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Targeted and non-targeted liquid chromatography-mass spectrometric workflows for identification of transformation products of emerging pollutants in the aquatic environment

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ABSTRACT

Identification of transformation products (TPs) of emerging pollutants is challenging, due to the vast number of compounds, mostly unknown, the complexity of the matrices and their often low concentrations, requiring highly selective, highly sensitive techniques. We compile background information on biotic and abiotic formation of TPs and analytical developments over the past five years. We present a database of biotic or abiotic TPs compiled from those identified in recent years. We discuss mass spectrometric (MS) techniques and workflows for target, suspect and non-target screening of TPs with emphasis on liquid chromatography coupled to MS (LC-MS). Both low- and high-resolution (HR) mass analyzers have been applied, but HR-MS is the technique of choice, due to its high confirmatory capabilities, derived from the high resolving power and the mass accuracy in MS and MS/MS modes, and the sophisticated software developed.

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1. Introduction

The term “emerging pollutants” (EPs) [or “emerging contaminants” (ECs)] refers to compounds and their metabolites that are not currently covered by existing water-quality regulations, have not been studied often, are overlooked and are thought to be

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potential threats to environmental ecosystems and human health and safety. According to NORMAN (Network of reference laboratories, research centers and related organizations for monitoring emerging environmental substances), they are compounds that are not included in routine environmental monitoring programs and may be candidates for future legislation due to their adverse effects and/or persistency (<http://www.norman-network.net/>). Most regulating and implementation bodies, responsible for water and wastewater treatment, are working on the assumption that the so-called priority pollutants are responsible for the most significant share of environmental, human health and economic risk, even though they represent a minor fraction of the universe of known and yet-to be identified chemicals [1].

EPs encompass a diverse group of compounds, including pharmaceuticals and personal-care products (PPCPs), drugs of abuse (DoAs) and their metabolites, steroids and hormones, endocrine-disrupting compounds, surfactants, perfluorinated compounds, phosphoric ester flame retardants, industrial additives and agents (e.g., benzotriazoles and benzothiazoles), siloxanes, artificial sweeteners, and gasoline additives.

Once released into the environment, EPs are subject to biotic and abiotic transformation processes that are responsible for their transformation and/or elimination, according to their persistence, transport, and ultimate destination. Various transformations can take place, producing compounds that, to some extent, differ in their environmental behavior and ecotoxicological profile from the parent compound. Formation of transformation products (TPs) occurs mainly through oxidation, hydroxylation, hydrolysis, conjugation, cleavage, dealkylation, methylation and demethylation. EPs and their TPs can move vertically through the soil profile to groundwater and away from the source site with mobile groundwater. They also have the potential to reach surface water when they travel laterally as surface run-off or through sub-soil tile drains, entering streams, major rivers, reservoirs, and ultimately estuaries and oceans [2].

Since there is a gap in the information on the occurrence and the toxicity of TPs in the environment, we are unable to evaluate their significance in risk assessment [3,4]. Standardized toxicity tests can provide quantitative information on the toxicity of the TP, compared to its parent compound, but these studies are limited [5–7]. In general, TPs are less toxic and more polar than the parent compounds. However, in some cases, they may be more persistent or exhibit higher toxicity or be present at much higher concentrations [8].

Although there is legislation regulating chemicals [e.g., pesticides, veterinary drugs, and persistent organic pollutants (POPs)], there is little mention of their TPs. Concerns over the TPs of pesticides in plants have been expressed since 1991 (European Directive 91/414/EEC), while the term “metabolite” appears in Regulation (EC) 1107/2009, concerning plant-protection products, and in Directives 2001/82/EC and 98/8/EC, concerning veterinary medical and biocidal products, respectively. European Medicines Agency (EMA, 2006) also referred to the need for assessment of potential environmental risks of human medicinal products. However, in all these documents, there is no clarification on the determination, limits and toxicological effects of metabolites or TPs.

In OECD guidelines, concerning the Aerobic and Anaerobic Transformation in Aquatic Sediment Systems, adopted in 2002, it is claimed that TPs detected at $\geq 10\%$ of the applied radioactivity should be identified. Meanwhile, EU Regulation 1907/2006 (REACH) requires identification of major TPs and degradation products for the registration of the substance. In the Regulation (EC) 850/2004 on POPs, a reference to their transformation processes also exists.

There is therefore a clear need to reveal the qualitative and quantitative occurrence of TPs in the environment, but this is only possible

with continual development of instrumental analysis. Thereby, the range of identifiable chemicals is extended, and the quantification limits are lowered. With respect to obtaining a holistic view of risk, target-based environmental monitoring should be accompanied by non-target analysis using high-resolution (HR) hybrid mass spectrometers. The development of these highly resolved, accurate, hybrid, tandem mass spectrometers, and improved sophisticated software, has enabled more reliable, selective target analysis of highly polar compounds, and screening for unknown pollutants. The major benefit of full-scan and HR, accurate MS is that, within a single analytical run, target, suspect and non-target compounds can be analyzed or identified.

In the analysis of EPs, HR mass spectrometry (HR-MS) has been widely reported [3,9–12]. Moreover, for identification of TPs in environmental, food and biological samples, hybrid HR mass analyzers [e.g., linear ion trap Orbitrap MS and quadrupole time-of-flight MS (Q-TOF)] have been used, following specific workflows [12–16]. More specifically, human and microbial metabolites, oxidation and photodegradation TPs of pharmaceuticals have been discussed often [17–21]. Similarly, TPs of pesticides in biological (human metabolism, phase II), food and environmental samples have been reviewed [22,23]. Furthermore, the TPs of anthelmintics [24], UV filters in the environment [25,26] and steroidal compounds in biological samples [27] are included in recent review papers. An interesting fact concerning the analysis of EPs and their TPs is enantioselective biotransformation. Chiral EPs or chiral TPs formed may have enantioselective activity or toxicity, making chiral chromatography indispensable [28].

Achievements, future trends and new developments in the analysis of EPs and their TPs were summarized by Farré et al. [29] and Fisher et al. [30]. Recently, highly sophisticated, comprehensive, step-wise workflows were also presented by Moschet et al. [31] and Hug et al. [32] for suspect and non-target screening of pesticides and EPs, including TPs in their suspect lists. However, it is still challenging to profile TPs in environment samples, since they are formed through many possible reactions, automatic workflows for the identification are not readily available, so manual data inspection is necessary, though time consuming, and, finally, there are no standards available.

The aim of this review is to compile the recent information regarding the background of (biotic and abiotic) transformation of EPs. We provide a brief overview of existing literature on transformation studies under biotic and abiotic conditions in recent years and we compile a list of all the EPs studied and comprehensive information for researchers in the field. We briefly summarize target analysis, since the development of accurate mass instruments and sophisticated computer tools has led to suspect and non-target analysis, even though all three procedures are indispensable parts of an integrated approach to determination of EPs and their TPs. We present the design of laboratory studies to facilitate identification of TPs by LC-MS and appropriate sample preparation. We thoroughly discuss target, suspect and non-target workflows using HR-MS/MS to identify new TPs.

2. Classification of transformation products (TPs)

TPs occurring in the environment can be classified into two main categories: biotransformation products formed by biotic or abiotic processes. This classification has subcategories that we describe in detail below, emphasizing the aquatic environment. The biotransformation products include human, animal and microbial metabolites in engineered and natural systems. The abiotic TPs are the outcome of hydrolysis, photolytic and photocatalytic degradation in the natural environment and water-treatment processes (e.g., chlorination, ozonation and advanced oxidation).

2.1. Transformation products formed by biotic reactions

TPs are formed by microbial activities in natural and engineered environmental compartments, such as soil, surface water or wastewater treatment. Enzymatic reactions are involved in microbial transformation [such as, oxidation (e.g., hydroxylation, N- and S-oxidation, and dealkylation) and reduction (e.g., dehalogenation, nitro reduction, and hydrolysis of amides and carboxyl esters)]. As mentioned, pharmaceuticals, drugs of abuse and other chemicals consumed by humans and other mammals can be metabolized and then released into the environment as metabolites. For mammals, besides oxidation and reduction, conjugation reactions also occur with endogenous molecules, such as carbohydrates, sulfate, glutathione and amino acids.

Some environmental pollutants are significantly accumulated and subsequently transformed in wildlife. In particular, aquatic organisms are considered the primary receptors, and might show qualitatively and quantitatively different metabolic pathways compared to microbes and humans. Hence, the metabolites formed in aquatic organisms and their toxicity are of increasing ecotoxicological concern.

In the sections below, we discuss biotransformation products, classified into three groups, and we present findings from recent studies, with a clearly stated identification workflow in Table 1. Table S1 (Supplementary Material) shows an extensive list of all the biotransformation products of EPs presented so far in the literature.

2.1.1. Transformation products formed by microbial metabolism

Due to the increasing occurrence of EPs in wastewaters, the formation of TPs during biological treatment and the mechanisms involved have been investigated for various classes of compounds (i.e., antibiotics [35,45,54], analgesics (painkillers) [39], anticonvulsants [37], anti-inflammatories [40,41], iodinated X-ray contrast media (ICM) [46,47] and anti-viral/-bacterial/-fungal agents [33,56,57]). Most of the studies were performed in batch systems with activated sludge.

In almost all the studies, oxidative reactions, such as hydroxylation, oxidation, and dealkylation, were observed as the primary biotransformation mechanisms. Hydroxylated metabolites were identified for triclosan [57], codeine [39], diclofenac [40,41], sulfapyridine [54], and a UV filter [48]. In some cases, oxidation of hydroxyl groups was followed by oxidative decarboxylation, deacetylation, and dealkylation taking place at the amide moieties [33,46,47]. Molecules with N-, O-, or S-alkyl groups were probably transformed to dealkylated forms, as observed for naproxen [53], triclosan [57], diclofenac [41] and ICM [46,47]. Other oxidative reactions included ring opening, oxidative deamination and oxidative dechlorination [34,45,50,56].

Helbling et al. intensively investigated microbial transformation of 30 xenobiotic compounds with amide groups and observed 53 TPs resulting from amide hydrolysis, N-dealkylation, hydroxylation, oxidation, dehalogenation, glutathione conjugation and many more pathways [34]. Moreover, the hydrolysis rate and the dominant reaction related to the degree of alkyl substitution of the amide group.

Other studies investigated predictive factors of the biotransformation reactions. Ammonia removal and ammonia monooxygenase (amoA)-transcript abundance can be associated with oxidative micropollutant-biotransformation reactions, without necessarily being catalyzed by amoA [58].

Meanwhile, reduction reactions predominantly take place under anaerobic conditions, such as reductive dechlorination of 5-chlorobenzotriazole and chlorpromazine and dehydration and hydration of testosterone [38,55]. However, nitro reduction of N,N-diethyl 1–4-nitrobenzamide was evident under aerobic conditions in a recent study [34].

Apart from common oxidation and reduction, other reactions, such as decarboxylation and deacetylation, were reported less frequently [46,47]. In addition, conjugation reactions, such as phosphorylation, succinylation and glutathione substitution, are also possible biotransformation mechanisms [34,45].

The biodegradability of perfluorinated compounds (PFCs) attracts the interest of scientists. A number of studies presented the degradability and the fate of PFC precursors, such as fluorotelomer alcohols in sediments resulting in perfluorinated carboxylic acids [43,44]. Nevertheless, no TPs of perfluorooctanoate (PFOA) have been reported, stressing that these compounds are very stable and hardly degradable [59,60].

2.1.2. Transformation products (metabolites) formed by human metabolism

Even though human metabolites of PPCPs and their mechanisms of formation have been extensively studied in pharmacology, limited information on their occurrence and stability in the environment is available. However, human and microbial metabolisms partly present the same metabolic reactions and thus the same metabolites, so their discrimination in environmental samples is sometimes difficult. Kern et al. stated that six pharmaceutical TPs found in surface-water samples were known human metabolites of metamizole, aminopyrine, carbamazepine and verapamil from registration files [51]. However, four of these six metabolites were formed through epoxidation, dihydroxylation and O-demethylation, which can also take place in microbial metabolism. Perez and Barceló reported that hydroxylation products of diclofenac and aceclofenac, known as both human and microbial metabolites, were measured in wastewater samples [41].

Mass balances of influent and effluent samples can clarify the origin of the TPs in more detail. In the tiered approach proposed by Kern et al., batch experiments with activated sludge can be used to verify the findings and to quantify transformation rates [52].

For estrogenic compounds, the metabolites formed are glucuronide and sulfate conjugates and are frequently detected in untreated wastewaters [61]. However, the conjugated estrogens are vulnerable in aerobic activated sludge and end up as free estrogens after de-conjugation [62]. The de-conjugation behavior is also observed for pharmaceuticals, resulting in negative removal efficiencies [63,64]. However, deconjugation reactions happen with different reaction rates. For example, for estrogens, sulfate conjugates are reported to be more persistent than glucuronides. In case of lamotrigine, N-glucuronide metabolite has frequently been detected in wastewater, surface-water and groundwater samples, unlike O-glucuronide, due to the difference in degradability of O- and N-glucuronide products [65].

2.1.3. Transformation products formed in wildlife

EPs are ubiquitous in the environment due to their low degradability, and probably accumulate in biota (bioaccumulation) and sediments. Biotransformation in organisms, as a subsequent process of bioaccumulation, is of great interest in order to clarify the fate and the toxicity of those compounds and their TPs. However, studies of drug metabolism in fish are extremely limited and the metabolic pathway and enzymes responsible for the metabolism of the drugs in fish are largely unknown [66].

Moreover, metabolites of POPs have been measured in various tissues (e.g., blood, blubber, fat, and bird eggs) and in marine organisms (including fish), but, in many cases, it is not known if the compounds formed are the result of *in-vivo* metabolism or are bioaccumulated from the environment [67].

In a study, oxidation reactions *in vitro* were hindered by the increased number of bromine substituents and hydroxylated metabolites and oxidative bond-cleavage products are formed in fish

Table 1
Identified biotransformation products of emerging contaminants in the aquatic environment

Parent compound/Group of substances	(Bio)assay/Test organism/ Degradation system	Identified TPs/Transformation reaction	Toxicity Information	Identification workflow			Ref.
				Sample preparation	Instrumental technique(s)	Software (prediction/ post data treatment)	
Acyclovir (ACV), Penciclovir (PCV)	Batch systems seeded with activated sludge	ACV: carboxy-ACV, PCV: 8 TPs (oxidation)	–	SPE (Isolute ENV+)	HPLC-LTQ-Orbitrap-MS ⁿ , 1D & 2D NMR	UM-PPS, Thermo XCalibur, fragmentation pattern	[33]
30 Amide-containing compounds	Batch system seeded with sludge from a pilot-scale membrane bioreactor	53 TPs: amide hydrolysis and N-dealkylation, hydroxylation, oxidation, ester hydrolysis, dehalogenation, nitro reduction, and glutathione conjugation/kinetic study	–	–	LC-MS Orbitrap, data dependent MS/MS acquisition	UM-PPS, Thermo XCalibur, post-acquisition data processing of the full-scan MS data (mass accuracy, isotope matching and MS/MS evaluation)	[34]
Amoxicillin	Laboratory alkaline & acidic hydrolysis study with wastewater and river water	4 TPs: b-lactam ring cleavage	–	SPE (OASIS TM HLB)	LC/QTOF-MS/MS	Agilent MassHunter Workstation, mass accuracy, XIC, fragmentation pattern	[35]
Benzotriazoles: 1H-BTR 5-CH ₃ -BTR 4-CH ₃ -BTR	Batch studies under aerobic conditions with sludge	42 candidate TPs – 4 confirmed TPs: 1-CH ₃ -BTR, 4-OH-BTR, 5-OH-BTR (from 1H-BTR) and 5-COOH-BTR (from 5-CH ₃ -BTR). Hydroxylation at the benzene ring was the major pathway	–	–	LC-MS Orbitrap, data dependent MS/MS acquisition	Suspect screening after UM-PPS, non-target screening; Thermo XCalibur, post-acquisition data processing of the full-scan MS data; In silico fragmentation by MetFrag; MOLGEN-MS/MS for structure annotation	[36]
Carbamazepine	Aqueous medium from air pulsed fluidized bioreactor and from culture broth	Acridone, acridine, 10,11-dihydro-10,11-epoxycarbamazepine & dihydroxycarbamazepine	Acute toxicity test: <i>Vibrio fischeri</i> luminescence reduction: nontoxic neither readily nor inherently biodegradable – toxic to anaerobic sludge	SPE (OASIS TM HLB)	UPLC/ESI-QqToF - HPLC/ESI-QqLIT -	Waters MassLynx, XIC, mass accuracy, fragmentation pattern	[37]
Chlorpromazine (tricyclic antipsychotic drug)	OECD: Closed Bottle test, Manometric Respirometry test, modified Zahn-Wellens test, anaerobic test	TPs: 3 aerobic & 1 anaerobic (hydroxylation, demethylation, acetylation)	–	–	HPLC-UV-Fluorescence-IT MS ⁿ	fragmentation & isotopic pattern, Bruker Esquire software, Metabolite Detect (Metabolite Tools), Data Analysis	[38]
Codeine (Opium Alkaloid)	Aerobic batch experiments seeded with activated sludge	8 TPs: double bond shifts, introduction of hydroxy groups, amine demethylation – transformation pathways suggested for structurally related opium alkaloids (morphine & dihydrocodeine)	–	SPE (OASIS TM MCX)	HPLC-LTQ-Orbitrap-MS ⁿ & HPLC-Qq-LIT-MS, data dependent acquisition, 1D & 2DNMR	fragmentation pattern, Thermo Xcalibur	[39]
Diclofenac	fungus <i>Trametes versicolor</i> – in vivo and in vitro experiments with cytochrome P450 and laccase pilot MBR	4-hydroxydiclofenac, 5-hydroxydiclofenac (hydroxylation) & 4-(2,6-dichlorophenylamino)-1,3-benzenedimethanol	Decrease in ecotoxicity (Microtox test)	–	HPLC-UV, NMR	–	[40]
Diclofenac, Aceclofenac	–	3 novel TPs: nitrosation, nitration, N-dealkylation and carboxylation	–	SPE (Oasis TM HLB, Isolute ENV+)	HPLC/ESI-QqLIT-MS, UPLC/ESI-QqTOF-MS, H/D-Exchange Experiments	fragmentation pattern, Waters MassLynx	[41]
Flame retardants: 4,4-dibromodiphenyl ether (BDE 15), 2,2,4,4-tetrabromo-diphenyl ether (BDE 47), tetrabromobisphenol A (TBBPA)	in vitro – liver microsomes and S9 fractions of crucian carp	BDE 15: Bromophenol, 2monohydroxylateddibromodiphenyl ethers TBBPA: 2,6-dibromo-4-isopropyl-phenol	–	LLE	HPLC-QqQ-MS/MS, GC-IT-MS/MS	fragmentation pattern, Thermo XCalibur	[42]
Fluorotelomer alcohols (6:2 FTOH)	Batch study with river sediment	5:3 acid, PFPeA, PFHxA, and PFBA, 5:2 sFTOH	–	ENVI TM -Carb (graphitized carbon)	LC-MS/MS (QqQ) -SRM technique	–	[43]

(continued on next page)

Table 1 (continued)

Parent compound/Group of substances	(Bio)assay/Test organism/Degradation system	Identified TPs/Transformation reaction	Toxicity Information	Identification workflow			Ref.
				Sample preparation	Instrumental technique(s)	Software (prediction/post data treatment)	
5:3 Polyfluorinated acid (major TP of 6:2 FTOH & 8:2 FTOH)	Activated sludge	4:3 acid, 3:3 acid, one-carbon removal pathways(-CF ₂ -), perfluoropentanoic acid (PFPeA), perfluorobutanoic acid (PFBA)	–	C18 cartridge, ENVI™-Carb	HPLC-LTQ Orbitrap MS/MS	Mass defect data filtration, mass accuracy	[44]
Fluoroquinolones (norfloxacin, ciprofloxacin) Macrolides (azithromycin, erythromycin, roxitromycin)	Wastewater effluents from Zenon hollow-fiber membrane bioreactor (MBR)	fluoroquinolones: conjugate compounds formed by phosphorylation, (phase II metabolites) macrolides: conjugates formed by succinylation of the piperazine ring & smaller metabolites formed by oxidative break-up of piperazine moiety	–	SPE (OASIS HLB)	UHPLC-QTOFMS, MS/MS acquisition	Waters MassLynx	[45]
Iodinated X-ray Contrast Media (iohexol, iomeprol, iopamidol)	Batch reactor seeded with secondary wastewater effluents	27 TPs: oxidation, oxidative decarboxylation, deacetylation, cleavage at the amide moieties	–	SPE (Isolute ENV+)	HPLC-Qq-LIT-MS, MS/MS, NMR	fragmentation pattern	[46]
Iodinated X-ray Contrast Media (diatrizoate, iohexol, iomeprol, iopamidol)	Aerobic soil-water and river sediment-water batch systems	7 novel TPs: Oxidation, cleavage of N-C bonds and decarboxylation	–	–	HPLC-Qq-LIT-MS, MS/MS	fragmentation pattern	[47]
4'-methylbenzylidene- camphor (4-MBC)	Fungus <i>Trametes versicolor</i>	Hydroxylated and pentose-conjugated TPs	No endocrine disruption and dioxin-like activity	Pressurized liquid extraction (PLE)	UPLC-QqTOF-MS/MS	fragmentation pattern, Waters MassLynx	[48]
Metformin	Closed Bottle test, Manometric Respiratory test, Zahn-Wellens test with activated sludge	Guanyl urea (dealkylation & oxidative deamination)	–	–	HPLC-Ion Trap MS ⁿ	Bruker Esquire	[49]
6 Pharmaceuticals & 6 Pesticides (atenolol, bezafibrate, diazepam, levetiracetam, oseltamivir, valsartan & carbetamide, clomazone, DEET, napropamide, propachlor, tebutam)	Batch reactors seeded with activated sludge	21 TPs from suspect screening & 26 TPs from non-targeted screening	–	–	HPLC-LTQ-Orbitrap, data dependent MS/MS acquisition	UM-PPS (suspects), Thermo XCalibur, detailed post-acquisition data processing of the full-scan MS data (target screening, non-target screening)	[50]
52 Pesticides, Biocides, Pharmaceuticals	Surface water	19 plausible TPs identified	–	SPE (100 mg Strata-X-AW, 100 mg Strata-X-CW, 150 mg Isolute ENV+, 200 mg Oasis HLB)	HPLC-LTQ-Orbitrap MS/MS	UM-PPS, Thermo Xcalibur, Mass Frontier, mass accuracy, fragmentation pattern, isotopic pattern	[51]
Pharmaceuticals: atenolol, bezafibrate, ketoprofen, metoprolol, ranitidine, valsartan, venlafaxine, carbendazim	Sludge-seeded batch reactors	12 TPs	–	SPE (Strata-X-AW, Strata-X-CW, Isolute ENV+, Oasis HLB)	HPLC-LTQ-Orbitrap, data dependent MS/MS acquisition	UM-PPS, Thermo Xcalibur, detailed workflow for the identification of biotransformation products	[52]
Acidic pharmaceuticals (ketoprofen, bezafibrate, naproxen, ibuprofen, diclofenac)	Activated sludge as inoculum under aerobic conditions	Ketoprofen: 2 novel TPs Bezafibrate: 4-chlorobenzoic acid Naproxen: O-desmethyl-naproxen Ibuprofen: 2 isomers of hydroxy-ibuprofen	–	ion-pair solid phase extraction (IP-SPE)	LC-ESI-MS/MS (QqQ), Full scan MS, Product ion scan, LC-UV	–	[53]
Sulfapyridine Sulfathiazole	Fluidized bed reactor with <i>Trametes versicolor</i> seeded with sterilized sewage sludge, in vitro and in vivo assays with purified laccase	7 TPs formylated and hydroxylated	–	–	UPLC-QqTOF-MS	mass accuracy, fragmentation pattern	[54]
Testosterone	Gamma proteobacterium <i>Steroidobacter denitrificans</i>	10 TPs (dehydrogenation, hydrogenation)	–	–	HPLC-APCI-LTQ-Orbitrap, MS/MS, LC-UV, LC – ion trap – MS, NMR	mass accuracy, fragmentation pattern	[55]
Triclosan	laccase in aqueous systems	2,4-dichlorophenol (2,4-DCP), dechlorinated forms of 2,4-DCP, 2-chlorohydroquinone	Detoxication by enzymatic transformation	–	GC-IT-MS, HPLC-ESI-QqQ, MS/MS, LC-UV	XIC	[56]

liver from 4,4'-dibromodiphenyl ether and tetrabromobisphenol A, but not from higher brominated flame retardants [42].

Recently, metabolites of polar organic pollutants formed in freshwater crustaceans, used in risk assessment, were identified [68,69]. Jeon et al. found 25 metabolites of irgarol, terbutryn, tramadol and venlafaxine in *Gammarus pulex* and *Daphnia magna*, formed via oxidation reactions including N- and O-demethylation, hydroxylation, N-oxidation and glutathione conjugation [68]. This shows the relevance of metabolism in wildlife.

2.2. Transformation products formed by abiotic processes in aquatic systems

Abiotic TPs are formed by water-treatment processes and in the aquatic environment by hydrolysis and photolysis. Table 2 shows research papers studying the formation of TPs from EPs under abiotic conditions through one of the above processes, from the past three years, with a clearly described identification workflow. Table S2 (Supplementary Material) shows a more extensive list of studies. Also, review papers provide information for TPs of antibiotics and estrogens already identified, covering a wide range of abiotic processes [76,77].

Oxidation processes, such as chlorination, chloramination, ozonation, and advanced oxidation by UV/H₂O₂ treatment, are the major processes used in advanced water treatment for disinfection and removal of ECs [8]. The oxidative reaction mechanisms often rely on the formation of reactive, short-lived oxygen-containing intermediates, such as hydroxyl radicals (\bullet OH). Generally, the TPs formed correlate with the process conditions [e.g., the physico-chemical properties of the matrix, and the specific conditions of treatment (e.g., time and medium)].

Ozone is a strong oxidant that can be used as a more selective agent for the removal of micropollutants. Ozonation may take place by the direct reaction of the ozone molecule with the target compound or by means of hydroxyl radicals produced from the decomposition of ozone in aqueous media. In practice, both direct and indirect reactions take place simultaneously. Ozone was recently used as a fourth full-scale treatment step in wastewater treatment [78]. Next to ozonation TPs, by-products formed by oxidation of matrix components, such as carcinogenic N-nitrosodimethylamine (NDMA) and bromate, have to be taken into the cost-benefit analysis of such technology [78].

Chlorination is a chemical process commonly used in water treatment for disinfection. In most cases, chlorination is not applied when oxidation of organic micropollutants is the goal, because it can produce biologically active TPs [79]. Especially when the inorganic content in the water matrix is very high, some reactive species, such as chloride or sulfate radicals, are produced, and they directly influence the formation of TPs. The chlorine radical (Cl \bullet) may lead to the formation of chlorinated organic compounds, which are known to be very harmful, and, in some cases, able to generate persistent substances [80]. In this disinfection process, hypochlorous acid (HClO) is the reagent mainly responsible for pathogen destruction, but both HClO and ClO⁻ react with organic compounds, giving addition, substitution, or oxidation products. One of the major concerns regarding disinfection byproducts from chlorination is that NaClO is known to produce genotoxic TPs and can thus increase the acute toxicity [75].

Photochemistry represents an important degradation process in the environment or as a light-related, advanced treatment of water. Many studies have been carried out on direct and indirect photolytic or photocatalytic degradation of EPs. For pesticides, mineralization of pesticides by photocatalytic degradation has been reported and the by-products and intermediates of organophosphate pesticides by photocatalytic degradation were recently presented [81]. Pharmaceutical compounds [19,82], endocrine-disrupting compounds [82], UV filters [83], and phenol [84] have

also been thoroughly surveyed for their fate and their TPs during photolysis.

3. Identification approaches – laboratory studies

Simulation of the transformation processes in batch experiments under well-defined conditions with appropriate controls is a very common first approach for the identification of TPs. Batch experiments can be applied under biotic and abiotic conditions at high concentrations of the parent EPs.

For biodegradation experiments, samples can be provided directly from a wastewater-treatment plant (WWTP) or a pilot-scale WWTP (ps-WWTP) or from natural waters [33,34,51]. Moreover, the ability of microorganisms to degrade EPs has been studied in Erlenmeyer flasks with various microorganisms under study, such as fungus *Trametes versicolor* and bacterial *Pseudomonas* strains [37,40]. Parameters, such as pH, dissolved oxygen (O₂), temperature (T) and total suspended solids (TSS), have to be monitored and adjusted to allow direct comparison with environmental conditions. In the case of batch reactors seeded with activated sludge or wastewater, a concentration of 3 g/L of total suspended solids (TSS) is desirable in order to avoid matrix interferences. During such experiments, spiked and non-spiked samples run in parallel, as do spiked autoclaved diluted sludge or autoclaved groundwater and ultrapure water in order to correct for abiotic processes [39]. Samples are collected periodically so that the reaction kinetics of the analytes can be determined sufficiently [33,50].

Whereas the question may arise if the use of batch experiments can simulate the biotransformation of EPs in full-scale WWTPs, previous studies with batch reactors have proved that, indeed, biotransformation reactions and kinetics can be observed reasonably well in a batch reactor [58]. In aerobic conditions, reactors are loosely capped and shaken or mixed in order to allow free transfer of oxygen, while, for anaerobic treatment, the process is conducted under an atmosphere of N₂. To maintain anaerobic conditions, the anaerobic chamber is flushed with a mixture of N₂/CO₂ gas.

Membrane bioreactors (MBRs), air-pulsed fluidized-bed bioreactors and sequencing batch reactors (SBRs) [37,50] are more realistic systems than batch reactors, because they better simulate the conditions in the full-scale system.

To assess the principal biodegradability and the formation of potential TPs, tests of the OECD series are referred to in the literature:

- the widely used Closed Bottle test (CBT, OECD 301 D) working with low bacterial density;
- the manometric respirometry test (MRT, OECD 301 F) working with medium bacterial density; and,
- the Zahn–Wellens test (ZWT, OECD 302 B) working with high bacterial density.

The CBT simulates conditions in surface water, while the other two simulate effluents [38]. However, those OECD tests usually work at very high concentrations and provide no reaction kinetics.

When preliminary studies in batch experiments are completed, verification of the results should then be carried out using real environmental samples. However, as concentrations are usually lower in the environmental systems, analytical methods have to be adapted. There are few studies with direct injection of environmental samples for the identification of TPs. More often, sediments, activated sludge, wastewater and river-water samples were collected and subjected to solid-phase extraction (SPE) or liquid-liquid extraction (LLE) prior to analysis, for enrichment of the analytes [35,44,51]. To achieve sufficient enrichment for a broad range of compounds, including TPs with different physical-chemical properties, Kern et al. used simultaneously four different SPE sorbents in one

Table 2
Identified transformation products (TPs) of emerging contaminants formed by abiotic processes (e.g., ozonation, photolysis and chlorination) in water

Parent compound/Group of substances	Type of study/ Degradation process	Identified TPs	Toxicity information	Identification workflow			Ref.
				Sample preparation	Instrumental technique(s)	Software (prediction/post data treatment)	
Azithromycin	Photodegradation: Simulated solar radiation	7 TPs – pathway suggested	–	–	UPLC/ESI-QqToF-MS, UPLC/ESI-QqQ-MS	Waters MassLynx, mass accuracy, fragmentation pattern, double-bond equivalents	[70]
Benzotriazoles	Ozonation	11 oxidation TPs (with and without ring cleavage)	–	SPE (Isolute ENV+)	HPLC-QTOF, UV-Vis, full scan mode HPLC-QqQ-MS/MS, HPTLC/AMD H/D Exchange, Derivatization experiments HPLC-UV-Fluorescence-Ion Trap-MS ⁿ	XIC, Agilent MassHunter Workstation, Molecular Formula Generator, Molecular Feature Extractor, Mass Profiler Professional Esquire software, Bruker Metabolite Tools & Data Analysis, fragmentation & isotopic pattern	[71]
Chlorpromazine (tricyclic antipsychotic drug)	Photodegradation: UV/VIS xenon lamp	28 TPs	–	–	–	–	[38]
Cocaine, benzoylecgonine (BE)	Hydrolysis, chlorination, photodegradation	16 TPs of Cocaine (3 already known), 10 TPs of BE (1 already known)	–	–	–	–	[72]
Fenofibric acid	Photodegradation: UV and UV/H ₂ O ₂	4-chloro-4'-(1-hydroxy-1-methylethyl) benzophenone, & minor chlorinated aromatics TPs	algal growth inhibition test using <i>Pseudokirchneriella subcapitata</i>	–	–	–	[6]
Imazalil	Ozonation	4 TPs	Acute toxicity test <i>Daphnia magna</i>	–	–	–	[5]
Ketoprofen	UV irradiation	22 TPs	–	SPE (Oasis™ HLB)	–	–	[73]
Quinclorac	Photolysis, Photocatalysis (TiO ₂)	14 TPs: Pyridine ring hydroxylation, ring opening and/or oxidative dechlorination	–	SPE (Oasis™ HLB)	–	–	[74]
8 Triazines (ametryn, atrazine, cyanazine, metrybuzine, prometryn, propazin, simazine, terbutryn)	Chlorination with sodium hypochlorite	4 TPs (1 never previously reported)	Acute toxicity test <i>Vibrio fischeri</i> : TPs higher toxicity	On line SPE (polymeric cartridge)	–	–	[75]

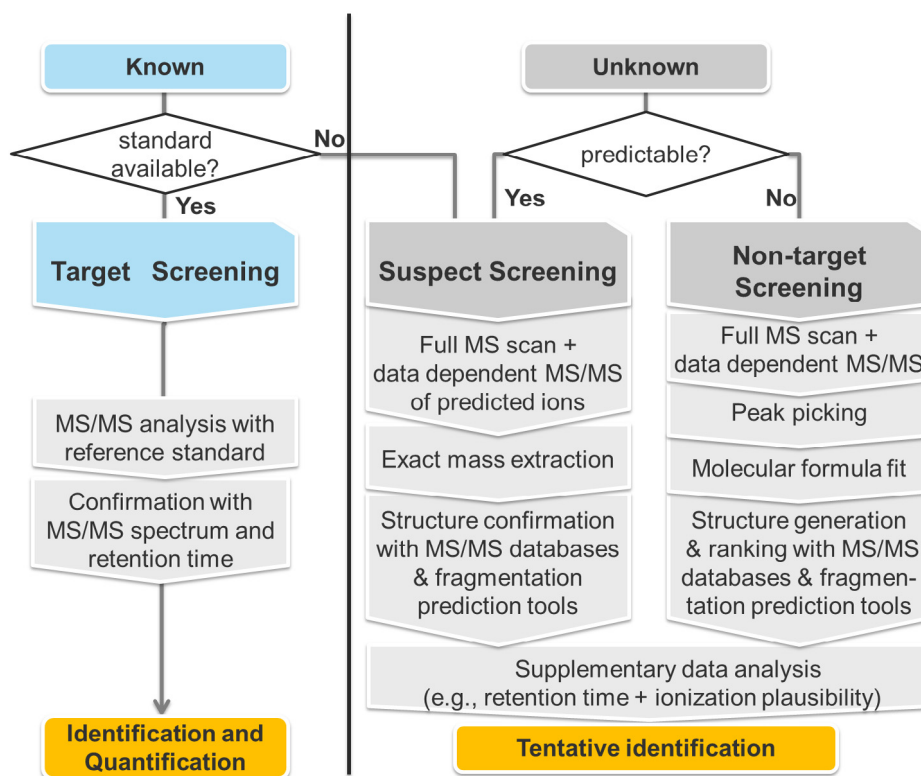


Fig. 1. Flow chart of screening procedure of transformation products (TPs). 'Known' TPs have been confirmed or confidently identified before, other TPs are considered 'Unknown'.

cartridge (weak anion, weak cation-exchange material, and non-polar and hydrophilic-lipophilic interaction sorbents) [5].

As identification is a time-consuming process and often a great number of TPs are formed, prioritization of the most relevant TPs for identification and assessment is necessary. There are two main approaches:

- the effect-directed analysis (EDA); and,
- the exposure-driven approach.

The success of each approach underlies on the selection of the sample together with the sample preparation.

EDA, which has a toxicological perspective, was first introduced for the identification of TPs by Escher and Fenner in 2010 [8]. Modified EDA is based on the relation of the toxicity change of an environmental sample to the decrease of the parent-compound concentration, undergoing a transformation process. If increased toxicity is observed, then toxic TPs are likely to be formed [8].

Furthermore, the US EPA applies a standardized protocol for toxicity identification and evaluation (TIE) (www.epa.gov). EDA is a useful tool to identify non-target and unknown toxicants based on their effects on the environment. This approach works with step-wise separation and simplification of the sample in order to isolate components with toxic activity. The manipulations are directed by bioassays; when toxicity increases, identification of the toxic compound through chemical analysis should be performed. The fractionation approach is a non-selective, non-destructive, clean-up methodology.

However, the more common approach is exposure driven, where the TPs are distinguished and singled out for identification according to their occurrence. As the concentration of the TP gets higher, it is considered more relevant to risk. This approach is commonly

used in the laboratory studies, followed by environmental fate assessment and toxicity testing [8].

The combination of both approaches is also possible:

- first, selection of samples with increased toxicity during transformation; and, subsequently,
- identification of the highest concentrated TPs in the sample without further separation.

4. Identification approaches – analytical techniques

Nowadays, liquid chromatography (LC) coupled to MS (LC-MS) using a variety of mass analyzers is the technique of choice for the investigation of EPs and TPs in environmental samples.

LC is a suitable chromatographic technique for polar, thermolabile compounds, thus for the identification of TPs, which are generally more polar than their parent molecules.

Mass analyzers commonly employed are triple quadrupole (QqQ), time-of-flight (TOF), ion-trap (IT), Orbitrap and hybrid [e.g., quadrupole time-of-flight (Q-TOF), quadrupole-linear ion trap (Q-LIT), linear ion trap-Orbitrap or quadrupole-Orbitrap].

There are various workflows in the literature for the identification of TPs, depending indispensably on the instrumentation and the available software. Fig. 1 shows the main outline:

- target analysis, which is based on the determination of already known TPs, and identification is carried out with standard solutions;
- suspect screening, with a list of possible TPs assembled from the literature or from prediction models, and the samples are screened for those candidates; and,

- (c) non-target screening, with identification of novel TPs being carried out with sophisticated post-acquisition data tools and supplementary analytical techniques.

The development and the use of powerful HR-MS is the driving force in development of novel analytical methodologies for the identification of TPs. Due to its sensitivity in full-scan acquisition mode and high mass accuracy, HR-MS is suitable for target and non-target analysis, pre- and post-acquisition processing, retrospective analysis and discovery of TPs.

4.1. Target analysis

In target analysis, as shown in Fig. 1, TPs are already known and standards are available, so that they can be included within a defined MS method and be monitored in routine analysis. LC coupled to triple quadrupole (LC-QqQ-MS/MS) is the workhorse in target analysis. The QqQ analyzer permits application of MS/MS modes [e.g., product-ion scan, precursor-ion scan, neutral-loss scan and selected reaction monitoring (SRM), which is the most predominant]. The SRM mode provides several advantages and interesting characteristics for target analysis, such as increased selectivity, reduced interferences and high sensitivity, which allows robust quantification. Another important point is the possibility of reducing the analysis time, including extraction and instrumental determination.

With the use of LC-QqQ-MS/MS, adequate results have been obtained for the analysis of ECs and the identification and the quantification of their TPs, especially in pesticides and pharmaceutical compounds, where standards are available.

HR-MS for target analysis offers promising solutions to the limitations of SRM analysis, which allows monitoring of only specific TPs. Virtually all compounds present in a sample can be determined simultaneously with HR-MS instruments operating in full-scan mode, making it unnecessary to pre-select compounds and associated SRM transitions. Target compounds included in a database are screened in the sample based on mass accuracy, isotopic pattern, retention time (t_r) and MS/MS fragments. Alternatively, hybrid instruments offer the possibility of data-dependent MS/MS acquisition, where MS/MS analysis is triggered if a compound from a target-ion list is detected in the full scan. Moreover, HR-MS instruments can differentiate isobaric compounds with the same nominal mass but different molecular formula due to their higher resolving power [3,12,13,15,16].

HR-MS outperforms LR-MS regarding the level of identification of an unknown compound, since, within Decision 2002/657/EC, it gains more identification points and can provide mass accuracy, even in full-scan mode. One ion in HR-MS gains two identification points, instead of one in LR-MS, whereas HR-MSⁿ transition products gain two-and-a-half instead of one-and-a-half. It is clear that in HR-MS full-scan mode, more than one ion is present in the mass spectra and evaluated.

4.2. Suspect screening – prediction of transformation products

Suspect screening is the technique of choice for the identification of TPs, when the confirmation of the analytes with a reference standard is impossible, but molecular formula and structure of suspected molecules can be predicted (Fig. 1) [10,13,15,50,51].

In suspect screening, an important step of the identification workflow is the prediction of possible TPs using computational (*in-silico*) prediction tools. Commercially available or freely accessible programs have been applied in the prediction step on environmental analysis, including:

- CATABOL (<http://oasis-lmc.org/products/models/environmental-fate-and-ecotoxicity/catabol-301c.aspx>);
- PathPred (<http://www.genome.jp/tools/pathpred/>); and,
- Meteor (<http://www.lhasalimited.org/products/meteor-nexus.htm>).

The prediction system should be properly selected by considering the organism or the system where TPs are formed.

Meteor was built based on mammalian biotransformation reactions of common functional groups and allows prediction of the most probable TPs, providing in parallel relevant literature references.

PathPred is a multi-step reaction prediction server for biodegradation pathways of xenobiotic compounds and biosynthesis pathways of secondary metabolites. It is linked to KEGG metabolic pathway maps and it has the potential to link the prediction result to genomic information.

CATABOL and UM-PPS predict microbial metabolic reactions based on biotransformation rules.

As UM-PPS is freely accessible and all rules applied are clearly assigned, it is the most common prediction tool in suspect screening, and many researchers have tried to evaluate and to improve its prediction power [34,50–52]. The prediction rules behind UM-PPS come from the University of Minnesota Biocatalysis/Biodegradation Database (UM-BBD) and literature [85]. Since UM-BBD has integrated data generated from pure microbial cultures, the predicted pathways may not be completely appropriate for environmental systems [34]. The relatively high false-positive rates of all prediction systems are of concern, since the inclusion of additional pathways increases the number of possible degradation products. In UM-PPS, combinatorial explosion can be limited by prioritizing the different rules using relative reasoning.

Prediction of TPs is followed by the HR-MS analysis; the exact mass for each of the predicted TPs is extracted from the chromatogram and checked by comparing it with control samples. An intensity-threshold value is applied to cut off unclear spectra. The plausibility of the chromatographic t_r , isotopic pattern, and ionization efficiency are used as further filters to narrow down the number of candidate peaks. Furthermore, using MS/MS or MSⁿ, structures of suspected TPs are suggested based on the observed fragmentation pattern.

Depending on the above criteria, there are different confidence levels of identification in HR-MS analysis of TPs. When all the above criteria are fulfilled, a probable structure is proposed based on a library-spectrum match or diagnostic evidence. Otherwise, tentative candidates or just unequivocal molecular formulas are the outcome of suspect screening [86].

One approach for processing the data would be the identification of key TPs in terms of persistence over the time of the experiment. It is carried out by a data-processing method that is established based on peak detection, time-trend filtration and structure assignment. Open-source software is used for peak peaking (e.g., MZmine) and processing of the chromatograms (e.g., enviMass), by noise removal and blank subtraction. Then, a meaningful time trend is acquired and the remaining candidate peaks are compared with a list from UM-PPS or literature for tentative identification [87].

Another approach to suspect screening is based on the use of characteristic fragmentation undergone by EPs during MS/MS fragmentation events [51]. It is based on the assumption that many TPs maintain a structure similar to the parent compound and therefore have common fragment ions. Thus, searching for specific fragment ions in MS/MS spectra throughout the chromatographic run could lead to new TPs. This is evident when applying production and neutral-loss scans, and other techniques, such as mass-defect filtering.

- University of Minnesota Pathway Prediction System (UM-PPS: <http://eawag-bbd.ethz.ch/>) [33,34];

4.3. Non-target screening

Non-target screening implies the identification of compounds for which there is no previous knowledge available and is usually carried out after target and suspect screening. Non-target screening becomes a challenging task, but, for TPs, further information of the parent compound (e.g., molecular formula, MS/MS spectrum, t_R and other physico-chemical data) may contribute to further ranking of possible structures and facilitate the identification process [68]. For non-target screening, HR-MS is strongly required in order to have mass accuracy for confirmation of the molecular formula and reliable interpretation of the MS/MS spectra [13,14].

The challenge with HR instruments is the generation of massive quantities of data and subsequently their evaluation and the export of results. Moreover, their ability to operate in full-scan and MS/MS modes simultaneously provides even more data in a single run. For this reason, post-acquisition data-processing tools are necessary; computer-aided techniques provide rapid, accurate and efficient data mining. There is a number of open-source and commercial software options for non-target screening, including:

- MZmine (<http://mzmine.sourceforge.net/>);
- XCMS (<https://xcmsonline.scripps.edu/>);
- EnviMass (<http://www.eawag.ch/forschung/uchem/software/enviMass1/>);
- Non-target, ACD MS/Workbook Suite; and,
- vendors' software, such as Bruker Metabolite Tools and ProfileAnalysis, Waters MassLynx and MetaboLynx, Thermo Scientific Networks and Sieve, Applied Biosystems Data Explorer (MDS-Sciex Analyst QS) and Agilent MassHunter.

The general procedure, as shown in Fig. 1, has several steps until it reaches the final result, which does not follow the same order in each software or workflow.

The first step is always peak picking. In this step, comparison of the sample with control or blank samples is important to exclude irrelevant peaks.

The removal of noise peaks, mass recalibration and componentization of isotopes and adducts is usually carried out automatically as the next step.

The assignment of the molecular formula to the accurate mass of the peak is performed using heuristic filters, such as the seven golden rules of Kind and Fiehn [88].

Exploration of databases, such as ChemSpider, PubChem, DAIOS database, NIST or structure generation, may lead to candidate structures [89,90]. Thereby, information on the parent compound (e.g., molecular formula, substructures) can help to restrict the search of databases and possible structures are likely to be proposed for the compound. However, databases contain mostly only EPs, but many TPs are not included yet.

One successful example of the application of a non-target workflow is the identification of biotransformation products of three benzotriazoles [36].

Even after filtering, strict criteria and thresholds in the above parameters, the number of peaks, which correspond to non-targets can exceed 1000. It is clear that elucidation of all those peaks would demand a great amount of time and effort; prioritization of the most intense peaks is a common strategy [91].

Similar to suspect screening, the observation of the presence or the absence of common characteristic ions in the fragmentation pattern of both the parent compound and the TPs, evidencing the stability or reactivity of certain parts of the molecule, can be helpful [3].

For ranking the candidate structures, information from MS/MS spectra has to be explored by comparing the fragmentation pattern

with *in-silico* mass spectral fragmentation or with spectra in libraries. There are a few databases with mass spectra, e.g.:

- MassBank (<http://massbank.ufz.de/MassBank/>); and,
- MetLin (<http://metlin.scripps.edu/index.php>).

However, most software do not take into account the fragmentation pattern. MOLGEN-MS (<http://www.molgen.de/>), ACD/MS Fragmenter (www.acdlabs.com/products/adh/ms/ms_frag) and MassFrontier (www.highchem.com/index.php/massfrontier) use fragmentation rules, whereas MetFrag offers a purely combinatorial approach based on bond energies only. Although the overall candidate ranking with MetFrag is not quite as good as that obtained with Mass Frontier and MOLGEN-MS, the scoring function used in MetFrag can improve the ranking significantly (<http://msbi.ipb-halle.de/MetFrag/>).

MetFusion, the newest development, combines MetFrag with spectral database searching (<http://msbi.ipb-halle.de/MetFusion/>).

The use of fragmentation trees as performed in SIRIUS is another approach for the structure elucidation (<http://bio.informatik.uni-jena.de/sirius2/>).

In any case, criteria must be established for the success of the identification of the TP by the accuracy of the molecular ion (e.g., mass error < 5 ppm, dependent on the mass accuracy) and the characteristic fragment ions in MS/MS mode (purity score \geq 65 recommended) [3].

Criteria also exist for the score of the isotopic pattern in most commercial software.

Müller et al. proposed another approach for non-target screening of TPs, focusing on relevant peaks (features). The sample is not regarded as an isolated specimen, but is rather evaluated in relation to a set of other samples based on considerations of, e.g., their temporal, spatial, or process-related connections. This also covers comparison of assays and controls, as carried out in evaluation of many transformation experiments. The features of the sample are considered as mathematical sets and treated with statistical tools [90].

Finally, orthogonal analytical approaches are frequently crucial for the successful unambiguous identification of TPs. Nuclear magnetic resonance (NMR) is one option for a complementary technique for identification and confirmation of TP structures as long as sufficient quantities can be isolated [33,39].

5. Future needs and trends

Development of generic and retrospective analytical techniques should permit the simultaneous determination of parent compounds and their TPs, within a single run. In the identification of TPs, the future lies in tiered approaches, which employ HR-MS, complementary techniques and advanced software tools. HR-MS outperforms LR-MS, regarding the level of identification of an unknown compound. Moreover, identification by HR-MS analysis has different levels of confidence, regarding the supporting evidence recorded, apart from the molecular ion {e.g., MS^n or library match [86]}.

However, we are at an early stage in studying the identification of TPs and the workflows applied, as instrumentation and software are still in progress and are getting more complete and easier to use. Specific, step-wise, automated workflows that take into consideration the advantages of HR-MS instruments are still missing for suspect and non-target analysis. Exclusion parameters and sufficient filtering are necessary for the prioritization of peaks and elimination of false-positive and false-negative results. In a more integrated workflow, prediction models should also be combined and propose TPs from the whole range of transformation rules and automatically integrate them in subsequent suspect screening.

Non-target screening should be an extra step for elucidating the rest of the unknown peaks, evaluating not only the most intense, but ideally the most relevant, also according to ecotoxicity. Therefore, new computational tools for toxicity prediction or coupling with high-throughput toxicity tests would be preferable.

Moreover, a universal t_R prediction model, which does not need unreasonable efforts for calibration and takes into consideration neutral and ionic compounds, would contribute a lot in further evaluation of the data [32]. A gap also exists for *in-silico* fragmentation models, lacking mainly in giving fragments, reasonable for each ionization mode and according to specific rules.

However, definite identification can be performed only using reference standards, and is difficult because, in most cases, reference standards are not commercially available. Additional and supplementary orthogonal techniques (e.g., NMR, different MS techniques or other analytical or separation techniques) could contribute in the structure elucidation of unknown TPs, and in confirmation. In this way, multiple physicochemical properties of the compound would be taken into account [92]. Although, most non-target compounds reported are not fully identified, their communication in the scientific community is of great importance, especially if the results are accompanied by an uncertainty or a confidence level [92].

Despite the demands for an identification process, sample preparation should not be forgotten, since environmental samples are complicated matrices and TPs are expected at very low concentrations. It is clear that sample preparation is very challenging in order to capture the wide range of polarities of the potential TPs and to take full advantage of the analytical instruments but, at the same time, to eliminate interfering matrix components.

Summing up, researchers, scientists and policy makers still have a long way to go in order to explore the field of TPs of EPs and to perform an integrated risk assessment. Research is ongoing on the development of high-throughput techniques, including extraction, analysis and data-evaluation steps.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.trac.2014.11.009.

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