, chemical quality assessment of water resources is based on chemical analysis of priority pollutants as laid down in the WFD. Forty-one compounds have been selected as priority pollutants in different priority categories. Concerning the quality of water resources used for drinking water production, maximal concentration has been established for several water contaminants (Council Directive 98/83/EC). Among these organic contaminants, the level of pesticides is particularly controlled. The regulation concerning drinking water production also includes some relevant metabolites or degradation products.

There is a tremendous discrepancy between the number of compounds potentially present in the environment and the number of regularly monitored priority pollutants. Actually, one of the difficulties encountered in accurately assessing the organic contamination of environmental systems is that there are multiple contaminants (multiple sources, multiple chemical classes) associated with both low concentration levels and considerable temporal and spatial variability. Analyzing several classes of compounds present at trace or ultra-trace concentrations (ppb or ppt) requires the use of different apparatus, protocols, and each protocol involves different sample preparation methods. These requirements are very time-consuming and costly. It clearly appears that the chemical monitoring of all compounds in every compartment is impossible and target analysis of preselected sets of pollutants often misses site-specific toxicants (that in addition remain unknown for many of them) and is therefore not able to explain ecotoxic effects of complex environmental samples.

Moreover, as chemical compounds, organic contaminants are reactive compounds that react in environmental and aquatic systems more or less rapidly. Well-known transformation reactions include physicochemical processes (hydrolysis, reactions induced by light, reactions with oxidants used in drinking water production) and biological processes (with free bacteria or biofilm, or metabolization). These transformation reactions lead to the formation of numerous transformation products for which, in most case, almost nothing is known. The formation of several transformation products arising from the numerous contaminants present in aquatic systems increases the complexity of the problem, notably because some of them can be more persistent and/or more toxic than their parent compounds. The universe of chemicals keeps expanding because of the need to include metabolites and transformation products when evaluating the risk from a certain chemical [1].

Numerous studies combining chemical and biological approaches to hazard assessment of complex environmental mixtures indicate that priority pollutant concentrations are a poor indicator of toxicity [2,3]. Thus, it is evident that the aim to assess and forecast the impact of chemical pollution cannot be met on the basis of priority pollutant analysis alone. Beside a chemical-driven strategy for assessing the ecological risk from pollutants, it is necessary to explore and to apply new strategies, combining both biological responses and chemical analyses, to identify toxic hot-spots, to characterize chemicals likely to cause adverse biological effects, and, finally, to assess the ecological risk of the identified chemicals at relevant spatial scales [4,5]. Combined biological and chemical-analytical approaches provide an important progress towards an estimation of the portion of an effect that can be explained by the analyzed chemicals. In particular, mechanism-based *in vitro* bioassays using recombinant cell systems in which specific biological effects are directly measured can provide valuable information about the expected total potency of the complex mixture of chemicals in an environmental sample [6]. Nevertheless, the link between the two approaches, i.e. linking chemical exposure and observed biological effects, is not straightforward when dealing with complex mixtures of contaminants. In this connection, integrated strategies such as effect-directed analysis (EDA) can be a powerful tool to elucidate unknown causative toxicants in complex environmental samples and their combined effects, thus improving environmental risk assessment. EDA approaches combine chemical and biological methods in order to direct chemical analyses to those compounds that actually cause effects [7,8]. The complexity of samples is sequentially reduced by removing non-toxic fractions while major toxicants are isolated and identified [7].

In addition, using passive sampling instead of classical spot water sampling could also help to improve environmental risk assessment; by their ability to concentrate bioavailable sediment and waterborne pollutants (and thus to take also into account of pollutants undetectable by conventional methods), these integrative passive devices allow increasing representativeness and reliability of the data obtained [9]. Furthermore, they can collect extracts of chemicals for either chemical analysis or bioanalysis and there are therefore advantages to be gained from combining the use of passive sampler extracts and bioanalyses, and in particular EDA.

In this paper, we describe through some outstanding examples the current limitations in the chemical-driven approach when confronted to new chemicals that have to be integrated in monitoring of water quality to take into consideration the continuous increase of used and produced chemicals and their transformation products and some of the integrated bioanalytical approaches as powerful tools to improve environmental risk assessment. Like many of our colleagues from the scientific community, we encourage the development of new monitoring strategies to respond more accurately to the current societal questions.

2. New challenging compounds: emerging contaminants and transformation products

The following paragraph does not aim at making an exhaustive review of what could be found as emerging contaminants or as transformation and/or by-products (TPs) but just focusses on some illustrative examples of new challenging compounds: polar forms that can be addressed in relation with LC and especially LC/MS (MS/MS and ToF-MS) progress, new forms of problematic pollution

(not due to classical persistence but to continuous introduction in the environment), and the need to identify and monitor TPs for a better environmental risk assessment of organic contaminants.

2.1. Emerging contaminants

Many environmental questions arising from the presence of pollutants in environmental and aquatic systems remain to be addressed, since new chemical compounds or classes of new compounds are continuously developed, brought to the market and at the end "arise" in the environment [10]. After having tackled priority pollutants from the seventies (mainly coming from technical processes and products), it is now crucial to also turn towards the emerging contaminants.

This group of organic compounds corresponds in most cases to unregulated contaminants, which may be candidates for future water-quality regulation depending on their potential health or ecotoxicological effects and on monitoring data regarding their occurrence. They encompass a diverse group of compounds, including drugs of abuse, pharmaceuticals, personal-care products (PCPs: fragrances, antimicrobials, UV screens, antioxidants and preservatives, and insect repellents), steroids and hormones, surfactants, perfluorinated compounds (PFCs), flame retardants, industrial additives and agents, and gasoline additives, as well as their transformation products (TPs). In addition, three new classes have to be added to the list of emerging pollutants: nanomaterials, 1,4-dioxane and swimming-pool disinfection by-products (DBPs) besides classical DBPs regulated in drinking water production such as THMs (trihalomethanes).

The issue of emerging contaminants is closely related to analytical performance concerning their monitoring in various environmental compartments. With the development of sophisticated and sensitive analytical apparatus and procedures (Section 3), more and more pollutants, in particular pharmaceuticals and personal-care products (PPCPs), can be detected at trace levels in the environment [11]. Consequently, a number of new or previously ignored and/or unrecognized contaminants have been brought under scrutiny. However, it is still necessary to further improve rapid and sensitive analytical procedures, in particular in two directions: on the one hand, high sensitivity at ultra-trace levels, and on the other hand, versatility in simultaneous screening for a wide variety of compounds with large differences in physicochemical properties (e.g., log K_{ow}, high water solubility, zwitterionic form according to pKa...) [12].

Of all the emerging contaminants, PPCPs are currently the most studied compounds. Several data concerning their monitoring in surface waters have been published in the last 15 years due to a significant improvement in analytical procedures and apparatus. The characteristic of these groups of contaminants is that they do not need to persist in the environment since they are continuously introduced via urban wastewaters. In this way, they can be considered as "pseudo-persistent" compounds. Numerous studies showed that pharmaceuticals are not completely eliminated in wastewater treatment plants (WWTPs) [13–16] and that some of them can be present at levels up to micrograms per liter, in rivers, streams, lakes, and even in groundwater [17–19]. The presence of PPCPs in WWTP effluents may also lead to the contamination of drinking water resources because of their quite polar structure and therefore high mobility [20]. Their occurrence has already been reported in drinking water of several large European cities as Berlin [21] or Milano [22]. The concentrations measured are far lower than those used in medical prescriptions but the daily consumption of drinking water leads to chronic exposure for which potential toxic effects are poorly known (e.g., bioresistance of bacteria to antibiotics...). With increasing knowledge of their environmental occurrence, there is a growing interest on their environmental fate and toxicological impact. Numerous studies dealing with hormonal steroids illustrate their potential implication in endocrine disruption phenomena but when considering the other pharmaceutical classes (e.g. antibiotics) information on their ecotoxicity, persistence and fate of pharmaceuticals and their transformation products in the environment are scarce [23–28]. All these substances have been designed to be biologically active but their potential action on aquatic organisms has not been described in detail until now. Thus there is a real need to document their potential ecotoxicological impact. A guideline has been recently published by the European Medicines Agency (EMEA) to describe the assessment of potential environmental risks of human and veterinary medicinal products, from general considerations to environmental risk assessment and safety measures to be considered [29,30]. However, some studies have already evidenced the toxicity of some pharmaceuticals [31–34] and some PCPs have been suspected endocrine-disrupting compounds (EDCs) [35–39]. In the terrestrial environment, the decline of the vulture population in Pakistan since the 1990s due to renal failure is the most important striking effect that has been reported for diclofenac (anti-inflammatory) [31]. Moreover, there is some evidence of potential interactive effects of PPCPs, so that low doses may lead to cumulative stress and synergic toxicity effects in exposed organisms [40,41]. As shown for some β-blockers, even when their toxicity as individual compounds is negligible, they might act in an additive manner in a mixture with other β-blockers exhibiting the same mode of action [42]. Also, antibiotics present in the environment could lead to selection of resistant bacterial strains [43].

Another aspect raised by Hogenboom et al. [44] in this field is that the European REACH legislation will possibly drive producers to develop newly designed chemicals that will be less persistent, bioaccumulative or toxic. If this innovation leads to an increased use of more hydrophilic chemicals, it may result in higher mobilities of chemicals in the aqueous environment and of course in water resources used for drinking water production. As a result, drinking water companies may face stronger demands on removal processes as the hydrophilic compounds are inherently more difficult to remove by adsorption onto active carbon (granular or powder) for example. Monitoring efforts will also experience a shift in focus to more water-soluble compounds [44]. It should be also noticed that REACH is for

the moment limited to industrial chemicals and does not consider other compounds such as pharmaceuticals and biocides.

The study of the ecotoxicity of an organic contaminant is hampered by the difficulty of knowing the nature and toxicity of its transformation products. Shortly, faced with a great number of emerging pollutants and the greatly larger number of metabolites and degradation products, there is a crucial lack of reliable data to assess their environmental and human health risks. For all these unknown compounds, very scarce information is available about their chemical and biochemical properties, and their potential interactive effects within complex mixtures. Currently, there is an increasing concern regarding the formation of transformation products since there is evidence indicating that they can be more toxic and persistent than parent compounds. In fact, transformation products are considered relevant within the group of emerging contaminants [45].

2.2. Transformation products

Some relatively inert molecules persist in the environment and are difficult to degrade. These compounds can be toxic and accumulate in food chains to present additional human and ecological risks. Others can be degraded to transformation products (TPs). Organic pollutants undergo various degrees of transformation in processes and in the environment, leading to a modification of the starting chemical structure and resulting in a cocktail of parent compounds and TPs in the environment.

Chemical transformations of the organic compounds can be biotic or abiotic. Photodegradation in sunlit aquatic systems can play a significant role beside biodegradation in eliminating those chemicals in the environment. For chemicals that are only poorly biodegraded, especially in biological treatments of WWTPs, photodegradation may represent a major route of degradation. On the contrary, whereas some compounds can evade photochemical reactions because they are not exposed to sunlight (e.g., when adsorbed onto particles or in the subsurface), microbial transformation processes dominate in removal of these organic compounds. As far as water resources are concerned, it is also necessary to distinguish between degradation products arising from naturally occurring processes (biodegradation, hydrolysis, sunlight photodegradation...) and degradation products arising from reaction in water treatment processes (biodegradation within biofilms, reactions with ozone and chlorine...). In the field of drinking water production, disinfection by-products (DBPs) are regulated for more than 30 years but the regulation only focused on small size, final DBPs such as trihalomethanes (THM), halogenated acetic acid (HAA), nitrosodimethylamine (NDMA) which mainly arise from reactions between disinfectants and dissolved organic material including organic pollutants [46]. Their toxic (carcinogenic and/or genotoxic) effects are well known [46]. However, these DBPs are formed after successive oxidation reactions (final transformation products) but intermediate transformation products from pesticides or PPCPs are rarely studied. Biological processes will similarly occur but other processes may give rise to specific transformation products according to the reagent involved.

In the environment, chemical transformation reactions mainly consist in hydrolysis and light-induced reactions. The phototransformation reactions can be distinguished in direct photoreactions when the contaminant absorbs light, and indirect reactions when absorption of light by water components, e.g. nitrate ions or organic matter generated reactive species (radicals, electrons...) able to provoke organic contaminants degradation. The set of degradation reactions is strongly dependant on the molecular structures of the pollutant and of the medium.

Hydrolysis processes are generally slow in natural aquatic medium with pH ranging between 6.5 and 8.5. Therefore, the formation of hydrolysis products is generally not considered. However, some compounds may react efficiency and their lifetime in water may be significantly impacted by the hydrolysis process. As an example, some carbamate (N-methyl carbamate) pesticides are only very stable in water: the half-life of oxamyl has been reported to be about 30 h in water at pH = 8 leading to the formation of oxime derivative [47]. For this pesticide, solar phototransformation will not occur.

Sunlight direct phototransformation processes firstly depend on the rate of light absorption of pollutants. Usually direct phototransformation is low, for example, for pesticides but several PPCPs and especially several pharmaceuticals undergo very rapid direct phototransformation. It would be very long to review the published results concerning the phototransformation of PPCPs but the several examples reported here show the complexity of the phenomenon. The famous anti-inflammatory diclofenac undergoes phototransformation very efficiently into carbazole derivative and hydroxyl-diclofenac as the main photoproducts [48]. There are a lot of data concerning the presence of diclofenac in river water but no data even qualitative is given concerning the presence of known photoproducts, although phototransformed diclofenac has been reported to be five times more toxic to green algae compared to the parent compound [49]. Naproxen (anti-inflammatory) also undergoes rapid solar phototransformation and the photoproducts were shown to be about ten-fold more toxic than the parent compound for *ceriodaphnia*, but they did not induce any genotoxicity [50]. On the opposite side, photoproducts of furosemide (diuretic agent) showed a mutagenic potential compared to the parent drug [51] and phototransformation of corticosteroids (prednisone, prednisolone, dexamethasone) were more toxic than parent drugs (*C. Dubia* reproduction 7d.) [52,53]. Some other PPCPs keep the properties of the parent compound (e.g., antibiotic activity) as demonstrated with some dehydrated products of tetracyclines [54] and photodegradation products of the fluoroquinolone antibiotic ofloxacin [55], or lose antimicrobial activity and toxicity. No general rules can be given concerning the formation of photoproducts since the efficiency and the reaction pathways strongly depend on product chemical structures.

Indirect phototransformation processes in water are mainly due to the presence of nitrate ions and natural

organic matter. Nitrate ions, even if their concentration is usually quite low, may provoke the formation of nitration products [56]. A few field studies reported low concentration of nitration products arising from these processes, especially with phenylurea and phenoxyacetic acid pesticides [57,58]. The formation of nitro derivatives may increase the long-term genotoxic impact of these pesticides [59].

All environment-relevant processes are simultaneous and therefore the competition will exist between all degradation processes. The case of reactions with reagents used in water treatment (ozone, chlorine) can be illustrated by several examples. The resin monomer bisphenol A (BPA) reacts very efficiently with chlorine to form chlorinated bisphenol A in the early stages of the reaction. The mixture shows strong estrogenic activity (24 times that of BPA itself) as demonstrated recently [60]. Similarly, the chlorination of acetaminophen (paracetamol) leads to the formation of toxic compounds such as benzoquinone and benzoquinone-imine [61].

Moreover, when the overall set of transformation reactions are taken into account, it is necessary to consider that some transformation processes will be successive according to the life cycle of a particular contaminant. Triclosan, a very common antimicrobial agent, can be classified in the group PPCPs. Triclosan, released in wastewaters, reacts with residual chlorine, leading to the formation of chlorinated derivatives. It has recently been demonstrated that the sunlight photodegradation of these derivatives, not eliminated by wastewater treatment plants, leads to the formation of toxic polychlordibenzo-p-dioxin [62]. Additionally, the sunlight phototransformation of triclosan is a well-known process [63] but it also generates chlorophenol derivatives [64].

Biotic processes commonly produce compounds of increased polarity whose different physicochemical properties result in distinct environmental behaviour. Plants and animals can detoxify or excrete contaminants after uptake, but accumulation in adipose tissue or the absence of appropriate enzyme systems necessary for biotransformation can hamper elimination of the contaminants. Organic pollutants can also be microbiologically transformed in WWTPs, which are not initially designed to reduce and to minimize the release of potentially harmful compounds into the aquatic environment. Many organic compounds are biodegraded by organisms that utilize the compounds as an energy source.

Either used for human consumption or as veterinary products, pharmaceuticals are excreted in their original form or as metabolites (free or conjugated) [65]. Some examples of metabolites of pharmaceuticals (human metabolites as well as microbial metabolites formed during environmental biodegradation) in the environment can be found in recent reviews [26,66]. In WWTPs, microorganisms play an important role in the transformation of emerging pollutants. In a recent study, Kosjek et al. demonstrated that the biodegradation of diclofenac leads to the formation of quinone-imine, nitro-diclofenac and hydroxyl-diclofenac in a reactor simulating a WWTP biological reactor [67]. The biological treatment of 17α-ethinylestradiol (EE2) and 17β-estradiol (E2) in WWTPs to reduce the estrogenicity of the effluent, prior to it being discharged into the environment, has been of great interest to many scientists and engineers [68]. Few studies on EE2 and E2 biodegradation (using activated-sludge systems, pure microorganism cultures or enzyme extracts) have identified some of their metabolites, mainly estrone (E1), but also some polar organic acids or keto-, hydroxyl-, and glucoside-derivatives [69–72]. Some metabolites of pollutants have also been shown to be more toxic and/or to be more persistent than parent compounds. As a good example, DDE, a DDT metabolite, is more persistent than DDT and was also shown to be more genotoxic than its parent compound in the Zebra mussel (*Dreissena polymorpha*). The herbicide diuron constitutes also good example, as demonstrated for its metabolite 3,4-dichloroaniline (DCA) versus *Vibrio Fisheri* [73]. The biodegradation of alkylphenol polyethoxylates (APEOs) in WWTPs leads to the formation of metabolites more toxic, more lipophilic, more estrogenic and more persistent than the parent substances [74,75].

Some metabolites can be also used as biomarkers of exposure to different pollutants, notably pesticides. For instance, the presence of TPs in farmers' human biological fluids has shown to be an indicator of occupational exposure to agrochemical compounds [76,77]. A very good example of the usefulness of monitoring pollutant metabolites is the use of polycyclic aromatic hydrocarbon (PAH) metabolites as PAH exposure biomarkers. To assess the significance of PAH contamination on organisms, the quantification of bioaccumulated PAHs in tissues is usually carried out. However, depending on the ability of organisms to metabolize PAHs, measurement of the bioaccumulated part of the absorbed PAHs can be restrictive and not representative of the environmental contamination [78]. Moreover, PAHs can be extensively metabolized into more toxic molecules than parent ones [79]. In this way, the study of PAH fate in marine organisms appears to be necessary in order to access bioavailability and toxicity of those contaminants. Moreover, biliary metabolites of PAHs have been shown to be particularly efficient biomarkers of PAH contamination in the environment [80,81]. The study of metabolites (not only hydroxylated PAHs but also phenol, dihydrodiol, quinone, and diol-epoxyde metabolites) could help to better understand the PAH exposure of organisms and also to establish a link between exposure and effects, as with DNA impairments for example. A study performed within the framework of the impact study of the oil spill of Erika on the French Atlantic coast clearly showed an exposure of fish *Solea solea* by the means of the biliary metabolites, when no accumulation in tissues was evidenced [81]. Similarly, measurements of PAH metabolites in human urine is the method of choice to determine occupational, environmental, medicinal and dietary sources exposures of PAHs [82,83].

As far as the behaviour of organic pollutants in the environment is concerned, the formation of degradation products must be taken into account even if it increases the complexity of the problem. Actually, the identification of main degradation products is requested in several regulations (pesticides in water resources used for drinking water production, biocides likely to be released in the

aquatic compartment). The first problem concerning TPs is their detection in the environment, and the second one is their toxic impact. Despite the improvement of analytical techniques (Section 3), it is impossible to characterize all xenobiotics in water because they are usually present at trace levels in rather complex mixtures and many of them remain unknown. For the same reasons, the impact of TPs is also difficult to take into account. The different examples mentioned previously show that the disappearance of chemicals is not synonymous with decontamination, since their degradation in the environment leads to a great variety of TPs that can be less biodegradable and/or more toxic than their parent compounds, thus increasing the toxicological risks. The complex nature of the multiple transformation reactions underscores the need to elucidate structures and potentially additive, antagonistic and synergetic ecotoxicological effects of intermediate TPs.

Actually, the lack of sufficient data in terms of exposure and toxic effects of emerging substances, as well as their transformation products, makes it impossible to achieve a correct risk assessment in relation to the use of these substances and their presence in the environment. The challenges in analyzing emerging contaminants and transformation products are the need for increased capabilities in environmental analysis, in terms of both the lowering of detection/quantification limits (due to their presence at low, but nevertheless potentially toxicologically relevant, concentrations in the ng/L range, which require enrichment, separation and sensitive detection) and the unequivocal identification without reference standards, which are often not available.

In addition, of all the emerging contaminants, pharmaceuticals are not necessarily of greatest concern; it should be mentioned that other compounds, especially polar metabolites and complex mixtures (chemical cocktails) have been actually identified as two of the major challenges for toxicologists [10,44,84].

3. Advances in analytical chemistry

Mass spectrometry (MS) has revolutionized environmental analytical chemistry by allowing the analysis of complex organic mixtures for trace amounts of analytes due to its intrinsic characteristics such as selectivity, sensitivity, and identification-confirmation capability. Currently, the detection of both organic contaminants and transformation products requires the use of chromatographic techniques (gas chromatography and liquid chromatography) hyphenated to MS using several analyzers, as quadrupole, time-of-flight or hybrid instruments [85].

Gas chromatography mass spectrometry methods have greatly contributed to the characterization of small apolar (except when a derivatization step is used) contaminants in water whereas liquid chromatography mass spectrometry methods have been more recently utilized to extend the investigation of water contaminants to non-volatile, (highly) polar, and thermally labile compounds, thus allowing the detection of analytes that were not routinely analyzed in the past, such as, for example, pharmaceuticals, pesticides, endocrine-disrupting compounds and personal-care products [86]. Nowadays, the major challenge is the analysis of highly polar compounds at trace concentration levels in aqueous environmental samples [44]. Compared to simple MS (e.g., single-stage quadrupole analyzer (Q)), the techniques of the tandem MS (MS/MS or MS^2; e.g., triple quadrupole mass detector (QqQ)) present both a very good specificity (selective detection) and an important sensitivity (low picogram injected level). In order to cover the widest possible range of identified compounds complementary ionization techniques have to be used, including GC-MS/MS (electronic impact, chemical ionization in positive and/or negative mode), LC-MS/MS (electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), photoionisation (APPI)...). The possibility to use a large variety of ionization techniques gives flexibility to ensure that most molecules present in a real sample will be ionized and thus detected and quantified. The identification of molecular structure is less easy in the field of environmental analysis since we have to deal with complex mixtures containing low concentration of organic compounds. A limit of these techniques is that they do not allow discovering, in most of the cases, molecules which are not specifically looked for. Moreover, even if the sensitivities of the methodologies have much increased, sometimes compounds are present at levels below detection limits of analytical methodologies but at levels that can lead to biological effects [87,88]. However, to tackle this limitation in sensitivity, integrative passive samplers can be used (Section 5).

The applicability of non-target analysis, i.e. full-scan screening, depends on current technical developments. Depending on the MS instrumentation available, two common strategies are employed to determine the identity of unknown compounds, based on: (1) structural information gained in tandem MS experiments; and (2) highly accurate molecular mass measurements [89].

Tandem MS using triple quadrupole mass detector (QqQ) provides a comprehensive dataset for structural elucidation. However, ion trap (IT) mass detectors have higher sensitivity in full-scan mode and the unique ability to isolate and to accumulate ions, resulting in a hypothetically infinite number of fragmentation patterns (i.e. MS^n) [90]. However, MS^2 fragmentation in QqQ and IT can be limited or insufficient for full structure elucidation [91], but allows proposing structural hypotheses for a newly formed compound. Further confirmation of the proposed identity or chemical formula of unknown compounds can be achieved by accurate-mass measurements using a time-of-fight (ToF-MS) system or other high-resolution mass analyzers. Current bench-top ToF-MS instruments can now achieve a low-femtomole (fmol) level sensitivity, high resolving power and mass accuracy [25]. However, structural elucidation is not always feasible (only for compounds that easily fragment "in-source" and/or have a characteristic isotopic pattern) [89]. Even more powerful alternatives in terms of confirmatory analysis are the hybrid quadrupole - ToF-MS (QqToF-MS) systems that acquire product-ion spectra with accurate-mass measurements of product ions (precision in the low-ppm range) [25,92,93]. Perez et al. [93] have reported the use of a hybrid quadrupole LIT (QqLIT) as a confirmation technique

in the structural characterization of enalapril and enalaprilat TPs (blood pressure regulators). The confirmation of TPs structures was based on the instrument's MS^3 ability, while the identification relied principally on the use of another hybrid instrument (QqToF), exploiting its ability to determine the accurate mass of (de)protonated molecule and product ions. The combined use of these two techniques (coupled to LC) can be thus a powerful way of elucidating unknown structures of metabolites and transformation products [94]. An alternative to ToF instruments is the recently launched LTQ Orbitrap that combines a conventional linear IT (LIT-MS) with an Orbitrap mass analyzer. This system provides outstanding mass accuracy, mass resolution (greatly higher compared to ToF) and reliable high-sensitivity MS^n performance [25]. However, due to the limited availability of Orbitrap instruments, there are still only very few applications. Finally, the latest advance in MS are LTQ Orbitrap and Fourier transform ion cyclotron resonance (FT-ICR)-MS (exhibiting unsurpassed mass accuracy) systems, but the latter is usually out of reach for most environmental applications due to its high cost [91].

The availability of sophisticated MS instruments (e.g., QqToF-MS enabling accurate-mass measurements, LIT-MS, and QqLIT-MS offering high-sensitivity multiple MS [MS^n] experiments, and LTQ Orbitrap combining both features) has enabled considerable progress to be made notably in characterizing transformation products in environmental samples [27]. Efficient extraction methods combined with selective MS detection allow not only confirmation of the identity of ultra-trace levels of parent compounds in complex matrices but also tracking of unknown compounds such as transformation products of organic pollutants. Furthermore, the lack of analytical reference standards means that accurate quantification of these degradation products is rarely possible [27]. However, even when having at our disposal such sophisticated analytical techniques, it has to be kept in mind that they do not allow for a complete characterization of all organic contaminants, including their TPs, in environmental matrices.

Beside a chemical-driven strategy for assessing the ecological risk from pollutants, it is necessary to explore and to apply new strategies, combining both biological responses and chemical analysis, to identify toxic hotspots, to characterize chemicals likely to cause adverse biological effects, and, finally, to assess the ecological risk of the identified chemicals at relevant spatial scales [4,5].

4. Bioanalytical tools

Linking biological effects to the exposure to specific active agents is often problematic due to the large numbers of compounds present in the environment. Bioassays likely constitute a solution to analyse activities in samples, but cannot identify compounds. At the same time, current chemical-analytical techniques provide excellent sensitivity in the analysis of known compounds, but they cannot give information on potency and will easily miss compounds that were not included in the specific quantification method [95]. In order to draw causal links between effects observed in the environment and to assess the results of chemical analysis, an increasing number of research groups have started to combine biological (mostly bioassays) and chemical techniques. Different types of combined studies can be distinguished [96]. The first type includes surveys based on target analysis of preselected compounds and correlation of the results with findings from biological analysis. In this approach, individual compounds are preselected, so that optimized and validated methods for chemical analysis can be used. The second type of study follows schemes such as Toxicity Identification Evaluation (TIE; established by the US EPA) and effect-directed analysis (EDA). The commonalities and differences between TIE and EDA are discussed comprehensively elsewhere [5]. Those studies aim at identifying chemical stressors without targeting specific compounds.

4.1. Mechanism-based bioassays

Bioassays based on the mechanism of action of target contaminants have emerged as bioanalytical tools to detect chemical toxicants in environmental samples [6]. Particularly, *in vitro* bioassays using cultured cells or microorganisms carrying a reporter gene coupled to specific intracellular receptors have been increasingly used to detect biologically active chemicals in the environment. In principle, such bioassays rely on the activation of an easily measurable reporter gene, whose expression is directly dependent on the concentration of active chemicals in culture medium; then, establishing dose-response curves enables (semi)quantification of contaminant load in a given sample. Practically, such *in vitro* assays are reputed as suitable and powerful screening tools as they are very specific and sensitive and their use in microplate format significantly reduces the volume of sample to be tested.

Development of bioanalytical tool based on bioassays needs a very good knowledge of the mechanisms of action of contaminants to be targeted, both in terms of active chemicals and of toxicological relevance of the measured response. Among the multiple mechanisms identified so far, ligand-receptor interaction leading to gene activation represents a key step in toxicological pathways induced by a number of environmental chemicals. Among the most studied and representative contaminants are for instance the dioxin-like compounds (DLCs), which exert their toxicity through the binding and activation of the aryl hydrocarbon receptor (AhR) and thereby induce a panel of toxicity events, including oxidative stress or genotoxicity. The use of *in vitro* AhR activation allows quantitative detection of DLCs within complex samples [97]. Another well-described example concerns endocrine-disrupting chemicals (EDCs) that mimic steroid hormones by binding to hormone receptors [e.g. estrogen (ER) or androgen receptors (AR)] and modulate expression of target genes involved in cellular hormonal response (reviewed by [98]). A variety of *in vitro* assays have been developed for the detection of DLCs and EDCs, and their application to quantify toxic-equivalent quantities in environmental samples has been successfully demonstrated [99–103]. However, ER and AR mediated activities do not take into

account large diversity of EDCs since several emerging contaminants, such as pharmaceutical compounds, are not ligands of these receptors. More recent studies have explored the potential of less studied nuclear receptors (NRs) to be used as bioanalytical xeno-sensors to detect other classes of contaminants, such as steroids, pharmaceuticals, pesticides, alkylphenols, polychlorobiphenyls (PCBs) and polybromodiethylethers (PBDEs). These include for instance the pregnane X receptor (PXR) [103] or the glucocorticoid receptor (GR) [104].

Generally, bioanalytical strategies are viewed as two-tiered approaches. First, the overall contamination load of whole mixture should be determined and characterised by mass-balance analysis. For this purpose, the use of integrated biological methods is particularly useful since they provide both qualitative information on the type of EDCs (i.e. by the screening of different NR-based bioassays) and quantitative information as amounts of biological toxic equivalents (Bio-TEQ) present in the samples. At this stage, performing targeted quantitative chemical analyses to derive chemistry-based toxic equivalents (Chem-TEQ) allow mass-balance calculation, which determines the overall contribution of targets pollutants on the biological activity of the sample. As a first step, mass-balance analysis is therefore necessary to characterise, at least partially, unknown active samples by determining the contribution of well-known priority pollutants as bioactive compounds. However, only a limited number of targeted ED pollutants can be addressed by this approach, which is often insufficient to formally identify EDCs in complex mixtures, and especially those for which there is no a priori information on the type of chemical classes to be monitored (i.e. ligands of new NRs like PXR).

Thus, the link between the toxic potential effect and the identity of the toxic agent(s) is not easy to establish. In several studies, correlation between toxic-equivalent quantities derived from bioassays and targeted chemical analyses has been often reported for certain classes of contaminants, such PAHs in sediments [101] or steroid estrogens in urban effluents [100]. Such correlation based on mass-balance calculations informs on the potential contribution of priority contaminants on the measured toxic responses, but it remains limited for risk analysis as it is restrained to targeted analyzed compounds and does not formally identify molecules that are indeed responsible for biological activity. Therefore, the structural characterisation of active compounds contained in complex matrices remains a key point to improve the environmental risk assessment. Thus, in the case where chemical analyses do not lead to identification of responsible compounds of activity at specific locations (i.e. mass-balance agreement), the EDA approach can be applied to elucidate unknown causative agents and their combined effects.

4.2. Effect-directed analysis (EDA)

One of the major requirements in the characterisation of the complex mixtures found in the environment is the identification of those compounds causing the effect. It is crucial to reduce the complexity of the mixture to a limited number of candidate compounds and finally to individual toxicants. EDA, combining biotesting, toxicity-based fractionation and chemical analysis, is the most innovating and promising approach to meet this requirement [7]. This approach enables one to detect and to identify both non-target known and unknown toxicant based on their effects on the environment.

EDA involves stepwise fractionation procedures (physicochemical separation methods) that systematically reduce the complexity of the sample by isolating groups of toxicants into individual fractions. At each fractionation step, bioassays identify active fractions, so that non-active fractions can be excluded from further processing. The manipulations are directed by bioassays until it is possible to identify the compounds responsible by chemical analysis. Then, advanced chemical identification techniques (based on mass spectrometry detection) reveal compounds responsible for the adverse effects quantified by the biological analysis. Finally, confirmation steps validate the findings [5]. Recent reviews gave excellent overviews of the possibilities and the limitations of EDA for identifying organic toxicants in the environment [5,105–108].

In this paper, our aim is not to review the EDA approaches, but to highlight the power of EDA compared to other approaches combining chemical and biological analyses through some outstanding examples.

As a first example, Keiter et al. [109] investigated the possible causes of the decline in fish populations in the Danube River, where priority PAHs are still found in high concentrations in the sediment. Using an aryl hydrocarbon receptor-based assay (AhR), they found AhR activity that could not be explained by priority pollutants. This is a case where wrong conclusions would have been drawn if only chemical analysis had been performed on the samples.

The EDA approach was also shown to be a powerful tool for identifying previously unknown toxicant and linking effects observed in bioassays to individual compounds, both in laboratory-test systems [4,7] and in the environment, although mainly focused on surface water quality [4]. In solid matrices, unregulated and oxidized PAHs have been identified as the predominant classes of compounds in the most mutagenic fractions obtained during bioassay-based chemical fractionation of various environmental samples (coastal and estuarine sediments and urban airbone particulate matter) [110–112].

However, while the EDA approach is increasingly utilized in hazard assessment, these studies often focus on few sites and on one or few ecotoxicological endpoints [4]. In the future, the biotesting strategy in EDA should be based upon a battery of bioassays that cover the largest range of modes of action as possible (e.g., the combined use of *in vitro* screening assays for genotoxicity, xenobiotic metabolism, gene expression alterations, and endocrine disruptive activities...), allowing the characterisation of potential alteration of different key biological functions in order to characterize the major toxicants present in studied samples and thereby direct their identification through analytical strategies. Another actual limitative factor in the identification of toxicants is the sensitivity of the analytical methods. Despite the

advances in analytical techniques (Section 3), the sensitivity achieved can be still insufficient to detect all the compounds present in the samples, in particular when regarding hormones, thus preventing the achievement of a quantitative agreement between chemically derived effect estimations and measured biological effects as a crucial basis for reliable conclusions [4]. This was demonstrated by a study assessing estrogenic compounds in complex environmental samples [3]. However, the lack of sensitivity of analytical methods can be overcome by the use of new emerging sampling tools, namely integrative passive samplers (Section 5).

The EDA approach can also be very useful in following the evolution of toxicity during the degradation of a chemical (in the environment or in water treatment processes), together with the identification of the most potent transformation product(s) responsible for the toxicity observed. This approach has been successfully applied for the identification of toxic phototransformation products of pollutants. Brack et al. [7] conducted irradiation of an anthracene suspension with simulated sunlight and a subsequent EDA of toxic photoproducts with respect to the inhibition of the bacterial energy metabolism of *Vibrio fischeri*, reproduction of the green algae *Scenedesmus vacuolatus*, and genotoxicity in the umuC test. The algal toxicity of anthracene was hardly modified by irradiation prior to testing and distributed over all fractions, with emphasis on the fractions containing anthracene-9,10-dione and a photoproduct, suggested to be 10-hydroxyanthrone. Bacterial toxicity and genotoxicity in contrast, emerged only when anthracene was irradiated. Anthracene-1,4-dione, a so-far-unknown trace photoproduct, was identified as a very potent toxicant dominating the toxicity of photomodified anthracene to *V. fischeri*. In genotoxic fractions, 1-hydroxyanthracene-9,10-dione and 1,4-dihydroxyanthracene-9,10-dione were identified and confirmed as genotoxicants. Schultze et al. [113] irradiated a standard solution of diclofenac (analgesic pharmaceutical) with simulated sunlight. Using EDA, they identified and subsequently confirmed 2-[2-(chlorophenyl)amino]-benzaldehyde (CPAB) as a transformation product with a ten-fold enhanced phytotoxicity to the green algae *Scenedesmus vacuolatus*.

When biotesting full extracts of a given sample, matrix effects can prevent a clear readout. Thus, assessing crude extracts by means of cell-based bioassays may be limited due to general (cyto)toxicity that can mask mechanism-specific effects [112,114,115]. In these situations, the issue of overlying toxicity in the crude extract can be overcome by fractionation approaches resulting in a separation of compounds with different modes of actions. In addition, fractionation allows one to highlight: (i) the presence of antagonists present in the full extract; (ii) the synergism/antagonism; and (iii) the concentration additivity of compounds with the same mode of action. As an example, Weiss et al. [116] reported a masking effect of anti-androgens on androgenic activity in European river sediments unveiled by EDA (using a reporter gene assay, the androgen receptor (AR) CALUX assay). Through the use of EDA fractionation strategies, it was demonstrated that androgenic (AR agonistic) and anti-androgenic (AR antagonistic) compounds present in a sediment sample influence each others' interaction with the androgen receptor. The AR agonistic effect appeared only following fractionation of the sample, whereas the crude sample exhibited a high anti-androgenic potency.

Considering sediment or suspended solid matter samples, exhaustive extraction in EDA studies is usually applied to samples in order to obtain information on all the compounds present, ignoring the fact that under normal conditions certain compounds may be more bioavailable than others. By disregarding bioavailability this approach may lead to a biased prioritisation of fractions and toxicants with respect to hazards and risks, resulting in the overestimation of hydrophobic toxicants relative to more hydrophilic ones that are more available. To overcome this problem, it is possible to use as a first step in EDA bioaccessibility-directed extraction methods (e.g., using mild solvent extraction or competitive adsorbants such as TENAX [117], cyclodextrins, etc.) and/or partition-based dosing techniques (i.e. passive samplers, such as SPME, silicon rods [118], SPMD, etc.; Section 5). In a study performed by Bandow et al. [118], the application of partition-based dosing had much influence, suggesting polar compounds such as triclosan (a very common antimicrobial agent) as key toxicants while PAH fractions did not exhibit significant effects. In contrast, conventional solvent dosing prioritized mainly PAHs. Using extraction methods that mimic bioavailability instead of total extraction may improve key toxicant prioritisation by considering exposure and effect rather than effect only, and thus improve the environmental realism of EDA [106]. In addition, EDA (or other bioanalysis approaches) can be combined with the use of passive samplers which allow for the concentration of bioavailable waterborne pollutants (the end of the following Section 5) to form an integrated EDA scheme for the detection and identification of readily bioconcentratable toxicants in waters.

These studies are excellent examples that stress the potential of EDA for integrated environmental assessment. Within the last decade, increasingly, attempts were made to improve EDA approaches [108], e.g., by: (i) its combination with the use of passive samplers [118,119] (see above and also Section 5); (ii) including fractionation methods for more polar compounds [119–121] or high-resolution fractionation (e.g., preparative capillary gas chromatography) [122]; (iii) methods for addressing the bioavailability within the fractionation approach [117,118]; (iv) incorporating structure generation and mass spectral classifiers for identifying of unknown substances [107] and structure-dependent effect prediction (e.g., QSAR), and the use of additional biological endpoints [103,123–125].

Establishment of causal links between chemical contamination and observed ecotoxic effects of environmental samples is a major challenge in ecotoxicology and the EDA approach is a powerful tool to take up this challenge. EDA not only helps to focus on samples that have an effect on a chosen endpoint, but more importantly allows testing to be performed without prior knowledge of which chemicals to expect [7]. Thus, the power of EDA lies in its suitability to reveal the emergence of novel environmental pollutants.

Chemical identification and correlation between effects and concentration will provide evidence of the main stressors and possible mitigation measures in order to improve the ecological status of different ecosystems [4].

5. Integrative passive sampling

Recent legislations (e.g., WFD, REACH) have increased the pressure to obtain better information than that provided by classical infrequent spot sampling. The growing concern among the public and researchers together with the implementation of these regulations in the European Union will increase the future demand on monitoring frequency, geographical distribution of the measurements and the number of sites included in monitoring programs and routine measurements. Thus, the need for simple, low cost strategies for monitoring and risk assessment of (polar notably) pollutants in aquatic environments will probably be high in the future. In the perspective to have at our disposal analytical methodologies compatible with the demands in terms of rapidity, quantification limits, number of treated samples, and reliability of modern environmental screening analysis, it is now possible to develop and use new integrative passive sampling devices.

Checking water quality compliance with regulatory provisions has been based on the chemical analysis of spot (bottle or spot) samples of water taken at a defined frequency [119]. This approach suffers from several drawbacks. Spot samples provide concentrations of pollutants only at the moment of sampling. Thus in water bodies characterized by marked temporal and spatial variability there is an increased risk of a false classification of the chemical status. On the other hand, the use of on-site automated sampling systems can be costly and difficult to maintain. Further, the laboratory methods commonly used for the analysis of spot samples of water are often not sensitive enough to fulfil the required minimum performance criteria associated with the current environmental quality standards for many priority pollutants. Moreover, these techniques are time-consuming and require a qualified staff [119].

A promising alternative method for monitoring pollutants in aquatic systems is based on the use of passive sampling techniques. In comparison with spot sampling techniques, passive samplers provide a more representative picture of the water quality. This is achieved by the integrative sampling of contaminants during sampler deployment periods up to several weeks. Passive sampling devices ensure continuous diffusion of the pollutant from the bulk water phase to the receiving phase in order to sample and to concentrate trace levels of pollutants. A range of passive samplers has been developed for monitoring organic pollutants in water. Their different designs and field performance have been reviewed [9,126–128]. Among the most widely-used passive samplers, it can be mentioned Semi-Permeable Membrane Devices (SPMDs) for hydrophobic organic compounds [129,130], Polar Organic Contaminant Integrative Sampler (POCIS) for hydrophilic organic compounds [131–133], Diffusive Gradient in Thin films (DGTs) for labile metals [134], and Chemcatchers for either hydrophilic organic compounds, or hydrophobic organic compounds, depending on the selected configuration [135,136].

Passive samplers present numerous advantages compared with conventional spot sampling of waters. First of all, they allow a simplified logistics which permits to increase the frequency of monitoring and/or the number of sampling sites: *in situ* extraction of compounds from water allowing one to avoid some difficulties in spot sampling (high sampling volumes, potential contamination or alteration of water samples, representativeness...); simplified and partially automated analytical protocols; low cost, no power requirement...).

Furthermore, where information on environmental levels, behaviour, and fate are needed, passive samplers can provide representative measurements of time-weighted average (TWA) concentrations, average concentrations that could be obtained by spot sampling only when used at a prohibitively expensive high frequency. An approach for providing a time-weighted average assessment is critical for an improved understanding of the consequences of prolonged exposure to environmental contaminant mixtures. Another advantage is that the masses of substances accumulated during deployment can ensure that the analytes fall within the range of quantification [137]. Hence, the detection of compounds present in water at concentrations lower than analytical detection (or quantification) limits, such as metabolites of some pollutants or steroid hormones though exhibiting toxicity at such levels [87,138] can be envisaged.

Passive samplers for non-polar organic compounds (SPMD, Chemcatcher) have been widely used to monitor PAHs and their derivatives, PCBs, persistent non-polar pesticides, or organotin compounds in many types of waters [87,139–145], as well as emerging contaminants such as musk xylenes and ketones, PBDEs or polychlorinated naphthalenes [146–148]. Passive samplers for polar organic compounds (POCIS, Chemcatcher) already provided valuable data on the identification of chemicals in water, in particular emerging contaminants (mostly using POCIS): numerous compounds among various pollutant classes have been detected in wastewaters and surface marine and freshwaters, such as pesticides [119,132,133,149], alkylphenols and their polyethoxylated derivatives [119,150–156], PAHs [119,157,158], pharmaceuticals [119,159–161], hormones [119,162], and personal-care products (PCPs, such as UV filters, musk fragrances, triclosan) [163]. In the near future, passive samplers will be used as identification tools; for example, POCIS samplers could be an adequate tool to get access to the transformation products. Thus, combining the high capability of integrative passive samplers to concentrate organic pollutants, in particular emerging contaminants and transformation products using POCIS, with molecular identification analytical techniques such as gas or liquid chromatography coupled with high-resolution mass spectrometry (Section 3) would allow one to have sensitive and rapid screening methodologies. As an example, POCIS deployed in fish farm cage systems combined with chemical analysis using LC-QLIT-MS for the screening of

micropollutants in marine aquaculture allowed the detection of several pesticides [164].

Moreover, passive sampling provides a measure of the freely dissolved and biologically available fraction of the substance. This is more ecotoxicologically relevant information than either total concentration in unfiltered spot samples, or filtered concentrations [137]. The latter are to a large extent defined by the filtration process used. Some field and laboratories have been designed to compare passive sampling techniques with biomonitoring [165–168]. As an example, POCIS has been shown to provide an integrated and biologically meaningful measure of estrogenicity in that they accumulate estrogens in a pattern similar to that of caged brown trout [166]. Passive samplers have the potential to replace the use of living organisms in assessing bioavailability since they have a number of advantages including lower cost, greater repeatability, smaller variability, and greater acceptability on ethical grounds [137]. Similarly, other passive sampling devices [e.g., silicone rubber, low density polyethylene strips (LDPE strips)] are a solution under development as an integrated tool in the assessment of micropollutant availability in sediments [169–171]. Deployed at the sediment-water interface, they provide information on both (bio)available amounts in sediment samples and also equivalent available aqueous concentrations of pollutants, via the determination of the non-exchangeable and the mobile fractions of pollutants accumulated in sediments.

Last but not least, in addition to instrumental analysis of pollutants, sampler extracts can be subjected to toxicity testing using bioassays that give information on toxic and ecotoxic risks associated with the sampled substances (substances being identified or not) [127]. Passive samplers have been used in combination with toxicity assays either to determine total toxicity of the pollutants in a water body [154,166,172–176], or in combination with an EDA approach to identify the toxic fractions among the many compounds accumulated during deployment. This latter approach has been applied to detect substances with estrogenic activity in a number of rivers in Germany and the UK [177] or to identify potential environmental hazards from compounds accumulated in POCIS samplers deployed in a French river by using several *in vitro* bioassays that detect endocrine-like and dioxin-like compounds [103,119].

Integrative passive samplers allow compensating for both the non-detection of low pollutant levels and the limitations of classical sampling. It may thus be possible to develop strategies based on passive sampling that will provide protection from possible environmental damage while minimising operational costs and improving representativeness and reliability of the data obtained [9]. Passive samplers have the potential to help in providing robust information on which decisions to approve registration applications can be based. Furthermore, they can collect extracts of chemicals for either chemical analysis or bioanalysis. As aforementioned, there are also advantages to be gained from combining the use of passive sampler extracts and bioanalyses, and in particular EDA.

6. Conclusion

One of the key challenges in environmental chemistry and ecotoxicology is to characterize and identify toxicants causing effects in the environment. However, many of the current bottlenecks in the assessment of organic contaminants in our environment concern the difficulty to evaluate diverse chemical classes and biological effects within complex mixtures. Classical chemical-analytical techniques are often not suitable to face this challenge since a vast number of chemicals, including "old" and emerging pollutants as well as their transformation products, can occur in the environment. Without prior knowledge about the toxicants present in a sample, their identification with chemical analysis alone is often prohibitively expensive or becomes rather a guessing game [44].

To tackle these analytical challenges, the bioanalytical concept has emerged during the last decade. In particular, mechanism-based *in vitro* bioassays using recombinant cell systems in which specific biological effects are directly measured can provide valuable information about the expected total potency of the complex mixture of chemicals in an environmental sample [6]. Combined biological and chemical-analytical approaches provide an important progress towards an estimation of the portion of an effect that can be explained by the analyzed chemicals. However, the approach does not provide a possibility to identify unknown causes of effects. The most promising approach to solve this problem is the sequential combination of toxic syndrome-related bioassays, fractionation procedures and chemical analysis referred to as EDA [4].

Recently, TIE and EDA approaches aiming to pinpoint the toxicant responsible for observed effects have gained increased interest in ecotoxicological studies and environmental risk assessment. The EDA approach can be applied to elucidate unknown causative agents and their combined effects. The identification of active agents is then a first and vital step towards the identification of their sources and a proper evaluation of their ecotoxicological potency in exposed organisms. EDA may also significantly help to implement the WFD by providing evidence on the main stressors and possible mitigation measures in order to improve the ecological status of aquatic ecosystems. Better understanding of causes is the only way to apply effective corrective measures and avoid waste of resources.

In addition, combining such integrated bioanalytical approaches with passive sampling instead of classical spot water sampling could also help to improve environmental risk assessment; by their ability to concentrate bioavailable sediment and waterborne pollutants (and thus to take also into account of pollutants undetectable by conventional methods), these integrative passive devices allow increasing representativeness and reliability of the data obtained. Further issues are the prioritisation of emerging substances, inclusion of transformation products and chemical mixtures in environmental risk assessment, the long-term presence of xenobiotics bound to soils and sediments, as well as an understanding of the ecological relevance of ecotoxicological end points [10,178–180].

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