Tina Kosjek

OCCURRENCE, FATE AND REMOVAL OF PHARMACEUTICAL RESIDUES IN WATER TREATMENT

Doctoral Dissertation

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Supervisor: Assist. Prof. Dr. Ester Heath

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Abstract

The presence of pharmaceutical residues in surface waters is an emerging environmental issue that provides a new challenge to treatment systems for potable water, wastewater and water reuse. Their principle pathways into the sewerage system are twofold: first, after being administered to patients they are normally excreted as various metabolites together with the unchanged parent compound; secondly, it is common practice to dispose outdated or unused medicines either down the drain or flush down the toilet. In either case, significant quantities of pharmaceutical residues eventually find their way to wastewater treatment plants and the occurrence of these residues in surface waters and groundwaters is due to the inefficient removal processes of conventional water treatment and emphasises the need for enhanced or new treatment technologies. The removal of pharmaceuticals and other organic micro-pollutants still only provides a partial indication of the efficiency of the various treatment methods because of the possible generation of nontargeted toxic intermediates more resistant to degradation. When it comes to pharmaceutical residues many gaps exist in our knowledge regarding degradation mechanisms, the identity of transformation products, and their impact on and their fate in the environment. Insights into this relatively unexplored field are hindered by the quantity and the variety of products present at various degrees of transformation. To resolve this will require the application of sophisticated instruments capable of providing complementary information for structural assignment and robust analytical methods, which require meticulous method development and validation.

This thesis describes an analytical method for determining key pharmaceutical representatives of nonsteroidal antiinflammatory drugs: ibuprofen, ketoprofen, naproxen and diclofenac, in aquatic matrices, and its validation at the intralaboratory and interlaboratory level. The analytical method is based on solid phase extraction and derivatisation followed by GC-MS. An intralaboratory validation revealed the method to be satisfactory in terms of linearity (r^2 ≥0.990), sensitivity, limits of detection (2 – 6 ng L^{-1}), and extraction efficiency (>84 %), thus proving its suitability for determining NSAIDs in aqueous environmental samples. The method was then applied to various Slovenian surface water, groundwater and tap water samples. The concentrations of pharmaceuticals (range 10 – 300 ng L^{-1}) determined were comparable to those found elsewhere in Europe. Furthermore, naproxen, when compared to the other selected pharmaceuticals is dispensed in the highest amounts, was detected in the majority of samples also in the highest concentrations. Interlaboratory validation involving GC-MS and LC-MS was also performed in two separate round robin studies. In summary, it was shown that the GC-MS method was superior to LC-MS method when determining ibuprofen, naproxen and ketoprofen in complex matrices, while neither of the two methods was satisfactory for determining diclofenac. In addition, the process of filtration and the filter material had no affect on the determination of NSAIDs.

Experiments involving the biological removal of pharmaceuticals with activated sludge revealed two groups: readily biodegradable compounds (removal ≥87 %) including ibuprofen, ketoprofen and naproxen, and recalcitrant compounds diclofenac, clofibric acid and carbamazepine, showing ≤59 %, ≤30 % and 16 % elimination, respectively. The study was performed in laboratory scale bioreactors, and the results were in agreement with actual data from municipal wastewater treatment plants. Because the activated sludge in the bioreactors was continuously exposed to the selected pharmaceuticals the effects on the makeup of the microbial community in the activated sludge were monitored. Overall, the pharmaceuticals in concentrations ≥50 μg L^{-1} resulted in a change in the microbial composition in the activated sludge, which became exaggerated with increasing concentration. The pharmaceuticals reduced the diversity in the activated sludge and especially worrying was the notable absence of *Nitrospira*, the bacteria responsible for 2nd stage nitrification.

This thesis also examines the biotransformation of certain pharmaceuticals. Interestingly, only the persistent pharmaceuticals diclofenac, clofibric acid and carbamazepine yielded any discernable biotransformation products. Among a number of diclofenac's biotransformation products identified was 1-(2,6-dichlorophenyl)-1,3-dihydro-2H-indol-2-one, hydroxy-diclofenac, a benzoquinone imine derivative

and a nitro-analogue of diclofenac. Further, 4-chlorophenol was identified in the bioreactor feed along with clofibric acid, and acridine and 9-acridone were formed during the biotransformation of carbamazepine. From the literature and according to this study certain transformation products are also formed *via* different mechanisms involving the abiotic breakdown or human and animal metabolism. Among the identified biotransformation products most notable is the incorporation of a nitro-group into the diclofenac molecule. This study also focused on the complementary use of mass spectrometric techniques including GC-IT, GC-Q and LC-QqTOF to detect as many transformation products as possible, and to apply different mass spectrometric techniques for cross-confirmation of their chemical structures. Among the available mass spectrometric techniques the LC-QqTOF was the most powerful in resolving the chemical structures of the transformation products because of its unique ability to perform both tandem mass fragmentation and accurate mass measurement.

Similar experiments under abiotic conditions, primarily UV irradiation and chlorine dioxide disinfection, were made. Out of the eight metabolites of carbamazepine detected, the structures of seven transformation products, formed during at least one of the treatment methods, were determined. In addition, one compound was generated by thermal decomposition during sample analysis. This study also compared treatment technologies according to the removal of carbamazepine and the production and decay of its transformation products. The most efficient at removing carbamazepine was UV treatment (93 %), while 76 % of acridine and <10 % of acridone were removed. Alternatively, acridine (≤92 %) and acridone (≤40 %) were more susceptible to biological treatment than carbamazepine (16 %). Therefore, based on the enhanced biodegradability of carbamazepine residues achieved by UV irradiation, a coupled treatment technology is proposed involving an initial UV treatment step followed by biological treatment. Similarly, chlorine dioxide degrades carbamazepine more efficiently (54 %) than acridine (38 %), and it may be applied prior to biological treatment.

Finally, at least three identified transformation products: 4-chlorophenol from clofibric acid; acridine and 9-acridone from carbamazepine, were found to be more toxic and hazardous than the parent compounds, a fact which supports the original thesis that considering only a parent pharmaceutical, more harm can be done by insufficient treatment than by completely avoiding it. Thus, with an aim of achieving complete mineralisation of both parent compounds and their transformation products, further development of treatment technologies, possibly involving additional and/or sequenced treatments, is needed. The efficiency of newly-developed treatment technologies, however, will require scale-up and evaluation, both from a scientific and economic perspective.

Povzetek

Prisotnost ostankov zdravilnih učinkovin v okolju, katerih vpliv je, v primerjavi z drugimi onesnažili, razmeroma nepreučen, povzroča naraščajočo zaskrbljenost in prinaša nov izziv za čiščenje pitnih in odpadnih vod ter za njihovo ponovno uporabo. Ostanki zdravil se po uporabi v humani ali veterinarski medicini običajno izločijo v obliki različnih metabolitov ali kot nespremenjene izhodne zdravilne učinkovine. Tako glavni vir tovrstnega onesnaženja predstavljajo odpadne vode, v katere preidejo ostanki zdravil z izločki bolnikov, nemalokrat pa je njihova prisotnost v odpadnih vodah tudi posledica nepravilnega odlaganja neporabljenih zdravil ali proizvodnih procesov v farmacevtski industriji. Odpadne vode se največkrat iztekajo v površinske vode z ali brez predhodnega čiščenja na čistilnih napravah. Sama učinkovitost čiščenja zdravilnih učinkovin na čistilnih napravah je vprašljiva, saj te niso prirejene za odstranjevanje tovrstnih spojin. Nekatere zdravilne učinkovine se tekom čiščenja pretvorijo v produkte razgradnje, katerih identiteta, kroženje in učinek na okolje in človeka so v večini primerov nepoznani, njihovo prepoznavanje pa temelji na uporabi sofisticiranih analiznih metod in instrumentov.

Predstavljeno doktorsko delo opisuje razvoj analizne metode za kvantitativno določanje najpomembnejših predstavnikov nesteroidnih protivnetnih učinkovin, ibuprofena, ketoprofena, naproksena in diklofenaka v vodnih matrikah ter njeno validacijo na notranjem in medlaboratorijskem nivoju. Razvita analizna metoda temelji na ekstrakciji na trdnem nosilcu, ki ji sledita derivatizacija in analiza s plinsko kromatografijo z masno spektrometrično detekcijo. Na osnovi validacijskih parametrov, linearnosti (r^2 ≥0.990), občutljivosti, meje zaznavnosti (2 – 6 ng L^{-1}) in izkoristka ekstrakcije (>84 %) za posamezen analit, sem potrdila primernost analizne metode za določanje nesteroidnih protivnetnih učinkovin v vodnih vzorcih. Razvito metodo sem testirala na vzorcih slovenskih površinskih in pitnih vod ter podtalnice. Izmerjene koncentracije nesteroidnih protivnetnih učinkovin (območje 10 – 300 ng L^{-1}) so bile primerljive z vrednostmi, ki so jih določili raziskovalci drugod po Evropi. Med temi spojinami je najbolj izstopala razmeroma visoka koncentracija naproksena, ki sem ga določila v večini analiziranih vzorcev, kar je lahko posledica izrazito velike porabe te nesteroidne protivnetne učinkovine v Sloveniji. Medlaboratorijska validacija, ki je vključevala analizne protokole z GC-MS in LC-MS določanjem, je bila izvedena v dveh krogih medlaboratorijskih primerjalnih analiz. Rezultati analiz so pokazali, da je GC-MS analizna metoda primernejša za določanje ibuprofena, naproksena in ketoprofena v kompleksnih matrikah, medtem ko se je analiza diklofenaka izkazala za težavnejšo in bi zato, pred uvajanjem na nivoju rutinske analize, potrebovala še dodatno optimizacijo. Medlaboratorijski primerjalni analizi sta potrdili tudi stabilnost nesteroidnih protivnetnih učinkovin v izbranih matricah in pokazali, da filtracija ne vpliva na določanje izbranih analitov.

V študijo biološkega čiščenja zdravilnih učinkovin na bioreaktorjih z aktivnim blatom sem vključila dve skupini zdravilnih učinkovin, biorazgradljive, pri katerih je odstranjevanje ≥87 % (ibuprofen, naproksen in ketoprofen) ter učinkovine, obstojne na biološko čiščenje (diklofenak, klofibrinska kislina in karbamazepin). Pri slednjih sem določila ≤59 %, ≤30 % in 16 % učinkovitost odstranitve z aktivnim blatom. Učinkovitost čiščenja obstojnih zdravilnih učinkovin na naših bioreaktorjih je primerljiva s podatki za komunalne čistilne naprave, medtem ko je učinkovitost čiščenja biorazgradljivih učinkovin nekoliko večja. Razlog za to je lahko adaptacija zaradi neprekinjene izpostavljenosti biomase izbranim zdravilnim učinkovinam ali podaljšan zadrževalni čas odpadne vode v bioreaktorjih. Tudi sicer je neprekinjena izpostavljenost zdravilnim učinkovinam vplivala na strukturo mikrobiološke združbe v bioreaktorjih. Tako so koncentracije ≥50 μg L^{-1} zmanjšale strukturno različnost predstavnikov, pri čemer je posebej pomembna odsotnost bakterij rodu *Nitrospira*, ki so odgovorne za drugo stopnjo nitrifikacije v dušikovem ciklu.

Ena izmed temeljnih nalog predstavljene doktorske disertacije je tudi prepoznavanje produktov razgradnje zdravilnih učinkovin tekom postopkov čiščenja odpadne ali pitne vode. Pri mikrobioloških pretvorbah je bilo produkte razgradnje mogoče zaznati le pri vseh treh obstojnih spojinah, diklofenaku, klofibrinski kislini in karbamazepinu. Med številnimi produkti biorazgradnje diklofenaka sem identificirala naslednje: 1-(2,6-diklorofenil)-1,3-dihidro-2H-indol-2-on, hidroksi-diklofenak, benzokinon imin derivat

diklofenaka in nitro analog diklofenaka. S prisotnostjo klofibrinske kisline v bioreaktorjih sem identificirala 4-klorofenol, medtem ko sta akridin in 9-akridon nastala med mikrobiološko razgradnjo karbamazepina. Sodeč po literaturnih podatkih lahko nekateri izmed produktov razgradnje nastanejo tudi po drugih poteh, kot so humani ali živalski metabolizem ali abiotska razgradnja. Med produkti mikrobiološke razgradnje zdravil, ki sem jih identificirala, vključitev nitro skupine v strukturo diklofenaka predstavlja najbolj nenavadno in nepričakovano pretvorbo. Za identifikacijo čim večjega števila produktov transformacije in navzkrižno potrjevanje njihovih struktur sem pri svojem delu uporabljala komplementarne kromatografske in masno-spektrometrične tehnike, med njimi GC-IT, GC-Q and LC-QqTOF. Med izbranimi instrumenti se je LC-QqTOF izkazal kot najprimernejši za reševanje kemijskih struktur neznanih spojin, saj omogoča tandemsko masno fragmentacijo, visoko ločljivost in natančnost masne meritve.

Predstavljena doktorska naloga vključuje tudi eksperimente z abiotsko razgradnjo zdravilnih učinkovin, predvsem UV razgradnjo in dezinfekcijo s klorovim dioksidom. Pri tem sem uspešno identificirala sedem produktov transformacije karbamazepina, še ena spojina pa je nastala med samo analizo kot posledica toplotnega razpada karbamazepina v injektorju plinskega kromatografa. Preučevanje učinkovitosti čiščenja karbamazepina in dveh izmed njegovih produktov razgradnje, akridina in akridona, je pokazalo, da je za odstranjevanje karbamazepina najučinkovitejša UV razgradnja (93 %), medtem ko je učinkovitost čiščenja akridina 76 %, akridona pa <10 %. V nasprotju s karbamazepinom (16 %) pa sem ugotovila uspešnejšo mikrobiološko razgradnjo akridina (≤92 %) in akridona (≤40 %). Na podlagi teh ugotovitev sem predlagala sklopljeno tehnologijo čiščenja, ki vključuje UV razgradnjo in nato mikrobiološko razgradnjo. Podobno se karbamazepin (54 %) uspešneje kot akridin (38 %) odstranjuje z oksidacijo s klorovim dioksidom, zaradi česar se v zaporedju z biološkim čiščenjem prav tako lahko uporabi za omejevanje oz. preprečevanje vstopa ostankov zdravilnih učinkovin v okolje. Predlagani tehnologiji bo potrebno preizkusiti na večjih pilotnih in realnih čistilnih napravah in oceniti njuno ekonomsko učinkovitost.

Literaturni podatki in raziskave znotraj te študije kažejo, da so vsaj trije izmed identificiranih produktov transformacije, 4-klorofenol, produkt razgradnje klofibrinske kisline, in akridin ter akridon, ki nastaneta iz karbamazepina, bolj toksični kot izhodne spojine, kar je zaskrbljujoče in še dodatno podkrepi zastavljene cilje po doseganju popolne mineralizacije obstojnih mikropolutantov tekom čiščenja odpadne in pitne vode in s tem znatno zmanjšanje obremenitve okolja in tveganja za zdravje.

Abbreviations

$^{\bullet}$OH	=	hydroxyl radicals
AOP	=	advanced oxidation process
APCI	=	atmospheric pressure chemical ionization
API	=	atmospheric pressure ionization techniques
APPI	=	atmospheric pressure photoionization
BOD	=	biological oxygen demand
BSTFA	=	N,O-bis(trimethylsilyl)-trifluoracetamide
CAS	=	conventional activated sludge treatment
CBZ	=	carbamazepine
CI	=	chemical ionization
CID	=	collision induced dissociation
CLA	=	clofibric acid
COD	=	chemical oxygen demand
CRM	=	certified reference material
DF	=	diclofenac
DF-Na	=	diclofenac sodium salt
DOM	=	dissolved organic material
EI	=	electron impact ionization
ESI	=	electrospray ionization
GC	=	gas chromatography
HPLC	=	high performance liquid chromatography
HRMS	=	high resolution mass spectrometry
HRT	=	hydraulic retention time
IB	=	ibuprofen
IPs	=	identification points
IT	=	ion trap mass detector
KP	=	ketoprofen
LC	=	liquid chromatography
LLE	=	liquid-liquid extraction
LOAEL	=	lowest observed adverse effect level
LOEL	=	lowest observed effect level
LOD	=	limit of detection
LOQ	=	limit of quantification
MBR	=	membrane bioreactors
MRM	=	multiple reaction monitoring
MS	=	mass spectrometry
MS/MS	=	tandem mass spectrometry
MS^n	=	multiple stage mass spectrometry
MSTFA	=	N-methyl-N-(trimethylsilyl)-trifluoracetamide
MTBSTFA	=	*N*,*N*-(*tert*butyldimethylsilyl)-trifluoroacetamide
NCI	=	negative chemical ionization
NF	=	nanofiltration
NIST	=	National Institute of Standards and Technology
NMR	=	nuclear magnetic resonance
NP	=	naproxen
OTC	=	over-the-counter pharmaceuticals
PFBBr	=	pentafluorobenzyl bromide
POCIS	=	Polar Organic Chemical Integrative Sampling

Q	=	quadrupole mass detector
QqLIT	=	quadrupole – linear ion trap
QqQ	=	triple quadrupole mass detector
QqTOF	=	quadrupole – time-of-flight mass detector
RRLC	=	rapid resolution liquid chromatography
RO	=	reverse osmosis
SPE	=	solid phase extraction
SPME	=	solid phase microextraction
SRM	=	selected reaction monitoring
SRT	=	sludge retention time
TIC	=	total ion chromatogram
TOF	=	time-of-flight mass detector
TP	=	transformation product
UPLC	=	ultra performance liquid chromatography
WW	=	wastewater
WWTP	=	wastewater treatment plant

1 Introduction

1.1 Pharmaceuticals in therapeutics

1.1.1 Pharmacokinetic and pharmacodynamic characteristics

Pharmaceuticals are an indispensable element of modern life. They are administered to humans and animals as an aid in the diagnosis, treatment or prevention of disease, for the relief of pain or suffering, or to control or improve physiologic or pathologic condition [1]. With an increasing population, increasing life expectancy and the continual development and introduction of new pharmaceuticals onto the market, their assortment and consumption continues to rise. As a mirror to the knowledge of their importance for our well-being is an increase in the awareness regarding their unwanted effects; many of which are only recognised long after pharmaceuticals have been launched on the market. To improve our knowledge in this field, their fate and behaviour need to be studied within and after being excreted from the target organism.

In this thesis six model compounds comprising four non-steroidal anti-inflammatory drugs, carbamazepine and clofibric acid (Table 1) were selected as model pharmaceuticals to study the occurrence, fate, removal and transformations of pharmaceuticals during water treatment and in the environment.

An important pharmacological group of drugs are the non-steroidal anti-inflammatory drugs (NSAIDs). Non-steroidal anti-inflammatory drugs suppress inflammation in a manner similar to steroids, but without the associated side effects, and are commonly used to relieve the symptoms of arthritis, gout, swelling, stiffness and joint pain. The pharmacological mode of action for NSAIDs is *via* the inhibition of the synthesis of prostaglandins, prostacyclins and leukotrienes involved in the inflammatory response. These pharmaceuticals, in particular those sold over-the-counter as non-prescription drugs, also have analgesic (pain-killing) and antipyretic (fever reducing) activities. Among the selected NSAIDs (Table 1), ibuprofen (IB) and naproxen (NP) are sold over-the-counter in most countries, while ketoprofen (KP) and diclofenac (DF) can only be obtained as prescription drugs [2,3].

Carbamazepine (CBZ) is an important drug for the treatment of epilepsy, which is, after stroke, the most common central nervous system disease. This drug is also used for the treatment of severe pain syndromes associated with neurological disorders (trigeminal neuralgia) and as a psychotropic agent. The drug has been introduced in clinical psychiatry for the treatment of schizophrenia, alcohol withdrawal, acute mania, and for prophylaxis against both the manic and depressive episodes of bipolar disorder [3]. It is administered chronically and usually in high dosages and hence its annual production is high [4,5,6].

Lipid regulating drugs, i.e. fibric acid derivatives (fibrates) and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins), are used to lower levels of blood cholesterol among people at risk of heart attack or stroke. Fibrates reduce the level of triglycerides and increase the levels of high-density lipoprotein, thereby decreasing the risk of cardiovascular events [3]. Among the fibrate antilipaemics, three compounds are known to transform into a pharmacologically active clofibric acid (CLA) during human metabolism.

Table 1: *Chemical classification of the studied pharmaceuticals*

Pharmaceutical	CAS	IUPAC name	Trade name	Molecular formula	Molecular weight
IB	15687-27-1	α-methyl-4-(2-methyl propyl)-benzene acetic acid	Bonifen® Ibuprofen®	$C_{13}H_{18}O_2$	206.283
NP	22204-53-1	6-methoxy-α-methyl-2-naphthalene acetic acid	Nalgesin® Naprosyn®	$C_{14}H_{14}O_3$	230.2616
KP	22071-15-4	3-benzoyl-α-methylbenzene acetic acid	Ketonal®	$C_{16}H_{14}O_3$	254.284
DF	15307-86-5	2-((2,6-dichlorophenyl)amino)benzene acetic acid	Naklofen® Voltaren®	$C_{14}H_{10}Cl_2NO_2$	296.152
CLA	882-09-7	2-(4-chloro phenoxy)-2-methyl propanoic acid		$C_{10}H_{11}O_3Cl$	214.647
CBZ	298-46-4	5H-dibenz(b,f)azepine-5-carboxamide	Tegretol®, Tegretol XR®, Equetro®, Carbatrol®	$C_{15}H_{12}N_2O$	236.273

The term ‘Drug metabolism’ refers to chemical alterations of a drug *in vivo* [7]. It takes place predominantly in the liver and in general, metabolism modifies the chemical structure of the active molecules into more polar and water-soluble derivatives, which in turn facilitates their excretion. Drugs are metabolized through a variety of mechanisms such as hydrolysis, oxidation, reduction, dealkylation, which are referred to as Phase I metabolic reactions and either introduce or expose a functional group on the parent compound. Phase I reactions generally result in the loss of pharmacological activity, although there are examples of retention or enhancement of the activity. In some cases, the compound may be a pro-drug, which is converted rapidly into its pharmacologically active form, usually during the first-pass liver metabolism. Phase II conjugation reactions lead to the formation of a covalent bond between a functional group (OH, COOH, NH_2 or SH) on the parent compound or a Phase I metabolite and D-glucuronic acid, sulphate, glutathione, amino acids, or acetate [7,8,9,10]. In general, conjugation diminishes pharmacological activity, though, it may be reversible and the phenomena of deconjugation in the environment or during municipal wastewater treatment can occur [4,11,12,13,14,15,16]. Certain drugs like CBZ are largely metabolized before they are excreted, while others, such as atenolol, are only poorly metabolized and others yet again, such as contrast media, are excreted completely intact. The compounds with a high metabolic rate in humans do not necessarily have a short lifetime in the aquatic environment [17].

1.1.2 Production and consumption

Pharmaceuticals are produced and used in increasing quantities each year. The available data (Table 2) show how the amount of IB, DF and CBZ prescribed in Germany (1995 – 1999) increased for 25, 70 and 44 %, respectively. The consumption and application of pharmaceuticals varies considerably from country to country, but in general the amount of dispensed pharmaceuticals is higher than those values given in Table 2, since these numbers do not include hospital applications and non-prescription i.e., so-called over-the-counter (OTC) drugs. The amounts consumed per person per year (Table 2) were calculated using the

data from CIA World Factbook [18]. Among the studied countries, Canada is the largest consumer of IB and NP per capita, in Slovenia most KP is dispensed, whereas in Germany most DF and CBZ per person is consumed.

Table 2: *Estimated prescription amounts of the selected pharmaceuticals.* *n.s.: not stated

Compound	Defined daily dose (adult, oral) (mg day^{-1})	Annual prescribed amount (t per year)	Country	Year	Amount per capita (mg $year^{-1}$)	Reference
IB	200 - 3200	250	Canada	2001	7972	[3]
		18	Switzerland	2001	2420	[19]
		140	Germany	1999	1691	[20]
		105	Germany	1995	1266	[4]
		6.696	Austria	n.s.	816	[21]
		0.945	Slovenia	2001	467	[22]
NP-Na	275 - 1100	45	Canada	2001	1435	[3]
		1.923	Slovenia	2001	950	[22]
KP	50 - 200	0.339	Slovenia	2001	167	[22]
		0.254	Switzerland	2001	34	[19]
DF-Na	75 - 200	250	Germany	1999	3021	[20]
		75	Germany	1995	904	[4]
		6.143	Austria	n.s.	748	[21]
		3.9	Switzerland	2001	524	[19]
		0.602	Slovenia	2001	297	[22]
		5	Canada	2001	159	[3]
CLA	Clofibrate: 2000 mg, 95–99 % transform.	16	Germany	1995	193	[4]
CBZ	100 - 2000	120	Germany	1999	1450	[20]
		30	Canada	2001	957	[3]
		80	Germany	1995	965	[4]
		6.344	Austria	n.s.	773	[21]

Due to their pharmacological activity and the amount used, pharmaceuticals, after they enter the environment, represent environmentally relevant compounds. In addition, pharmaceuticals are produced and administered with the aim of causing a biological effect, their occurrence, fate and effects on the environment are not only of scientific but also of public interest. Besides the active substances, the pharmaceutical formulations may also incorporate adjuvants and in some instances pigments and dyes, but in terms of the possible impact on the environment, these are of minor importance compared to the pharmacologically active substances themselves [8].

1.2 Pharmaceuticals in the environment

1.2.1 Sources

In human and veterinary medicine, pharmaceuticals are administered orally, parenterally, topically, or rectally and are used extensively in hospitals and domestically. Together with their metabolites they enter the municipal sewer network and either reach sewage treatment plants, or in rural households directly release in to septic tanks [23]. Because the pharmaceutical industry follows Good Manufacturing Practice regulations (GMPs), actual emissions during manufacturing are low and only in the case of accidents may emissions occur locally; therefore, point source emissions are of minor importance [8].

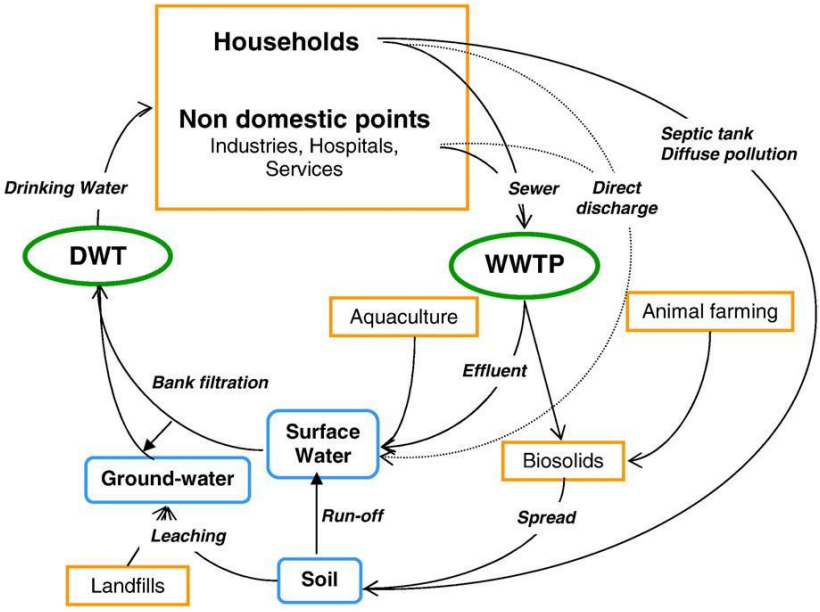


Figure 1: *Sources, distribution and sinks of pharmaceuticals in the environment* [24]. (DWT: drinking water treatment, WWTP: wastewater treatment plant)

Because pharmaceuticals are not totally eliminated during wastewater (WW) treatment they enter the aquatic environment, where they can leach into underlying groundwater, which in turn may be used as a source of potable water (Figure 1) [16,25,26]. In addition, outdated and excess medicines are often disposed of with household waste or down the drain. It is reported that approximately one third of the total volume of pharmaceuticals sold in Germany and USA [27,28] and about 25 % of those sold in Austria [29] is disposed of in this manner. In the United Kingdom the predominant method of disposal is in the household waste, which is of concern since medicines deposited in their original form in landfill bypass metabolism in the human body and degradation in WWTPs [30], and landfill leachate can be a major source of groundwater contamination [31,32,33,34].

Drugs used in animal husbandry, either for veterinary purposes or as growth promoters, are together with their metabolites excreted with manure [35]. Farmers use manure and sewage sludge as fertilizer allowing drug residues to reach the soil and to enter the water environment in runoff after a heavy rain. Application of pharmaceuticals in aquaculture can result in a direct input of these compounds into environmental water or to sediments [8] (Figure 1). Pharmaceuticals with sorption properties can enter the soil and the subsurface by river bank filtration, artificial groundwater recharge and leaky sewerage systems [36]. In addition, groundwater and surface water are intimately linked, and as a result can contaminate one another [24]. The passage of pharmaceuticals in raw water resources through drinking water treatment plants is their ultimate elimination step before potable water distribution [24].

1.2.2 Occurrence

The important factors determining the occurrence of pharmaceutical residues in the environment are their overall consumption, their disposal and the fate of each individual compound in the human body, in the WWTPs, and in the aquatic environment itself. Pharmaceuticals occur as persistent residues at the ng to µg per liter level in WWTP effluents, which are then discharged into surface waters, where they are detected in the several hundred ng L^{-1} level (Table 3). In surface waters pharmaceuticals are subjected to natural attenuation processes, which diminish them over time and distance [37]. However, certain compounds can persist. These include CLA and CBZ [38], which prove to be excellent anthropogenic markers, as well as markers for sewage contamination in surface and groundwaters [16,39,40]. Whenever bank filtration or other methods for groundwater recharge are used for potable water production, persistent pharmaceuticals may leach from contaminated watercourses into the groundwater aquifers. Depending on the methodologies used for potable water production, pharmaceutical residues may also appear at trace-level concentrations in tap water. For example, investigations by Stan et al. [41] and Stan and Linkerhägner [42] show that samples taken from various districts in Berlin all contained CLA in high concentrations (165 and 270 ng L^{-1}). Table 3 shows the levels of IB, KP, DF and CBZ determined in potable water. However, these compounds in potable water occur only sporadically because of their removal in the environment or during water treatment. One reason might be the lack of systematic monitoring programs, as currently there are no statutory maximum contaminant levels for pharmaceuticals in potable water and no regulatory requirement to monitor them. Another reason is the insufficient detection limits of the analytical methods used, since pharmaceuticals in potable water usually occur in the sub ng L^{-1} level or below [43].

Table 3: *Occurrence of selected pharmaceuticals in the environment: Concentration range, median and maximum determined concentrations*

Target compound	Surface waters (ng L^{-1})			Groundwater	Potable water
	Conc. range (ng L^{-1})	Med (ng L^{-1})	Max (ng L^{-1})	Max (ng L^{-1})	Max (ng L^{-1})
IB	4.9-32 [44] 60-152 [45] 80-220 [17]	60 [46] 297 [47] 826 [48]	80 [49] 150 [46] 146 [39] 5044 [48]	200 [50]	3 [43] 8.5 [51]
NP	10-400 [49] 90-250 [17]	70 [45] 33 [46]	50 [46,52] 32 [39]		
KP	10-70 [17]		5 [49]		8.0 [51] 3.0 [53]
DF	20-150 [49] 26-67 [44] 26-72 [45] 10-120 [17]	29 [46]	60 [52] 60 [46] 69 [39] 568 [48]	590 [25] 300 [50]	6 [43]
CLA	10-20 [46] 2.4-7.6 [44] 24-35 [45]	11 [46]	22 [39] 25 [49] 30 [52]	7300 [50]	165 [41] 270 [43]
CBZ	30-250 [49] 100-500 [17]	30 [46] 1200 [39]	110 [46] 2500 [39]	900 [25]	30 [54] 258 [55]

The occurrence of pharmaceuticals in surface waters is presented in Table 3 as a concentration range, maximum and/or median concentration (ng L^{-1}). In groundwater and potable water the concentrations of pharmaceuticals are determined less frequently and are often below the detection limit, therefore the available literature only provides the maximum determined concentrations. When interpreting the data, such as those collected in Table 3, we need to take into account that not all the studies considered daily and seasonal fluctuations in concentration of pharmaceuticals, and so Table 3 probably does not present a clear picture of pharmaceutical pollution in the aquatic environment. To fully estimate their environmental occurrence, load and discharge from WWTPs, their levels would have to be monitored for an appropriate period of time and suitable sampling conditions applied. Use of grab or composite sampling does not provide a true average of the concentrations of pharmaceuticals present [47]. Samples collected using these

methods only represent a snapshot in time and do not mimic the continuous exposure of organisms to these chemicals. An alternative way to achieve a time weighted average concentration is to use passive samplers, such as "Polar Organic Chemical Integrative Samplers" (POCIS) [56,57,58].

1.2.3 Fate and behaviour

Two factors that attenuate pharmaceuticals in environmental waters are dispersion and dilution. Although these processes do not result in chemical transformation, they effectively decrease the peak and average concentrations of a compound. As a consequence, the ambient concentration may not be sufficient to elicit an enzymatic or biological response in aquatic organisms [37]. The dispersion and dilution factors principally depend on weather and flow conditions.

Alternatively, those parameters that determine the fate and distribution into different environmental compartments, like sorption, aquatic mobility, (bio)accumulation, bio- and photo- degradation, volatility, and environmental persistence, can to some extent, be predicted from their chemical structures (Figure 2) and physico-chemical properties (Table 4).

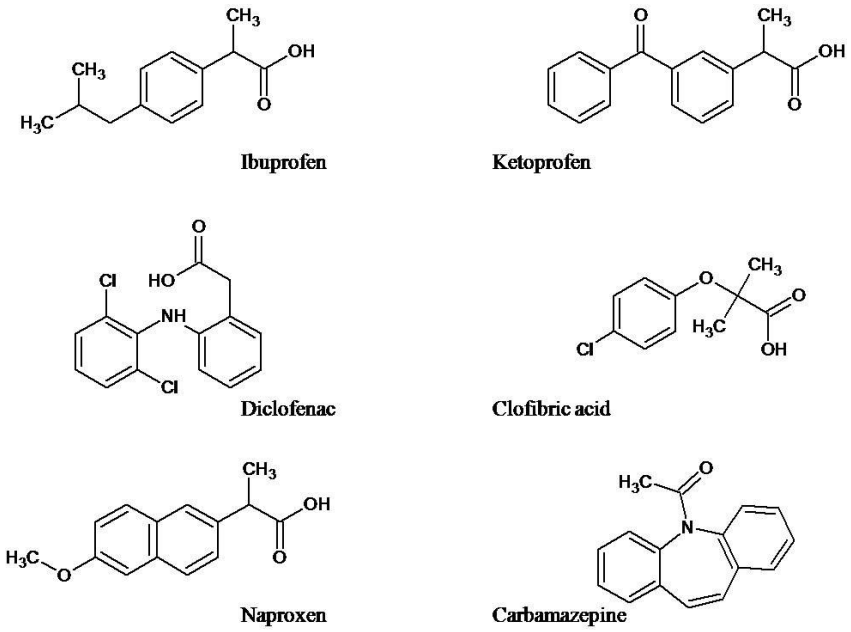


Figure 2: *Chemical structures of the studied compounds*

Table 4: *Physical and chemical properties of the test compounds: Solubility, octanol-water partition coefficient (K_{OW}), dissociation constant (pK_a), organic carbon partition coefficient (K_{OC}), Henry coefficient (K_H)*

Compound	Solubility (mg L^{-1})	logK_{OW}	pK_a (20 °C)	logK_{OC}	K_H (Pa m^3/mol)
IB	21 [59]	3.5 [60] - 3.79 [61]	4.52 [62]	2596 [63]	1.5×10^{-2} [64]
NP	15.9 [64]	3.10 [61] - 3.18 [46]	4.15 [46]	-	3.4×10^{-5} [64]
KP	51 [64]	3.00 [61] - 3.12 [46]	4.45 [46]	-	2.2×10^{-6} [64]
DF	2.37 [65]	4.02 [61] - 4.51 [66]	4.16 [62]	2921 [63]	4.8×10^{-7} [64]
CLA	582.5 [71]	2.84 [61]	3.18 [67]	1640 [63]	2.2×10^{-3} [64]
CBZ	112 [5]	2.25 [61]	14.0 [36]	3588 [63]	1.1×10^{-5} [64]

Table 4 shows how the solubility of the test pharmaceuticals is notably higher than their actual concentrations in the aquatic environment (Table 3) and therefore the solubility does not limit their environmental occurrence. Also, because of the low values for the Henry coefficient (K_H) (Table 4), the

fraction removed by volatilization is negligible [68].

Two types of coefficients are commonly used to determine the sorption and affinity of a given substance to organic matter: octanol-water partition coefficient (K_{OW}) and the organic carbon partition coefficient (K_{OC}) [69], derived from the n-octanol/water distribution coefficient (D_{OW}) and the solid-water distribution coefficient (K_d), respectively. D_{OW} is defined as the ratio of the concentration of a chemical in two phases, n-octanol (a surrogate for lipids) and water, when the phases are in equilibrium with one another and the test chemical is in dilute solution in both phases. The D_{OW} indicates the tendency of an organic chemical to:

- distribute between lipids and fats;
- sorb to particulates such as soils or sediments;
- sorb to biomass and sludge;
- distribute among environmental compartments.

However, in most cases the D_{OW} can only be applied to neutral industrial chemicals and pesticides, while it does not appear to be as applicable to pharmaceuticals and their transformation products (TPs), which are primarily complex, multifunctional organic compounds that are ionized in the aquatic environment at environmentally relevant pH levels. Since pH governs the degree of ionization, for ionisable compounds, D_{OW} is usually determined at pH 5.7 and 9, while for an environmental risk assessment pH 7 is used [7]. In general, logD_{OW} values < 1 indicate that a chemical is unlikely to significantly bioconcentrate or sorb onto organic matter. A logD_{OW} value ≥ 3 indicates that a compound may bioaccumulate or sorb significantly. The n-octanol/water distribution coefficient (D_{OW}) may be corrected for the ionization of the compound so that only the concentration of the un-ionised species is considered (K_{OW}). The K_{OW} is given by [70]:

$$K_{OW} = D_{OW}(1+10\times|pH - pK_a|) \qquad \text{Equation 1.}$$

LogK_{OW} is often represented as log$P$ (Table 4). Where available, both logK_{OW} estimated using the SRC Database [61] and the experimentally determined logK_{OW} values show only a slight deviation between each other. DF in the form of carboxylic acid exhibits a substantially higher logK_{OW} and lower solubility, which may result in significant sorption of the neutral drug. In therapeutics, low solubility may be a problem, and therefore, the bioavailability of the compound is improved by producing a sodium salt of DF, which exhibits a substantially higher solubility (2425 mg L^{-1}) [71] and lower logKOW (0.57) [61], than the undissociated compound (Table 4).

Besides flow conditions and persistence, sorption is key factor controlling input, transport, and the transformation of pharmaceuticals in the aquatic environment [36]. Sorption (either adsorption or absorption) is a process in which compounds become associated with solid phases. The equilibrium distribution of a compound between the solids and the solution depends on concentration and is described by a sorption isotherm - most commonly the Freundlich isotherm:

$$c_{sorb} = K_F \times c_w^{1/n} \qquad \text{Equation 2,}$$

where c_{sorb} is the concentration of a chemical in the solid phase (mg kg^{-1}), K_F the Freundlich constant, c_w the concentration of the chemical in water (mg L^{-1}) and $1/n$ the linearity parameter. The simplest case of the Freundlich isotherm occurs when the linearity parameter $1/n = 1$, where the isotherm becomes linear and is termed the Nernst partitioning (K_d). The solid-water distribution coefficient K_d is the sum of the following sorption mechanisms: absorption and adsorption to natural organic matter, intermolecular interactions (van der Waals, dipole-dipole forces, H-bonding), specific bonding of reactive moieties and solid surface groups, and the ionic interactions between charged species [72].A significant reduction in the variability of the sorption coefficients is achieved by normalizing it to the organic carbon content (fraction of organic carbon f_{OC}), giving the organic carbon normalized sorption coefficient K_{OC} according to

$$K_{OC} = \frac{K_F}{f_{OC}} \qquad \text{Equation 3.}$$

The mechanism and magnitude of sorption is defined by the compound's chemical structure. Sorption can be an equilibrium process, except when chemical or biological degradation follows, or continued diffusion into deeper sediments occurs. At equilibrium, the net uptake of the sediments will cease, unless microorganisms adapt and transform the contaminants. In the absence of sediment-mediated (biological or chemical) transformation, sediments can be sources of contaminants [37,73].

The dissociation constant (pK_a) also affects sorption. The pK_a is an equilibrium constant that describes the degree of ionization of a compound at a known pH. The significance of the dissociation constant is in the relationship between pK_a and pH and the resulting distribution of a pharmaceutical in the environment. The degree of ionization at a particular pH will affect its bioavailability, its chemical and physical reactivity, and its ultimate fate. Among the studied pharmaceuticals, five (IB, NP, KP, DF and CLA) are carboxylic acids with a pK_a of approx. 4 (Table 4), and are negatively charged at an environmental pH (6-9 [74]). This suggests a much higher water mobility than that proposed by calculating K_{OC}. Alternatively, CBZ with a pK_a of 14.0 (Table 4) will be present in the environment in an undissociated form, and its $\log K_{OW}$ equals $\log D_{OW}$. Thus, hydrophobic sorption could be the potential sorption mechanism for CBZ [36,75], but is negligible due to its high polarity (Table 4). In addition, Reemtsma et al. [76] propose that the sorption of polar pollutants to WW solids by hydrophobic interaction can be neglected, but ionic interactions may be important, especially for organic cations; the latter is not the case when considering the selected five pharmaceuticals. In general, due to their low sorption properties all the tested compounds tend to remain in the aqueous phase, which favours their mobility through WWTP and into the receiving waters [77].

1.2.4 Effects

Pharmaceuticals are designed to produce an effect on living organisms, either to treat humans and animals, or to kill microorganisms. It is therefore expected that their environmental impact will be based on their

- activity against bacteria,
- activity against fungi,
- activity against (non)target higher organisms, or
- persistence [8].

However, the actual concentration levels of pharmaceuticals in the environment are three to four orders of magnitude lower than that needed to produce a human pharmacological effect; therefore, the likelihood of any acute health risk in humans is low [78]. This is exemplified in the case of CBZ, where according to the maximum possible intake of CBZ (Table 3) *via* potable water over a lifetime (assuming an intake of 2 L per day of water for 70 years) is 13 mg, whereas a single therapeutic dose is 100 mg or higher (Table 2). The lowest observed effect level (LOEL) is in the case of pharmaceuticals usually the lowest therapeutic level, which is considerably lower than the lowest observed adverse effect level (LOAEL), i.e. the toxic dose of CBZ. Therefore, any risk of ingesting a toxic dose of CBZ *via* acute exposure from potable water is negligible. However, most studies on the therapeutic effects of pharmaceuticals are based on the short-term ingestion of relatively high doses, while little is known about potential health effects associated with long term chronic exposure [9]. Moreover, the criteria for potable water is currently based on the toxicity of individual compounds and not on a combination of compounds; the possibility that exposure to multiple organic compounds, even at low concentrations, may have a synergistic human-health consequence, which should not be ignored when making a risk assessment [55].

While the effects of pharmaceuticals as therapeutics have been studied in detail, and are documented in pharmaceutical dossiers, little information is available on their effects on either the aquatic or terrestrial environment [9]. High concentrations of certain compounds, i.e. in mg per liter range have been found to produce acute effects in environmental organisms. However, because pharmaceuticals are continually being introduced into the environment, questions about potential chronic effects on biota have been raised. Thus, data concerning chronic effects based on more specific endpoints and/or more realistic tests, e.g., biomarkers, long-term exposure, and multigeneration tests, should be included in any risk assessment [71].

However, the effect of antibiotics on *Daphnia*, algae and bacteria has been demonstrated using low concentrations in chronic tests [79,80,81,82,83,84,85,86]. The presence of antibiotics in the aquatic environment is of particular concern because of fears that they may stimulate dissemination of antibacterial resistance among native bacterial populations [87]. It is known that antibiotics in sub-inhibitory

concentrations can have an impact on cell functions and change the genetic expression of virulence factors or the transfer of antibiotic resistance [88,89]. Antimicrobials exhibit a different activity spectra and mechanisms of action. Therefore, they can affect different bacterial populations in different ways and to a different extent. *In vitro* experiments show that 100 μg L^{-1} of gentamicin increases the transfer rate of resistance in staphylococci, but did not select resistant bacteria. However, other substances, such as macrolides, quinolones or vancomycin did not have such an impact [90]. When a complex mixture of bacteria is exposed to antibiotics an increased activity can be observed in some cases [81,91]. Single and multiple antibiotic-resistant bacteria have been detected in municipal wastewater effluents [92], sewage-affected surface water systems [93,94], and even potable water [94]. The detection in aquatic environmental systems of resistant bacteria and the antibiotics to which they are resistant, clearly merits a great deal of concern regarding the fate of antibiotics in relevant water treatment processes.

Besides antimicrobial resistance, other toxic effects of pharmaceuticals in the environment have been reported. For example, DF has been associated with hepatotoxicity in humans, which was caused independently of the administered dose [95] and was possibly related to the formation of pharmacologically active metabolites [96]. Diclofenac is reported to be ecotoxic in rainbow trout (*Oncorhynchus mykiss*) [97,98] and was found responsible for an unusually high death rate among Asian vultures, fed with diclofenac treated livestock [99], which suggests that its pharmacological response may be species specific. In addition, it is important to consider bioaccumulation, possible additive or synergistic effects and especially, the toxicity of their TPs compared to the parent compound [100]. Ferrari et al. [71] investigated the toxicity of DF, CLA and CBZ to organisms from a range of different trophic levels by means of standard ecotoxicity methods. The acute tests using bacteria (*V. fisheri*) and crustaceans (*D. magna, C. dubia*) show the following hierarchy, in decreasing order of toxicity: diclofenac > carbamazepine > clofibric acid. Chronic tests on algae (*P. subcapitata*), crustaceans (*C. dubia*) and early life stage fish (*D. rerio*) display a higher toxicity than acute tests. Based on the lowest observed effect concentration (LOEC) obtained for each pharmaceutical, the toxicity ranking in decreasing order of chronic toxicity was carbamazepine > clofibric acid > diclofenac, which contrasts with the global hierarchy of acute toxicity [71].

1.3 Pharmaceuticals in water treatment

1.3.1 Occurrence

Studies on the occurrence of pharmaceuticals in wastewaters report their presence in influents and effluents of numerous WWTPs across the Europe and America. In Table 5 the available data are presented. It is important to observe that individual studies usually provide only limited data, i.e. either concentration range, median or maximum concentration. Furthermore, even though all data were obtained from peer reviewed publications, the quality of sampling and analyses differed, which hinders the comparability of such data. Table 5 shows that IB, DF and CBZ are frequently detected in μg L^{-1} concentrations. In a few cases, there is no considerable decrease between the median influent and the median effluent concentrations. Such examples involve CBZ [16,17,46], DF [16,17,46] and KP [16], while for CLA [16] and NP [46] an increase in concentration is observed. One reason may be that in the complex influent samples the MS/MS detector signal is suppressed by a high concentration of organic matter, as has been reported [101,102,103,104,105]. It has been hypothesized that this may also arise from deconjugation of conjugated metabolites during the treatment process [4,15,16]. Alternatively, the reason may be an inappropriate sampling strategy, which does not take into account the fluctuations in concentration. Nevertheless, this phenomenon most commonly occurs in case of CBZ and DF, both of which are resistant to conventional wastewater treatment [16,19,122,123].

Table 5: *Occurrence of pharmaceuticals in WWTPs: concentration range, median and maximum concentrations in ng L^{-1}*

Target compound	WWTP effluent			WWTP influent		
	Conc. range	Med (ng L^{-1})	Max (ng L^{-1})	Conc. range	Med (ng L^{-1})	Max (ng L^{-1})
IB	40-800 [46] 5-1500 [49] 18-1860 [45]	266 [46] 1885 [3] 2972 [47] 150 [17] 100 [16] 3086 [48]	24600 [3] 680 [106] 27256 [48]	37-860 [45]	516 [46] 3590 [17]	900 [46] 1660 [106]
NP	100-3500 [49]	108 [46] 168 [3] 625 [45] 250 [17] 80 [16]	160 [46] 855 [3]	109-455 [45]	99 [46] 3650 [17] 440 [16]	190 [46]
KP	130-620 [46]	318 [46] 130 [3] 330 [17] 230 [16]	200 [49] 130 [3]	160-970 [46]	451 [46] 131 [45] 940 [17] 300 [16]	
DF	100-700 [49] 32-1420 [45]	215 [46] 359 [3] 289 [47] 120 [17] 2510 [16] 424 [48]	390 [46] 28400 [3] 1680 [106] 4700 [16] 2349 [48]	50-540 [46] 21-148 [45]	250 [46] 160 [17] 3020 [16] 1500 [76]	4470 [106] 7100 [16]
CLA	20-30 [46] 22-107 [45]	28 [46] 30 [3] 44 [47] 480 [16]	60 [49] 76 [3] 110 [106] 730 [16]	25-58 [45]	72 [46] 460 [16]	110 [46] 170 [106] 950 [16]
CBZ	100-800 [49]	410 [46] 107 [3] 1180 [17] 1630 [16]	630 [46] 2300 [3] 5000 [16]		420 [46] 1680 [17] 1780 [16] 1200 [76]	950 [46] 3800 [16]

Pharmaceuticals may occur in WWTP effluents, either because they are truly persistent under the conditions of an activated sludge process, or because their microbial degradation was not fast enough to be completed within the typical hydraulic retention time (HRT) of 15 hrs. A partial degradation in the WWTP implies, however, that this compound may be further degraded after its discharge into the receiving water body. The mere presence of a polar pollutant in WWTP effluents does not imply that it would spread in the aquatic environment, but only when it is in a stable form, such a risk must be considered [76,107]. Therefore, a moderate effluent concentration of a poorly degradable compound that does not undergo a significant decrease in concentration in a WWTP is more problematic than the same effluent concentration of a compound that was degraded extensively from a much higher influent concentration. In this sense, an evaluation of a pharmaceutical or any other polar WW constituent with respect to its potential to spread in an aquatic environment may be based on the ratio of its WWTP effluent concentration (c_{OUT}) and its normalized removal in municipal wastewater treatment ((c_{IN} - c_{OUT})/c_{IN}). This ratio is called the 'water cycle spreading index' (*WCSI*) [76]:

$$WCSI = \frac{effluent\ \ concentration}{normalized\ \ removal\ in\ WWTP} = \frac{c_{IN} \times c_{OUT}}{(c_{IN} - c_{OUT})} \qquad \text{Equation 4}$$

This index has the dimension of a concentration. A higher *WCSI* indicates a higher potential of a compound to spread in the aquatic environment and through the water cycle, and, thus, higher environmental concentrations are expected, as compared to a compound with a low *WCSI*. When the *WCSI* is calculated from the data of only one WWTP, it is site specific, as the amount of a compound discharged into a sewer

system, the extent of its removal in a WWTP, and thus its final discharge into receiving waters may differ from one plant to another. Poor treatment thus leads to a drastically increased *WCSI*. To assess the spreading of polar compounds in larger catchments, however, the influent and effluent concentration data of a larger number of properly operated WWTPs should be considered to calculate the *WCSI* of a compound. The *WCSI* data are suitable to prioritize pharmaceuticals in terms of their spreading potential, as they may actually reflect the two most critical aspects of a water contaminant, i.e. its amount released into the system and the persistence in that system, but it should be further noted that the *WCSI* does not consider pharmacological effects toward aquatic organisms or humans [76].

1.3.2 Removal

Conventional WW treatment is usually a combination of physical and biological processes designed to remove organic matter from solution [108]. The first step in conventional treatment is a sedimentation process, which removes the settled and floating organic matter. Secondary treatment is biological treatment, where cultures of microorganisms (activated sludge) metabolize the biodegradable organic matter from the wastewater. A few municipalities employ a tertiary (advanced or chemical) wastewater treatment, where specific chemicals are removed from the partially already purified water, before final disinfection. Depending upon the nature of the pollutant, tertiary treatment can include chemical and physical processes, such as adsorption of nonpolar organic molecules onto activated carbon, removal of phosphate by precipitation as the calcium salt, heavy metal removal by the addition of hydroxide or sulphide, iron removal by aeration at a high pH to oxidize it to its insoluble Fe^{3+} state, desalination by reverse osmosis, electrodialysis or ion exchange [109].

The conventional treatment of groundwater for the production of potable water follows similar stages to wastewater treatment, though the quality of the final product meets much stricter quality standards compared to WWTP effluent [110]. The technology commonly comprises aeration, flocculation / filtration or slow-sand filtration, and disinfection. Aeration removes dissolved gases and volatile organic compounds and is followed by precipitation and settling of colloidal particles. This is achieved by adding Al or Fe salts (aluminium sulphate, ferric chloride), which form gelatinous hydroxides at neutral and alkaline pH values, and physically incorporate the colloidal particles in a removable precipitate. If the content of Ca^{2+} and Mg^{2+} salts is too high, the water is softened by adding Na_2CO_3 or NaOH, while to remove colour or odour, adsorption on granulated or powdered activated carbon is applied. Disinfection is applied as a last step, where the chlorination, UVC radiation or ozonation are most commonly used [43,109].

Conventional water treatment is designed to remove nutrients, particulate matter, dissolved gases, odorous substances, colorants and pathogens. However, literature data reveal that conventional treatment processes poorly remove persistent pharmaceuticals [111]. Developments in chemical water treatment have led to an improvement in oxidative degradation procedures for the removal of persistent pollutants in applying catalytic and photochemical methods, which are referred to as advanced oxidation processes (AOP) [112]. The majority of AOPs involve the generation of significant amounts of hydroxyl radicals ($^{\bullet}OH$), which are an effective nonselective oxidizing agent in aqueous solution [109]. Since the generation of $^{\bullet}OH$ radicals is a relatively expensive process, AOPs are especially useful in two cases: (*i*) as a pre-treatment to transform recalcitrant pollutants into more biodegradable compounds; or (*ii*) as a post-treatment, to polish waters before their discharge to the receptor bodies [113].

The removal efficiencies of pharmaceuticals vary between different WWTPs and depend on the design and operation of the treatment systems. The important process conditions for removing pharmaceuticals are hydraulic retention time (HRT), sludge retention time (SRT) and temperature [21,51,78]. The effect of weather conditions on the elimination rate can also play a role. Such an example is the elimination efficiency of the readily biodegradable IB or KP, which in municipal WWTP decreases during wet periods compared to dry [19]. For further studies in the area of mitigation, attention should be focused on optimizing the WWTP design and operation, possibly involving a multiple-stage treatment, in order to improve the removal efficiencies of pharmaceuticals. However, the abatement of parent pharmaceuticals only provides a partial indication of the efficiency of the various treatment methods and the possible generation of toxic intermediates more resilient to degradation must not be overlooked [114].

1.3.2.1 Physico-chemical processes

The effectiveness of physico-chemical processes, i.e. coagulation-flocculation and flotation, principally depends on physico-chemical characteristics and the chemical structure of each pharmaceutical [69]. Generally, coagulation-flocculation processes enhance the removal of suspended solids and colloids, because the addition of metal salts causes the agglomeration of these particles, thus allowing their elimination by decantation or filtration [115]. Lipophilic trace pollutants in water and wastewater treatment systems and, based on the Van der Waals bonds, the positively charged molecules, are likely to be found associated with colloids and may thus be removed together with the agglomerates [116]. Literature information on pharmaceuticals removal by coagulation-flocculation processes is scarce and sometimes contradictory. Westerhoff et al. [117] reported <20 % removal of sulphametoxazole, CBZ, DF and iopromide with Fe and Al salts. Alternatively, ferric chloride was found to significantly contribute to the improved removal efficiencies of tetracycline [78]. In addition, coagulation-flocculation experiments show a 70 % removal of DF, resulting from its possible adsorption to agglomerates *via* electrostatic interactions. In the same study, NP is reduced to a lesser extent (up to 25 %), while IB and CBZ show no removal by coagulation-flocculation [69].

With flotation techniques, finely suspended particles are separated by adhering to the surface of rising bubbles. The removal of pharmaceuticals by flotation depends on their solubility in lipid fractions or sorption to small aggregates [69]. For instance, Praxeus [118] associates the removal of CBZ with an unusually high content of silicone oil in wastewaters. Accordingly, Carballa et al. [69] show how the removal of CBZ, IB, NP and DF by flotation improves in a high fat wastewater.

1.3.2.2 Biological removal

The activated sludge process is a continuous or semi-continuous aerobic treatment method through which wastewater undergoes nitrification [119]. Its performance and characteristics are evaluated by pH, RedOx potential, HRT, SRT, chemical oxygen demand (COD) in mg L^{-1}, biochemical oxygen demand (BOD) in mg L^{-1}, nitrates in mg L^{-1} and phosphates in mg L^{-1} [120].

Figure 3 illustrates the removal efficiencies of test pharmaceuticals determined in municipal WWTPs. In all cases the WWTPs utilised classical treatment methods, based on conventional activated sludge (CAS) treatment. Figure 3 shows that CLA and CBZ are poorly removed (<51 % and <30 %, respectively) from most WWTPS, while the removal of DF is highly variable (0 to 75 %). The removal of IB, NP and KP is satisfactory, but again, as expected, it varies from plant to plant.

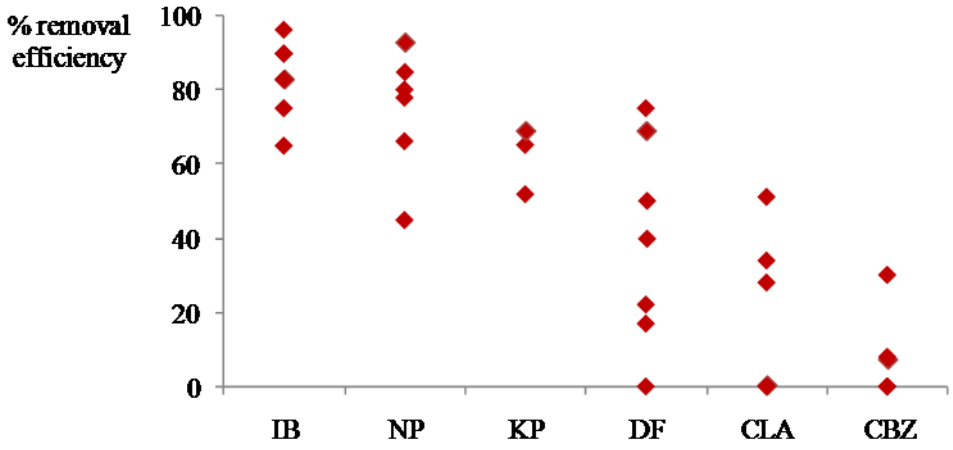


Figure 3: *Removal efficiencies of the target compounds in different CAS WWTP systems* [4,16,17,19,47,52,121,122,123]

Membrane bioreactor (MBR) treatment has gained significant popularity as an advanced wastewater

treatment. The technique uses ultrafiltration or microfiltration membranes for the complete retention of the biomass, as well as of suspended solids [21]. Consequently, the high biomass concentration in the reactor results in a highly efficient biological degradation process with reduced sludge production [124,125]. MBRs are characterized by having a long SRT, which gives the biomass time to adapt to more recalcitrant micropollutants and consequently improves their removal [126]. This is supported by González et al. [127], who show that a lag period is needed for microorganisms to start degrading persistent pharmaceuticals, such as DF. Alternatively, an improved removal of readily biodegradable pharmaceuticals in the MBR is explained by the smaller flock size of the sludge, which enhances mass transfer by diffusion and therefore increases elimination [123]. Literature data on removal by MBR is still rather contradictory. Membrane bioreactors are proven to be more effective in eliminating pharmaceuticals, such as KP and NP, when compared to the CAS [126]. Alternatively, neither MBR, nor CAS result in the satisfactory removal of either DF, CLA [126] or CBZ [21,123], while the elimination of readily biodegradable IB was higher than 90 % [126]. Further, Clara et al. [128] compare MBR with CAS and find comparable removal rates for DF for both systems running at a similar SRT, whereas Radjenović et al. [123] find an improvement in elimination by MBR for the majority of the test compounds, including DF (87 %) and CLA (72 %).

1.3.2.3 Advanced oxidation processes

Advanced oxidation processes comprise a range of water reclamation strategies aiming to achieve the complete mineralization of organic pollutants using highly reactive oxygen species generated by different techniques, including photochemical systems (UV/H_2O_2, photo-assisted Fenton, TiO_2 photocatalysis, UV/O_3) or ˙OH-generating systems (O_3/H_2O_2, Fenton: Table 6) [129]. Such techniques, making use of different reaction systems, are all characterized by the same chemical feature: production of OH radicals (˙OH). These are highly reactive and nonselective species, which is an advantageous attribute for an oxidant used for the removal of a wide range of organic micropollutants in water treatment. The versatility of the AOP is also enhanced by the many ways of generating ˙OH, thus allowing greater compliance with the specific treatment requirements [130]. A suitable application of AOP must consider the cost of reactants, such as H_2O_2 and/or O_3, which currently means that they cannot compete with established treatment technologies, such as biological degradation. Table 6 gives a list of different AOP.

Table 6: *Most common advanced oxidation processes* (adopted from [130])

AOP		AOP name
Photochemical systems	H_2O_2 / Fe^{2+} (Fe^{3+}) / UV	Photo-assisted Fenton
	TiO_2 / UV / O_2	Heterogeneous photocatalysis
	O_3 / UV	
	H_2O_2 / UV	
Other ˙OH generating systems	O_3 / H_2O_2	
	H_2O_2 / Fe^{2+}	Fenton
	H_2O_2 / Fe^{3+}	Fenton-like

Albeit individual UV treatment does not accurately fit into the definition of an AOP, during indirect photolysis it makes use of ˙OH radicals and is therefore discussed under this chapter. UV treatment is a popular method for disinfecting potable water; however, it is not applicable for the removal of pharmaceuticals in WW treatment systems [131]. The effectiveness of UV for removal of pharmaceuticals varies depending on the target compound. Kim and Tanaka [131] classified KP and DF as easily photodegradable, while NP and CBZ belong to a group of compounds slowly degraded by UV. Also, because the degradation rate increases with the production of ˙OH radicals, the use of AOP systems could improve the removal of slowly-degrading compounds [131]. In a similar study, Packer et al. [67] demonstrate that DF and NP are photolabile and that direct photolysis is the dominant process for removing these two pharmaceuticals. On the contrary, the photoresistant CLA and IB are minimally affected by direct irradiation, whereas the radical mediated processes are found to be the dominant photochemical loss mechanisms [67].

Many AOPs involve UV radiation energy as an important step in the synthesis of ˙OH radicals, because UV energy can increase the reaction rate of AOPs in comparison with the same technology in the absence of illumination [112]. The UV-radiation induced oxidation with ozone and/or hydrogen peroxide and the

photo Fenton reaction are carried out in the homogeneous phase. The disadvantage of homogeneous catalytic processes is the necessity to recover the dissolved catalyst. In the Fenton process, production of ˙OH occurs by means of the addition of H_2O_2 to Fe^{2+} salts:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \qquad \text{Equation 5}$$

This reactant is an attractive oxidative system for wastewater treatment since iron is both abundant and non-toxic and because hydrogen peroxide is easy to handle and environmentally safe [130]. The Fenton-like process (Table 6) occurs at the adoption of pH to 2.7 – 2.8 [132], which results in the reduction of Fe^{3+} to Fe^{2+} and thus its regeneration, which makes the iron act as a catalyst [130]. The rate of degradation of organic pollutant with Fenton and Fenton-like reagents is strongly accelerated by UV-VIS light [133,134], where the ˙OH radicals are produced at a higher rate (photo-assisted Fenton process). Accordingly, Pérez-Estrada et al. [135] were able to show a fast decay of DF from the solution using the "dark" Fenton treatment, whereas the photo-Fenton treatment leads to complete mineralization (disappearance of dissolved organic carbon).

Similarly, it is shown by Andreozzi et al. [136] that UV direct photolysis yields negligible effects in terms of substrate disappearance as compared to the UV/H_2O_2 process. The initial step for the UV/H_2O_2 oxidation process is represented by the photolysis of hydrogen peroxide and the generation of ˙OH radicals [136]:

$$H_2O_2 \xrightarrow{h\nu} 2{}^{\bullet}OH \qquad \text{Equation 6}$$

H_2O_2 photocatalysis and ozonation have both reached a high level of development and have found application on an industrial scale [136]. In general, the reaction of ozone with organic compounds can be classified into the direct reactions of ozone with the target molecule and ˙OH radical mediated reactions [137]. Oxidation with ozone is commonly applied in water treatment for disinfection or for aesthetic reasons, i.e. for removal or coloured and odorous substances, as well as to remove organic micropollutants. In comparison with ˙OH radicals, direct oxidation reactions with O_3 are highly selective. As a result, rate constants range over ten orders of magnitude [138]. Such high reaction rates are observed for pharmaceuticals containing double bonds (CBZ), activated aromatic structures like a phenolic group, nitrogen heterocycles, or amino (DF, sulfamethoxazole) and sulfur groups. The reaction with ozone is also pH dependent, as deprotonated groups are stronger nucleophiles and therefore react more rapidly with electrophilic ozone than their protonated forms. The pharmaceuticals lacking the ozone reactive moieties (CLA, IB, KP, NP) exhibit low rate constants with ozone. Here direct reactions with ozone play a minor role during the ozonation process and the oxidation of such pharmaceuticals is caused by ˙OH radicals originating from ozone decomposition in water [43,138,139]. An alternative solution for removing pharmaceuticals are hydroxyl radical-based AOPs. Thus, the combined application of H_2O_2 and O_3 will enhance the O_3 decomposition by the formation of ˙OH radicals [130]. Zwiener and Frimmel studied the removal of IB, CLA and DF using O_3/H_2O_2 [137]. In distilled water IB and CLA were degraded to half of their initial concentration under the experimental conditions, while DF was quantitatively degraded. However, in the case of a river water matrix the degradation efficiency of CLA and IB was significantly reduced. This they explain by the presence of radical scavengers (dissolved organic matter, DOM) that compete with the pharmaceuticals for the ˙OH radicals. In this sense, the oxidant concentration required for water treatment depends on both, reaction kinetics of a specific pharmaceutical and the OH-scavenging capacity of the matrix [137].

In contrast with the homogeneous photocatalysis, in the heterogeneous photocatalysis two phases are present, and the active component is fixed at the surface of the catalyst [140]. Heterogeneous photocatalytic processes make use of a semiconductor metal oxide like titanium dioxide (TiO_2) as a catalyst and oxygen as an oxidizing agent [141]. TiO_2 is reactive, inexpensive, non-toxic and chemically stable over a wide pH range, and it is not subject to photo-corrosion. The electron/hole pair (e^-/h^+) generated under light illumination reacts with water oxidized by photoholes (h^+) and gives rise to the generation of ˙OH radicals, responsible for the complete decomposition of the chemical substances [142]. Doll and Frimmel [143] report that heterogeneous photocatalysis using TiO_2 is a promising technology for removing persistent pharmaceuticals, CLA and CBZ. Furthermore, TiO_2 photocatalysis results in the complete removal of KP, DF and NP under the applied experimental conditions [144]. Thus, heterogeneous photocatalysis may find its use as a part of sequential treatment technology, either to remove the persistent organic pollutants,

which survived the biological treatment, or as a pre-treatment step, to enhance the biodegradability of pollutants [143]. The latter is less favourable from an economic perspective.

1.3.2.4 Disinfection

Direct UV irradiation and O_3 are used for removing of pathogens, but the best known and historically most important disinfection agents are chlorinating agents (e.g., molecular chlorine, sodium hypochlorite, chloroamine and chlorine dioxide). Molecular chlorine is more reactive in oxidation reactions and in reactions with double bonds, while sodium hypochlorite has a higher activity in electrophilic aromatic substitutions [145]. However, trihalomethanes and halogenated acetic acids are generated during Cl_2 and NaClO treatment, and carcinogenic dimethylnitrosamine is also formed from chloroamine. European legislation [146] limits the content of these substances in potable water, and, to minimize their formation, the use of chlorine, sodium hypochlorite and chloramine for disinfection of potable water is being gradually replaced by alternative disinfection agents worldwide. Alternatively, chlorine dioxide does not yield halogenated disinfection by-products, nor does nitrosamine under the correct operating conditions, and, in this sense, appears to be the chlorinating agent of choice [145,147]. Chlorine dioxide is an oxidant used for the disinfection of relatively high quality water, such as groundwater or treated surface water. Appropriate dosing of chlorine dioxide to treated water provides residual concentrations, which protects the potable water distribution network from microbiological contamination and fouling. In Europe, chlorine dioxide residuals are kept at $< 0.05 – 0.1$ mg L^{-1} [148]. Chemically, chlorine dioxide is a stable free radical that reacts with other water matrix components and micropollutants through a one electron reaction. It is a highly selective oxidant with respect to specific functional groups, such as phenolic moieties or tertiary amino groups. A comparison of NSAIDs, CLA and CBZ reactivity with chlorine dioxide shows an appreciable decay only for DF, which is reasoned by the presence of an amine group in its chemical structure [147]. Compared to ozone and hypochlorous acid, chlorine dioxide reacts more slowly and with fewer compounds, but still appears to be a more powerful oxidizing agent than molecular chlorine. Overall, chlorine dioxide can be applied only as a partial barrier for pharmaceuticals [147] and complementary treatment methods are needed to achieve their complete mineralisation.

1.3.2.5 Physical methods

Adsorption on activated carbon depends on the non-polar character of uncharged compounds. The K_{OW} has thus proved useful for predicting removal efficiency, offering a good correlation between the percentage removal and logK_{OW} values [117]. Exceptions include N-heterocyclic compounds, such as CBZ, pentoxifylline and trimethoprim, where, because of specific interactions with activated carbon, their removal efficiencies are higher than might be expected from their K_{OW} values. In contrast, removal efficiencies for compounds involving carboxyl groups (e.g. CLA, NSAIDs) are much lower; this is because in water they dissociate, yielding anionic compounds. The adsorption of charged pharmaceuticals is much lower than what would be expected from their logK_{OW} values [117].

Reverse osmosis (RO) and nanofiltration (NF) are an effective means of removing pharmaceuticals from potable water [149]. Reverse osmosis is a physical separation process in which properly pretreated source water is delivered against a semipermeable membrane, which rejects most solute ions and molecules, while allowing water of very low mineral content to pass through. This process also works as an absolute barrier for bacteria and viruses. The process produces a concentrated reject stream and a clean permeate product and has been applied to saline groundwaters, seawater and for removing inorganic and other organic pollutants. Because the source water needs to pass through very narrow passages in the membrane module, larger suspended solids must be removed during the initial treatment phase (pretreatment). Nanofiltration is a lower pressure RO technology with lower monovalent ion rejection properties, making it more suitable to treat waters with low salinity [150]. Because of the low operation pressures necessary for the nanofiltration, the latter is a more economic option. The rejection efficiency, however, also correlates with the concentration of dissolved pharmaceuticals and requires greater effort in lower concentrations. Also, negatively charged compounds (CLA, NSAIDs) can be rejected very effectively in comparison to non-charged compounds, which tend to adsorb to the membrane materials [43,149].

1.4 Transformation of pharmaceuticals during water treatment and in the environment

Physical processes, such as sorption, dilution, dispersion and filtration, all contribute to the natural attenuation of pharmaceuticals, but without causing a change in their chemical structure [37]. However, the majority of the attenuation processes, either taking place in the environment or as a part of water treatment, alter the chemical structure of the substrate micropollutants. It is possible to divide the structural transformation processes that organic chemicals undergo into three major categories: chemical, photochemical, and biologically mediated transformation reactions. The former two are commonly referred to as abiotic transformation processes. Chemical reactions encompass all reactions that occur in the dark and without mediation of organisms. In the presence of light a compound undergoes transformation either as a consequence of direct absorption of light (direct photolysis), or by reacting with highly reactive oxygen species (e.g., free radicals or singlet oxygen) that are formed as a result of the incidence of light on sensitized chemical species (indirect or sensitized photolysis). Finally, xenobiotic organic chemicals can be transformed by microorganisms. Many types of chemical or photochemical reactions can also be performed by microorganisms, and therefore it may not always be clear, whether, in a given environmental or treatment system, a reaction occurs strictly abiotically, whether it is mediated by microorganisms, or whether both types of processes play a role [151].

In comparison to environmental breakdown, the transformation processes involved in mitigation technologies are far more intense, albeit in principle, the underlying reaction mechanisms only imitate the natural processes that take place in the environment. Table 7 lists the most common techniques applied in WW treatment or potable water production and naturally occurring processes involving similar reaction mechanisms.

Table 7: *Transformation processes in the environment and water treatment*

Reaction mechanism	Water treatment	Environmental process
Microbial transformation reactions	- CAS (WW) - biological membrane (WW) - MBR (WW) - sand filter (potable water)	- microbial degradation in soils, sediments
Photo-transformation reactions: direct and indirect photolysis	- UV irradiation	- exposure to sunlight
Photocatalytic processes (enhanced production of $^{\bullet}OH$)	Photoassisted AOP: - O_3 / UV - H_2O_2 / UV - H_2O_2 / Fe^{2+} (Fe^{3+}) / UV - TiO_2 / UV / O_2	- indirect photolysis with dissolved organic matter (DOM)
Chemical oxidation (including the reactions involving the reactive oxygen species)	- chlorination - chloramination - ClO_2 treatment - ozonation (direct oxidation and the decay of O_3 to $^{\bullet}OH$) - O_3 / H_2O_2 - H_2O_2 / Fe^{2+} (Fe^{3+})	- chemical oxidation by natural or anthropogenic oxidants present in the environment - indirect photolysis with DOM

For transformation processes, especially the environmental chemical and photochemical reactions are complex and constantly overlapping, it is therefore impossible to clearly distinguish between them. This chapter in particular discusses the transformation reactions leading to a structural alteration of the parent pharmaceutical molecule, without respect to where the transformation process takes place (i.e. environmental process or mitigation technology).

1.4.1 Abiotic transformation

The following chapter discusses abiotic transformation reactions occurring in the environment or as a part of water treatment, where photochemical transformation is addressed. Two different types of photochemical processes may lead to the transformation of organic pollutants in the aquatic environment: (i) direct photolysis, when a pollutant absorbs a photon and consequently undergoes transformation, and (ii) indirect photolysis, which occurs due to the energy transfer from another excited species (photosensitization) or reactive oxygen species (e.g. hydroxyl radicals, peroxy radicals, singlet oxygen) that are induced by the presence of light. When a chemical species has been promoted to an excited state, it may undergo various physical or chemical processes, as summarised in Figure 4. As indicated, there are several physical processes by which an excited species may return to a ground state; that is, it is not structurally altered by these processes. Thus, an excited species may return to a ground state by giving off energy as either heat or light (luminescence), or an excited species may transfer its excess energy to another molecule in a process called photosensitization. Alternatively, there are a variety of chemical reactions, which result in a transformation as referred to as direct photolysis of organic pollutants [152].

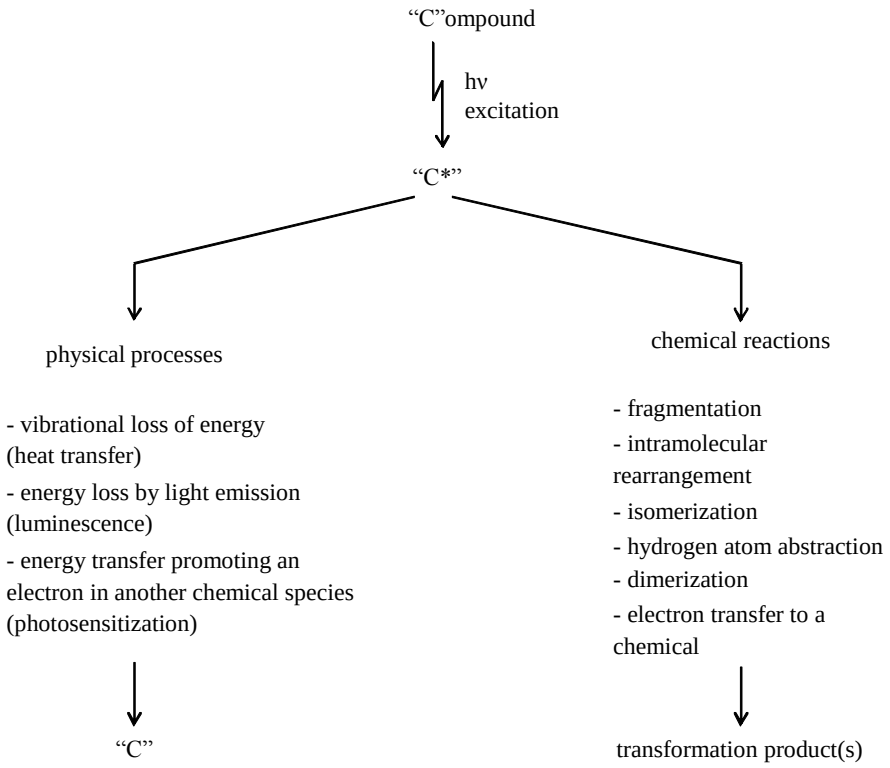


Figure 4: *Physical processes and chemical reactions of a photochemically excited organic species* (adopted from [152])

The second group of processes initiated through light absorption by other chemicals present in the system are commonly referred to as indirect or sensitized photolysis. Figure 5 depicts the most important physical (i.e. sensitized C path, singlet oxygen $^1O_2$ path) and chemical (i.e. formation of radical species, "solvated electrons", e_{aq}^-) pathways that may lead to the transformation of an organic chemical (C) as a consequence of the excitation of an unknown chromophore (UC) present in DOM. By far the most important acceptor of UCs is molecular oxygen in its triplet state, which promotes into its excited state, singlet oxygen $^1O_2$. The latter may then react with organic pollutants. Organic pollutants are in competition with $^3O_2$ for the available absorbed light energy; therefore, energy transfer from the ^{3}UC* (the triplet state of the excited DOM chromophores formed by intersystem crossing from the singlet states, ^{1}UC*) to a given organic pollutant is most important in waters of low oxygen concentration. In addition to energy transfer to

either molecular oxygen or to organic compounds (including organic pollutants), chemical reactions of excited UCs (^{1}UC*, ^{3}UC*) may lead to other reactive species that react with organic pollutants (Figure 5). Such processes include the formation of reactive DOM species (DOM*), the reaction with $^3O_2$ to form DOM-derived peroxy radicals (ROO˙), the transfer of an electron to $^3O_2$ to form superoxide anions ($O_2^{-\cdot}$), the formation of solvated electrons (e_{aq}^-) and the formation of hydroxyl radicals ˙OH [152]. In case of hydroxyl radicals, however, DOM also reacts with hydroxyl radicals thus acting as a radical scavenger.

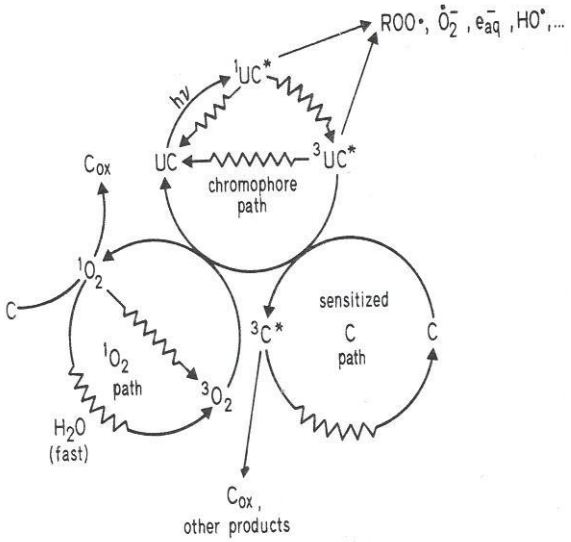
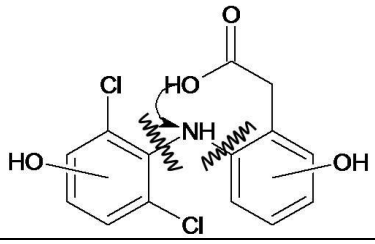
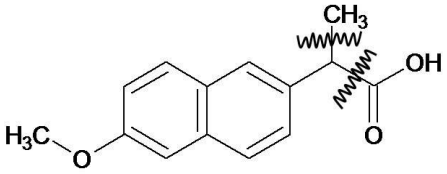
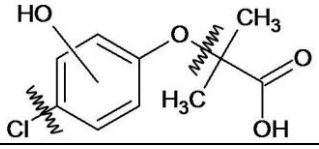
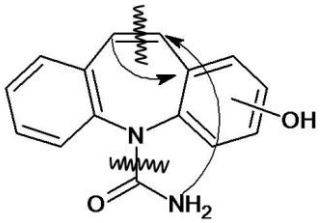


Figure 5: *Pathways for indirect photolysis of organic chemical (C).* UC refers to unknown chromophores. Wavy arrows symbolize radiationless transition [152].

The hydroxyl radical is highly reactive and a nonselective oxidant, which plays a major role in AOPs in water treatment and is generated by various means (Table 6), H_2O_2, or, in the surface waters from reactions of excited humic acids, nitrite, and nitrate as the major source [152]. During treatment the transformation rate of a given pharmaceutical depends primarily on the OH-scavenging capacity of the matrix [137], while in the environment, it also includes the latitudes and varying seasons [153]. Table 8 illustrates the most common transformations reported as a result of photochemical reactions on NSAIDs, CLA and CBZ.

Table 8: *Abiotic transformation reactions reported on selected pharmaceuticals*

Pharmaceutical	Primary structural transformation	Chemical structures with active sites	Reference
DF	- aromatic hydroxylation, - cleavage of C-N bond		[140,154]
NP	- dimerization, - oxidation of α-propionic acid moiety		[155]
CLA	- aromatic hydroxylation, - cleavage of ether bond, - substitution of chlorine with hydroxyl group		[156]
CBZ	- ring contraction to yield aromaticy, - formation of quinazoline derivatives, - cleavage of carbamyl side chain, - aromatic hydroxylation		[143,157]

Breakdown as a result of photochemical processes depends on the physicochemical characteristics of the compounds. As derived from Table 8, it typically involves hydroxylation of the aromatic ring by an electrophilic attack from the ˙OH radicals, cleavage of C-O or C-N bond and cleavage on the α-position from the aromatic moiety [158]. The subsequent breakdown of the aromatic structures generally leads to oxidative ring cleavage and the production of carboxylic acid fragments *via* classical degradation pathways [154].

1.4.2 Biological transformation

As for chemical and photochemical reactions, the biochemical processes change the structure of an organic micropollutant, thereby removing that particular compound from the system. The resulting product(s), e.g. TPs, exhibit their own properties, reactivity, fate, and effects. Similarly to the abiotic transformations, the biologically mediated transformations do not necessarily end up in complete mineralization. Organisms enable biological transformations *via* two important approaches. The first approach employs enzymes that serve as catalysts, and thereby reduce the activation energy that determines the transformation rate [159]. Secondly, organisms may convert the matrix compounds into activated species, such as $ROO^{\cdot}$, $O_2^{\cdot-}$, or ˙OH, which evolve further reactions with organic micropollutants.

Biodegradability studies in WWTPs may be useful in determining the species entering the environment after biotransformation or biodegradation through microbial metabolism of the substrate or other mechanisms [7]. Studies show that the formation of TPs can vary in wastewater treatment works, depending on the composition of the sewage, weather conditions, and the design and operation of the treatment process [4,160]. In many cases, the metabolites formed during biodegradation exhibit a higher polarity than the parent drugs [161], which, combined with low biodegradability, results in the metabolite

passing through the treatment systems and high aquatic mobility. In contrast to human metabolism of pharmaceuticals, which has to be studied in detail prior to pharmaceuticals being approved, their microbial degradation, their transformation pathways and products are still yet to be recognized [162]. Due to the different enzyme systems involved, the enzymatic transformations in waste and environmental waters are generally not comparable to those in mammals. This assumption was supported by Jjemba [163], who shows that drugs highly metabolized in target organisms (and therefore excreted in low proportions) may have an inherently low environmental (bio)degradability. Alternatively, studies [164,165] report similar compounds originating from enzymatic biotransformation and human metabolism. The structure of the microbial community is critical for different biological conversions and the formation of TPs largely depends on the bacterial diversity present in the biological treatment system. Different chemicals present in a given system also affect the microbial community structure and thus govern the transformation of these compounds. Besides, the presence of chemicals may also cause a microorganism to change the production rate of enzyme units suited to degrading a substance, which happens through enzyme induction, depression, or mutation [159].

1.5 Determination of pharmaceutical residues in the environment

This chapter reviews the state-of-the-art in the analysis of selected pharmaceuticals in aquatic environmental matrices and wastewater. It describes common methods of sample preparation and discusses various aspects of current gas chromatography - mass spectrometry (GC-MS/(MS)) and liquid chromatography - mass spectrometry (LC-MS/MS) in quantitative analysis. Furthermore, it focuses on qualitative determination, i.e. identification of pharmaceutical TPs, where the potentials or different mass spectrometric technologies are compared with respect to their applicability for identification or confirmation. Finally, the validation of analytical methods is discussed and basic parameters explained.

1.5.1 Sample preparation

The procedure for pharmaceutical residue analysis in water samples typically includes an enrichment step followed by separation of the target analytes and detection. Nowadays, few enrichment methods are based on classical techniques, like liquid-liquid extraction (LLE) [166,167]. Solid phase extraction (SPE) has gradually replaced LLE becoming the most common sample preparation technique in environmental analysis. SPE offers the following advantages over LLE:

1. higher recoveries;
2. improved selectivity, specificity and reproducibility;
3. no emulsion formation;
4. reduced organic solvent usage;
5. shorter sample preparation time; and,
6. easier operation and the possibility of automation [63].

In SPE, the analytes are partitioned between a solid phase and a liquid phase, and should exhibit a greater affinity for the sorbent than for the liquid matrix. The choice of a sorbent is the key to successful SPE, since it controls parameters, such as selectivity, affinity and capacity [168]. Classical SPE sorbents include chemically-bonded silica with C8 and C18 organic group and ion-exchange materials, polymeric materials, immunosorbents and molecularly-imprinted polymers [63]. Polymer-based sorbents are the most versatile enabling extraction of a wide range of analytes or performing analysis under different matrix conditions [168]. Currently, one of the most widely used sorbents for SPE of pharmaceuticals is a copolymer of divinylbenzene and vinylpyrrolidone [46,121,169,170,171,172]. In a recent comparison of seven polymeric SPE sorbents, this phase showed a superior performance for extracting acidic pharmaceuticals (IB, DF and CLA) [44]. In other studies, a polydivinylbenzene resin sorbent containing piperidone groups also gave excellent extraction recoveries for pharmaceuticals [173,174,175]. To improve the retention on sorbents, a sufficient hydrophobicity of the analytes should be ensured by avoiding the deprotonation of the acidic compounds and the protonation of basic compounds. Acidic pharmaceuticals should therefore be extracted under acidic conditions, opposite to basic analytes [169]. However, Gómez et al. [172] showed that polymeric cartridges offer the possibility to perform multi-residue analysis work at

neutral pH, which greatly simplifies the sample-handling procedure and also avoids the risk of acidic hydrolysis [176].

Figure 6 shows how the next crucial step following sorption is elution of the analytes from the sorbent. The choice of elution solvent used to desorb the target compounds from the SPE cartridge depends on the physico-chemical properties of the analytes and the elution strength of the solvent. The type and the volume of the elution solvent are important factors that affect the recovery [176]. Ethyl acetate, acetone and methanol, having different elution strengths and polarities, are examples of commonly used solvents [177]. Figure 6 shows the general SPE procedure.

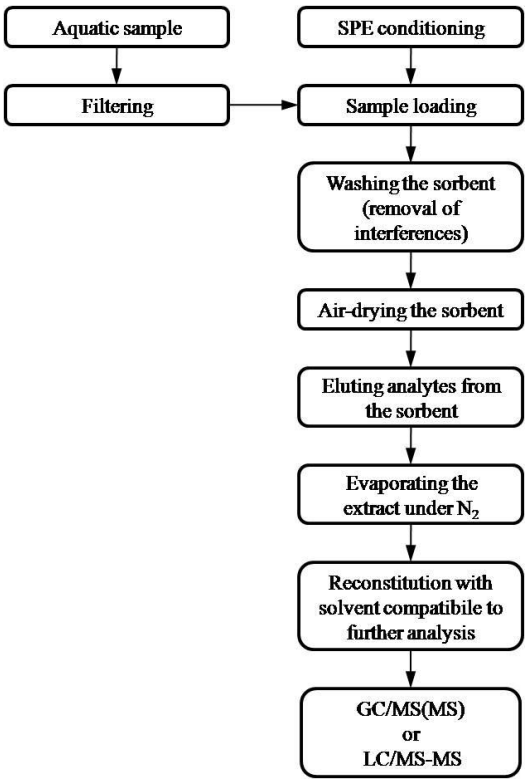


Figure 6: *Common steps in the SPE procedure* [63]

Solid phase microextraction (SPME) differs from SPE in terms of sorbent volume. It involves using a fused silica fiber coated with a thin film of a polymeric stationary phase [63]. The technique has several advantages over SPE; i.e. it requires less sampling volume, it is solvent free, allowing high enrichment factors, and is easily automated [178,179]. When SPME is coupled to GC, analytes are thermally desorbed from the fiber in the GC injector, while coupling SPME to HPLC requires a special interface allowing the desorption with a small amount of solvent [180]. There are some drawbacks of using SPME, and these include limited robustness, often limited sorption capacity and insufficient limits of detection (LOD) and quantification (LOQ) [63,176].

1.5.2 Quantitative analysis

1.5.2.1 GC-MS based procedures

An advantage of GC hyphenated to MS is that the usual ionization modes, such as electron impact (EI) and chemical ionization (CI) are less affected by the matrix suppression compared to ionization modes commonly used in LC-MS [169]. Thus, for some families of pharmaceuticals GC-MS(MS) based methods can offer lower detection limits than LC-MS [181].

In reality many pharmaceuticals lack sufficient volatility to be directly compatible with GC, and

derivatisation is often necessary after sample clean-up and pre-concentration. The purpose of derivatisation is to convert polar substances into less polar analogues with increased volatility and thermal stability. Although derivatisation procedures may be time consuming and may introduce errors due to incomplete reactions, insufficient stability of derivatives or side-reactions during derivatisation, they are still widely used for routine analyses. Derivatisation reactions can be affected by several factors, such as solvent, derivatisation time, temperature and reagent dose [121]. A typical derivatisation reagent for NSAIDs, CLA and CBZ is the alkyl halide pentafluorobenzyl bromide (PFBBr) in the presence of a catalyst (triethylamine) [19,25,182,183], which produces an electron rich derivative that can be analyzed by GC with an electron capture detector (GC-ECD) or GC-MS using negative chemical ionization (NCI) [181]. Among the available alkylating reagents, diazomethane [49,153] is commonly used for analyzing acidic pharmaceuticals. However, on account of its toxicity, carcinogenicity and explosiveness it is being replaced by much safer silylating agents. The two main classes are: N-methyl-N-(trimethylsilyl)-trifluoracetamide (MSTFA) [52,184], N,O-bis(trimethylsilyl)-trifluoracetamide (BSTFA) [185], which produce trimethylsilyl derivatives (TMS), and N,N-(tertbutyldimethylsilyl)-trifluoroacetamide (MTBSTFA), yielding tert-buthyldimethylsilyl derivatives (MTBS). MTBSTFA is the most commonly applied silylating agent [69,121,182,186,187], probably due to the greater thermal and hydrolytic stability of its derivatives. Furthermore, EI-MS spectra of the MTBS derivatives show a characteristic MS fragmentation, which together with a higher molecular mass, improves the reliability and detectability of the analyses [188]. A major drawback is its lower reactivity compared to the TMS producing reagents [181].

1.5.2.2 LC-MS based procedures

Since the introduction of atmospheric pressure ionization (API) techniques, LC-MS has played an increasingly important role in environmental analysis. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) can analyze a broad range of compounds, including non-volatile, thermally-labile and polar species. In addition, ESI and APCI provide high sensitivity, which is essential for environmental analysis where contaminants are in ng L^{-1} to µg L^{-1} levels [189,190]. However, the sensitivity is approximately 10-fold higher in the ESI mode than in the APCI mode [191]. In addition, when using ESI, matrix effects can be reduced by decreasing the flow directed into the ion source, which decreases the droplet size as well as the number of molecules ionized in a given time [181]. Although single quadrupole instruments have been used for pharmaceutical residue analysis during the early-stage LC-MS development [192], more sophisticated mass analysers are nowadays considered state-of-the-art. Among them, triple quadrupole (QqQ) [169], ion trap (IT) [193] and time-of-flight (TOF) [194] mass detectors hyphenated to LC have been applied for the analysis of pharmaceuticals. The QqQ and IT instruments enable tandem MS operation, and thus help to avoid false positive determinations, if the ions of at least two ion-ion transitions are used in combination with at least one ion intensity ratio. Still, for the purpose of quantitative determination of trace level micropollutants the QqQ-MS, provides superior performance in terms of dynamic range, selectivity and sensitivity, by enabling multiple reaction monitoring (MRM) transitions between the precursor and product ions. The potentials and capabilities of mass detectors are in detail discussed in Section 1.5.3.

Besides avoiding the derivatisation step and the high sensitivity and selectivity, an additional benefit of LC-MS techniques is that the analytes do not have to be completely separated. Alternatively, a good chromatographic separation reduces matrix effects, and improves both detectability and reproducibility. As mobile phases, mixtures of polar solvents, such as acetonitrile, methanol and water, are generally used. In LC-MS, there are three strategies for attaining good peak shapes and to achieve sufficient retention of weakly acidic compounds in reversed-phase HPLC. First, the pH value of the eluant can be decreased until the analyte reaches its undissociated state, by using formic or acetic acid, depending on the pH required. However, the acidification and the transformation of an anion into its non-dissociated form may decrease signal intensity, when using ESI in a negative mode. Alternatively, ammonium formate can be added to form an ion pair with the negatively charged analyte anion, which exhibits a good peak shape and dissociates easily in the ionization source to release the molecular ion. Finally, in case of very small and polar analytes (salicylic acid), a stronger retention in the chromatographic column can be achieved by using organic amines in their protonated form. An example is the ion-pairing agent tri-*n*-butylamine, which notably increases the signal intensity and thus decreases their detection limits [195]. Alternatively, for analysing pharmaceuticals with basic character (β-blockers, antidepressants) neutral pH and positive ionization mode are preferable.

1.5.3 Qualitative analysis

To make a relevant environmental risk assessment, it is important to identify the relevant chemical species that are likely to enter the environment. To date studies that address risk assessment of pharmaceuticals have dealt only with the parent compounds, but ignored their TPs [71,196]. However, for a comprehensive environmental risk assessment, it is important to include in the test protocols also the stable TPs, due to their possible pharmacological activity, which may add up in an additive or synergistic manner to total risk related with the presence of the pharmaceutical residues in the environment.

1.5.3.1 Principles and potentials of mass spectrometry

Mass spectrometry has revolutionized environmental analytical chemistry by allowing the analysis of complex organic mixtures for trace amounts of analytes. It can not only generate informative fragmentation patterns that give an organic compound a unique molecular signature, which can be resolved using the principles of physical organic chemistry to reveal its chemical structure. It may also give an accurate mass to confirm the presence of a target compound (targeted analysis), or to reveal an elemental composition of an unknown (nontargeted analysis).

The coupling of a chromatograph with a mass spectrometer requires an efficient interface, which connects both components of a GC-MS or LC-MS system. Most common ionization methods in GC-MS systems are EI and CI. Whereas EI can result in a loss of a compound's molecular ion in a mass spectrum, CI is a much "softer", lower energy alternative to EI that uses a reagent gas (usually ammonia or methane) in the ion chamber. The result is a reduction in the residual energy of the charged molecules, so that the fragmentation is greatly reduced and the molecular ion in the mass spectra is more prominent [135,197]. The coupling of HPLC with MS has proved more challenging, since the aqueous HPLC effluent containing polar analytes must be converted to gas-phase molecules. Fortunately, this physical hurdle was overcome by the development of API techniques [198]. API gives ''soft'' ionization with high efficiency, thus providing molecular mass information and excellent sensitivity. However, poor fragmentation still makes identification of unknown compound a challenge [199]. The most common API interfaces are ESI and APCI, while the more recently developed atmospheric pressure photoionization (APPI) is yet to be applied for identification. From the literature, it is mainly ESI that is used for determining TPs [135,197]. Generally, ESI enhances the analysis of more polar compounds, while less polar and thermally-inert compounds are more amenable to APCI (Figure 7). Both techniques are based on the consecutive process of nebulisation and vaporization of the sample solution, the ionization of the analyte molecules in the ion sampling nozzle, ion transportation in ion transfer optics and mass analysis in a mass spectrometer. These processes allow solvent evaporation, efficient ionization of organic compounds and optimal introduction of ions into the mass analyser. In this case mostly protonated $[M+H]^+$ or deprotonated $[M-H]^-$ ions are formed in a strong electric field. In ESI, multiple charged ions are favoured, while using APCI the corona discharged electrode allows the formation of only singly charged ions.

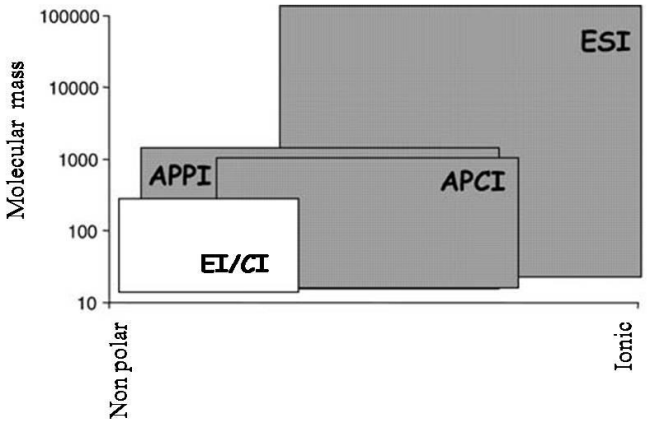


Figure 7: *Application range of different GC-MS and LC–MS interfaces as a function of compound polarity and molecular mass* (adopted from [200])

The simplest mass spectrometer is a single-stage quadrupole (Q), which, used to be the most widely used analyser due to its ease of use, mass range covered, good linearity for quantitative work, resolution, quality of mass spectra and a relatively accessible price. The Q is composed of two pairs of metallic rods. One set of rods is at a positive electrical potential, and the other one at a negative potential. A combination of DC (direct current) and AC (alternating current) voltages is applied on each set. The positive pair of rods is acting as a high mass filter; the other pair is acting as a low mass filter (Figure 8).

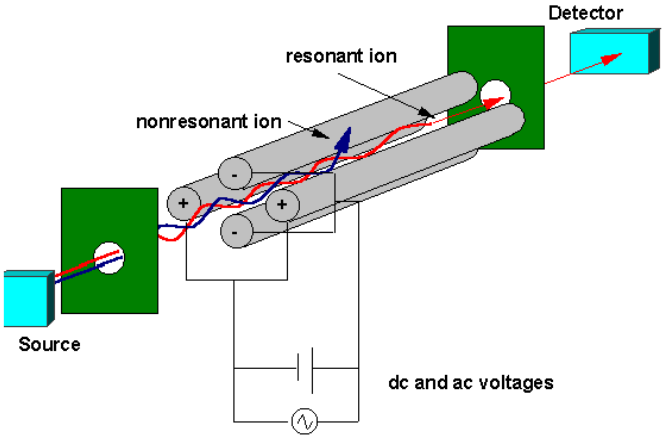


Figure 8: *Scheme of single quadrupole mass analyser.* Red arrow shows a pathway of a resonant ion passing the quadrupole and thus reaching the detector; whereas the blue arrow represents a nonresonant ion destabilised by hitting the rods [201].

In the Q mass analyzer, both analyte and matrix ions generated in the source undergo fragmentation, which results in complex, ambiguous spectral data and hence in non-selectivity, which is its main disadvantage [198]. This nonselectivity of the Q is overcome by tandem mass analyzers, which, due to their high specificity, can reduce ‘‘chemical noise’’.

A triple-quadrupole (QqQ) mass detector is a ‘‘tandem in-space’’ instrument, comprising two mass analyzers with a collision chamber in between, in which collision induced dissociation (CID) occurs. The QqQ, by allowing multiple-reaction monitoring (MRM), precursor-ion scans and constant neutral-loss scans, affords much greater experimental flexibility and precision, when compared to the single Q. However, the product ion scan in QqQ shows lower sensitivity compared to IT or TOF instruments, which is unfavourable for the structural elucidation of TPs. Thus, while the QqQ represents primarily an instrument of choice in targeted quantitative analysis and possesses excellent sensitivity, wide dynamic range and repeatability of analyses, its popularity has been overtaken by other mass analysers for

qualitative analysis. Such an example is the IT mass detector, e.g. the quadrupole ion trap and linear quadrupole ion trap. Its unique ability to isolate and to accumulate ions – by iterating ion trapping and scanning – allows the generation of CID spectra of the parent and fragment ions (and their fragment ions), resulting in a hypothetically infinite number of fragmentation patterns (i.e. MS^n) [198]. Combined with knowledge of the functional group fragmentation behaviour, the MS^n spectra greatly facilitate the elucidation of the fragmentation mechanism of unknown species, and increase the level of confidence in assigning a particular structure [202]. IT-MS uses three electrodes (a ring electrode and two end cap electrodes; Figure 9) to trap ions, where a mass spectrum is generated by changing the electrode voltages to eject ions from the trap. In general, the ITs have high sensitivity in the scan mode, but neutral loss scans are not possible with this technique, and the quantification is less reliable than MRM with triple quadrupole instrument [189].

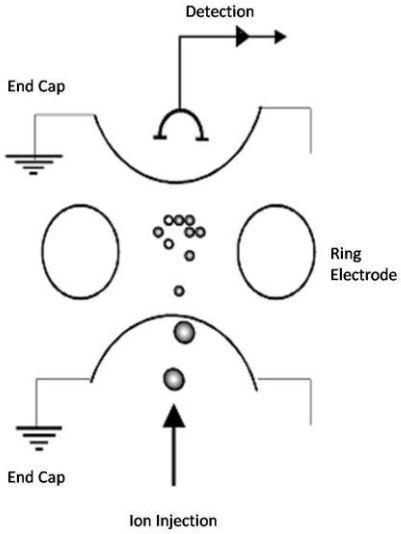


Figure 9: *Scheme of IT mass detector*

TOF instrument measures the mass-dependent time it takes ions of different mass-to-charge ratios to move from the entrance of the analyzer, where they are orthogonally accelerated in a pulsed fashion, to the detector (Figure 10, right). These instruments can now reach resolutions between 10000 and 20000 FWHM, and have to a large extent replaced traditional high resolution instruments, such as sector field or Fourier-transform ion-cyclotron resonance mass analyzers [203]. The instruments are characterized by good mass assignment accuracy (< 3ppm), high sensitivity, and allow rapid mass scanning in a theoretically limitless scan range [204]. Accurate-mass determination provided by TOF instruments allows specific information to be obtained for a given molecule and enables almost unequivocal confirmation of the identity of the compound. However, structural elucidation of unknown compounds is feasible primarily for compounds with easy in-source fragmentation or compounds having a characteristic isotopic pattern [202].

To gain sufficient data for reliable structure elucidation, a combinatory analysis should be performed, combining tandem MS techniques for fragmentation with high resolution mass spectrometry (HRMS) for accurate mass measurements. An established combination of mass spectrometric techniques is IT-MS and TOF-MS [205,206]. For instance, the ability to conduct multiple stages of fragmentation in the IT-MS can generate MS^n spectra with large amounts of structural information that allows the identification of an unknown TP. Without multiple-stage MS, isomers may not be distinguishable from each other. Further confirmation of the proposed identity of a TP can be achieved by accurate mass measurements using TOF-MS or other high resolution MS instrument [205,206].

As an alternative, different types of mass analyzers can be coupled into a hybrid tandem mass spectrometer. Many different combinations of mass analyzers offer to find the tandem mass spectrometer of choice with good resolving power and rapid switching between various MS/MS modes. Maximum versatility is achieved usually with a combination of two analyzers and a collision cell to form an efficient hybrid tandem mass spectrometer. The collision cell is usually mounted in the field free region between mass analyzers. Introduction of an inert gas into this cell is the most widely used approach for CID. As a result of collision of ions and gas molecules fragmentation is achieved. An example is the hybrid QqTOF.

In Figure 10, Q refers to the mass-resolving quadrupole, q refers to a collision cell and TOF refers to a time-of-flight mass spectrometer [207]. Basically, in QqTOF the final resolving mass filter of a QqQ is replaced by a TOF analyzer [158]. Having this configuration the QqTOF not only allows MS^2 operation but also has the high accuracy and the resolution necessary to give exact-mass measurements in a single instrument. However, due to the price and the lower sensitivity of QqTOF instruments, the number of environmental applications remains low. Despite this, developments expected in QqTOF instruments may bring an improved sensitivity and linear dynamic range, which will contribute to a wider acceptability of these instruments in the environmental analysis.

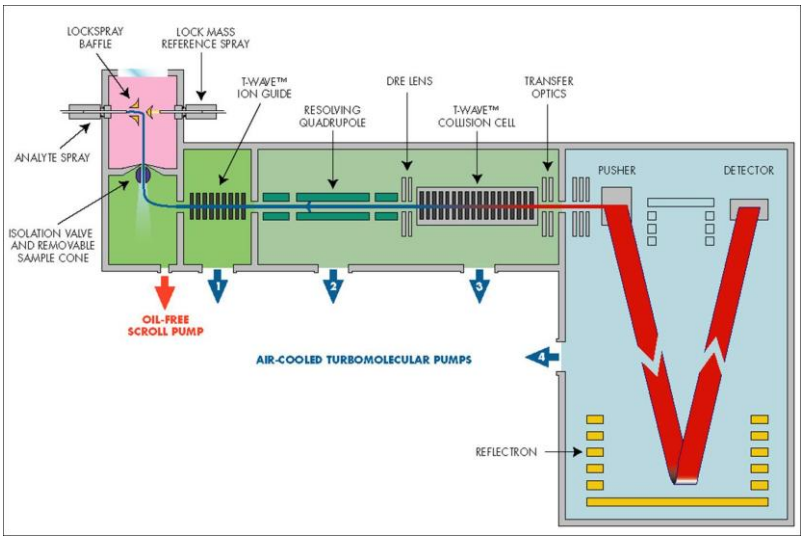


Figure 10: *Scheme of QqTOF hybrid tandem mass analyser*. Left: quadrupole segment; Right: TOF analyser.

Another promising hybrid mass spectrometer for the identification of TPs is a quadrupole – linear ion trap (QqLIT). The QqLIT configuration is based on an ion path of a QqQ mass spectrometer using the collision cell of the final mass analyser as the LIT. The LIT has two major advantages over conventional three-dimensional IT, i.e. a larger ion-storage capacity and a higher trapping efficiency, which in turn, increases the sensitivity [204].

Recently, another hybrid MS system has been launched, which may represent an alternative to QqTOF. LTQ-MS^n-FT Orbitrap combines LIT with the Orbitrap analyser, using Fourier-transform MS to attain HRMS spectra. The instrument may become important in the field of nontargeted environmental analysis, due to its high resolution and high mass accuracy and to its wide dynamic range, when compared to that of the QqTOF.

1.5.3.2 Nontargeted identification

The main drawback of a conventional analytical approach is target compound monitoring, which is often insufficient to assess the environmental relevance of emerging contaminants. Generally, there is a lack of information on the comprehensive list of TPs formed under various conditions, so, in the open literature there is a dearth of data on their occurrence. There are several reasons for this, including that not all the TPs are commercially available [208]. This means that in-house chemical synthesis of authentic TP standards is often the only option available.

The detection and identification of TPs require the application of sophisticated instrumentation, of which MS is considered to be a head of the field, both in terms of technology development and application [202,209]. At the same time, improvements in separation techniques, such as ultra performance liquid chromatography (UPLC) or rapid resolution liquid chromatography (RRLC), make this technology more attractive and powerful, when combined with MS [194,210,211]. Because of the data intensive nature of LC–MS, considerable time and effort are required to interrogate the data in order to extract the results needed to identify unknowns. However, holding a certain structural relationship with their parent compound, TPs are not complete unknowns. In structure elucidation of pharmaceutical abiotic degradation products, a few studies have been published recently [158]. Due to the greater complexity of the task (e.g.,

screening for trace levels of unknown chemical structures in complex media (wastewater) that involve biomass activity), there have been fewer studies involving the identification of their biodegradation products [212].

In GC-MS the detection of TPs can generally be performed by direct comparison of total ion chromatograms (TIC) of untreated (control) and treated samples, where any new-formed peaks in the treated samples are considered as potential TPs and subjected to further investigation. In LC analysis, a direct comparison of the TIC of the treated and the control samples is, however, not possible. Instead, the TPs may be automatically detected by applying the spectral and chromatographic search algorithms, such as MetaboLynx™ from Waters, Analyst/MetaboliteID™ from Applied Biosystems, Xcalibur/MetWorks™ from Thermo Fisher or MassHunter™ from Agilent. Such an algorithm searches the extracted mass chromatograms for expected metabolites based on predicted or unpredicted molecular changes relative to the parent compound and thus aids in the detection and identification of unknowns, particularly those buried within the spectral noise. The software compares mass spectral chromatograms of a control versus that of metabolised, stressed or treated sample, and automates the detection, identification and reporting of metabolites [213]. Such approach has been used before in a number of studies dealing with identification of drug metabolites in *'in vivo'* metabolism [198] and degradation products in food and environmental analytical chemistry [214,215,216,217].

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 (MS^n) experiments and

2) highly accurate molecular mass measurements [202].

Often however, MS alone is insufficient to identify the exact position of oxidation, to differentiate isomers, or to provide the precise structure of unusual and/or unstable TPs. In addition, other substances present in environmental samples can suppress ionization, complicating metabolite identification. In such cases, multiple analytical and wet-chemistry techniques, such as LC with nuclear magnetic resonance (NMR), chemical derivatization, and hydrogen/deuterium-exchange combined with MS are used to characterize the novel and isomeric TPs of drug candidates [198,158]. Also, having authentic standards available, ultraviolet-visible (UV-Vis) spectroscopy is often applied, as it allows rapid and simple analyses of TPs. Another possible confirmatory method is matching against spectra of authentic compounds, which may be found in extensive GC-MS libraries, i.e. NIST (National Institute of Standards and Techonlogy) [218]. This ability to match analytical data with mass spectral libraries is particularly feasible when using EI ionization. EI is normally performed at 70 eV, thus yielding mass spectra which are identical over time and between instruments for a given compound, which is not the case with the API ionisation techniques.

1.5.3.3 Targeted identification

The term "targeted identification" refers to identity confirmation of previously known (targeted) organic residues and contaminants, which are being screened for in complex environmental matrices. The 2002/657/EC [219] European Commission Decision set up the quality criteria for the spectrometric identification and confirmation of these compounds, based on the use of identification points (IPs). The European Guidelines requires a minimum of 4 IPs for banned compounds and 3 for others to satisfactorily confirm their identity. In general, when using LC or GC coupled to MS/MS (QqQ) an excellent sensitivity is obtained in the selected reaction monitoring (SRM) mode. Confirmation of the identity of the target compound is achieved by monitoring two characteristic precursor-product transitions, which earns 4 IPs and fulfils the requirements of 2002/657/EC [219,220]. The sensitivity, selectivity, and mass accuracy of different MS techniques, and the number of IPs earned for each ion are compared in Table 9.

Table 9: *Comparison of different MS techniques with respect to sensitivity, selectivity, mass accuracy, dynamic range and number of IPs.* Adopted from Petrović and Barceló [220] and Hernández et al. [221].

MS technique	Sensitivity	Selectivity	Mass accuracy	Dynamic range	IPs earned for each ion
Q	medium (SIM)	low	low	high	1 per ion
QqQ	medium (full scan)	high	low	high	1 for precursor ion
	high (SRM)				1.5 for transition ion
TOF	high	low	high	low	2 per ion
QqTOF	medium	high	high	medium	2 for precursor ion
					2.5 for transition ion
IT	medium (MS^2)	high	low	medium	1 for precursor ion
					1.5 for MS^2 and MS^3 product ions
QqLIT	high (SRM)	high	low	medium-high	1 for precursor ion
					1.5 for MS^2 and MS^3 product ions

Occasionally, however, the fragmentation of a target compound is insufficient (less than two transitions, low intensity transitions) to meet the EU Guidelines [219]. As proposed in Table 9 the selectivity of the analysis can be increased by using HRMS instruments (TOF and QqTOF) that enable accurate mass measurements and, especially when the mass measurements are performed at an error less than 2 mDa [221], a high total amount of IPs is achieved. This results in increased certainty in the identification of target compounds [220].

1.5.4 Validation of analytical methods

In any scientific work, unreliable data may lead to over- and underestimation of effects, to false interpretations, and to unwarranted conclusions. If such errors are not obvious, they may remain undetected, and may be multiplied within the scientific community or become a part of a general accepted knowledge. The basis for achieving high quality data are reliable analytical methods, which requires a thorough validation of careful method development. The validation of analytical method can objectively demonstrate its inherent quality and thus prove its applicability for the stipulated purpose. This is especially true in the context of quality management and accreditation, which have become matters of significant relevance in the environmental analytical chemistry in recent years [222]. Owing to the importance of method validation in the whole field of analytical chemistry, a number of guidance documents have been issued [223,224,225,226,227].

Environmental analytical methods are used for screening and identification of unknown pollutants and transformation products (nontargeted analysis), or for identity confirmation and quantification of predetermined analytes (targeted analysis). Whereas for qualitative procedures, a general validation guideline is currently not available [228], Figure 11 shows the parameters that should be involved in the validation of quantitative analytical procedures.

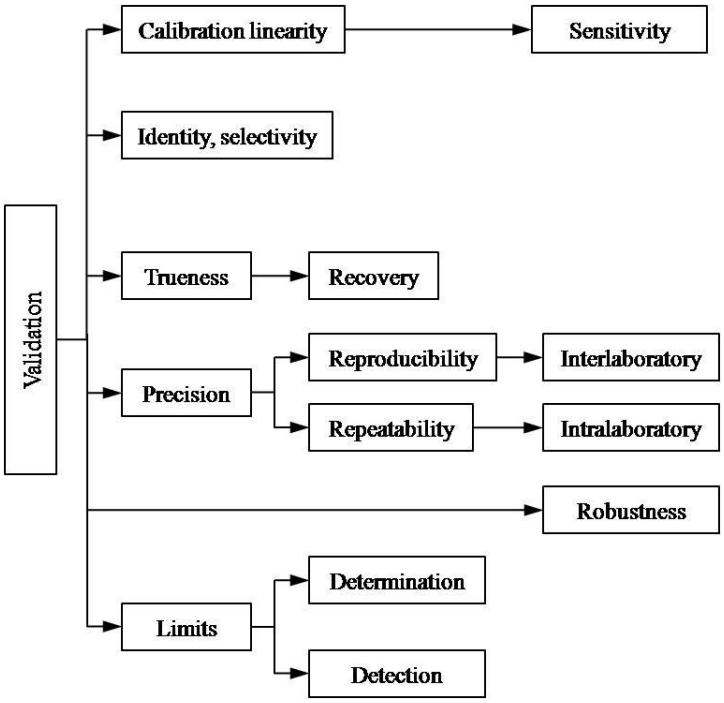


Figure 11: *Aspects of an analytical method that may be assessed during a method validation.* Adopted from [229].

The validation parameters are explained as follows:

- Linearity is the ability of a method to obtain test results proportional to the concentration of analyte, within a given concentration range. Calibration is performed to determine the relationship between the concentration of analyte and the corresponding response of a detector. The relationship is described by the linear calibration model, which is normally derived as a least squares regression model with a known confidence level [230].
- Sensitivity is the change in response of a measuring instrument divided by the corresponding change in the stimulus. The sensitivity is arbitrarily determined by the slope of the calibration curve [230].
- Selectivity is the ability of an analytical method to measure unequivocally and to differentiate the analytes in the presence of components, which may be expected to be present in a sample matrix (metabolites, TPs, matrix components) [226]. One approach to establish method selectivity is to prove the lack of response in a blank matrix, i.e. there are no signals interfering with the signal of the analyte or the internal standard.
- Trueness is the closeness of agreement between the expectation of the test result (expected mean value) and an accepted reference value (true value). When no certified reference material (CRM) is available, the trueness is expressed as a % recovery of a known spiked amount of analyte [230].
- Precision is the closeness of agreement between independent test results obtained under stipulated conditions and is reported as repeatability and reproducibility. ISO 5725-1 [231] defines repeatability as "the precision under repeatability conditions", i.e. the conditions, where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time. Reproducibility is defined by ISO as the "precision under reproducibility conditions", i.e. where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment [231].
- Robustness is a measure of capacity of a method to remain unaffected by small variations in method-performance parameters [230].
- LOD is the lowest concentration of analyte that can be reliably distinguished from zero [230].
- LOQ is the lowest amount of analyte that can be determined quantitatively with an acceptable level

of repeatability, precision and trueness [230].

Besides the intralaboratory or internal method validation, interlaboratory studies can serve this purpose. The aims of method performance (validation) studies are to estimate the repeatability and reproducibility of a method, which, for a method precision study is predetermined and unique for all participant laboratories. Besides method validation, the interlaboratory studies also allow the assessment of the proficiency of individual laboratories, estimation of the measurement uncertainty and certification of reference materials [232].

2 Hypothesis and aims

To date, commonly used pharmaceuticals have been detected in waste, surface and potable waters. The factors influencing their occurrence are their overall consumption and the fate of an individual compound during metabolism in the human or animal organism, during the water treatment and in the environment. Thus, besides consumption rate, metabolic conjugation and de-conjugation pathways, information about their biotic and abiotic degradability, sorption and persistence are needed to predict their behaviour in the environment.

Municipal and hospital WWTP, as well as discharges from pharmaceutical industry, have been found to be the major sources of pharmaceuticals in the environment. Pharmaceuticals subjected to the water treatment can be, depending on their physical-chemical characteristics, removed by various mechanisms, e.g. degradation (biotic or abiotic) or sorption to biomass, soils, sediments and/or on the surfaces of holding bodies. By recognizing the response of model pharmaceuticals to potential factors influencing their degradation (e.g. UV light, biological treatment and chemical oxidation) it is possible to develop a treatment method and integrate it into an existing water treatment process. The idea is to contribute to the development of an efficient, sustainable and cost-effective wastewater treatment technology, which will set new standards of effluent quality by successfully removing pharmaceuticals and other emerging contaminants. In this study pharmaceuticals are used as model compounds, i.e. representatives of widely applied environmentally toxic and / or persistent emerging contaminants. Thus, with efficient treatment of the model pharmaceuticals, numerous other persistent trace contaminants are believed to follow a simultaneous removal.

For a comprehensive assessment of water treatment (wastewater or potable water) efficiency, pharmaceutical TPs formed during treatment must be accounted for. The properties of TPs are unlikely to resemble the metabolic products formed in the human body and are yet to be identified and recognized as environmental trace-contaminants. To our knowledge, no data exists regarding their toxicity, synergistic and additive effects to the environment. For this reason this study attempts to identify pharmaceutical TPs and evaluate the wastewater and potable water treatment efficiency with respect to both, removal of parent pharmaceuticals, and the formation and the decay of their TPs.

The aims of the thesis are as follows:

- to develop analytical methods for the determination of selected pharmaceuticals and their TPs in waste and environmental water samples;
- to compare and to assess the performance of the developed analytical method by participating an international interlaboratory exercise;
- to determine the elimination efficiency of model pharmaceuticals during biological water treatment;
- to investigate effects on activated sludge arising from continuous exposure to pharmaceuticals;
- to research abiotic treatment methods for elimination of persistent pharmaceuticals (e.g. ClO_2 disinfection and UV irradiation) and to suggest improvements to existing biological treatment in laboratory scale bioreactors by integrating advanced treatment technologies;
- to identify (bio)transformation products formed during treatment and to estimate the performance of different treatment methods according to their production and decay.

3 Publications

3.1 Development and validation of analytical method for determination of NSAIDs in aquatic samples

Pollution with NSAIDs in European surface waters is widespread and their concentrations often reach a level of few hundred ng L^{-1} (Table 3). As no data exist on the occurrence of pharmaceuticals in the Slovenian aquatic environment, we developed an analytical method for determination of these compounds in aquatic matrices. Principally, the analytical method involved a sample pretreatment step (acidifying and filtration), enrichment and clean-up by applying SPE, derivatisation with MSTFA and finally, the analysis using GC-MS. The intralaboratory validation of the analytical method was performed, which involved testing the linearity and sensitivity, determination of instrumental limits of detection, method limits of detection, limits of quantification and the extraction efficiency. Overall, the validation parameters demonstrated that the developed and optimized analytical method was applicable to environmental samples. The developed method was tested on wastewater, river and tap water. While no traces of NSAIDs were found in tap and well water samples, the majority of river water samples contained NP and/or DF, while KP occurred at concentrations below LOQ. IB was not detected in any of the samples. In general, it can be concluded that the concentrations of NSAIDs determined in Slovenia are comparable with those found elsewhere in EU surface waters (Table 3). Among the analytes NP was determined in highest concentrations, which corresponds to the fact that in Slovenia it is dispensed in highest amounts (Table 2). Further, a notably elevated concentration of NP and KP was observed in a sample taken downstream from a pharmaceutical manufacturing site. This indicates that the sources of pollution with pharmaceuticals are not only attributed to diffusely distributed municipalities, but also point sources. However, in order to assess the extent of the pharmaceutical contamination in natural Slovene waters their levels would have to be monitored for a sufficient period of time. In this sense, a more comprehensive study taking into account more sampling points, different seasonal conditions and an improved sampling strategy, preferably including POCIS, should be made. Also the occurrence of NSAIDs in wastewaters was assessed, but this is a part of another research and will be published separately.

The description of development, optimization and intralaboratory validation of analytical method for determination of NSAIDs in the aquatic environment, and their determination in environmental samples are presented in the paper:

- Determination of non-steroidal anti-inflammatory drug (NSAIDs) residues in water samples (Environment International, 2007)

The first step in validation of an analytical method is generally performed on the intralaboratory level, while, to assure the transferability of a method to another expert laboratory, an external (interlaboratory) validation should be made. Besides validation itself, the objectives of interlaboratory studies are also harmonization and dissemination of the analytical method, which improves the quality and comparability of data on pollution with target analytes [233]. In this sense, two interlaboratory studies on the determination of NSAIDs in the aquatic environment were organized within the EU FP6 project NORMAN (Network of reference laboratories for monitoring of emerging environmental pollutants). The 1st study was performed using GC-MS or LC-MS/MS, both of which employed SPE as a purification and concentration step. The applied analytical protocols were developed individually by each participant laboratory and differed in sample pretreatment, SPE sorbents, elution and reconstitution solvents, and, in case of GC-MS protocol, also in derivatising agents. The 2nd round required the use of predetermined GC-MS and LC-MS/MS procedures, which resulted in a notable reduction in the total number of outliers. This improvement was particularly on account of the GC based analytical protocol, where the number of outliers decreased up to five-folds in the 2nd round, which is a likely consequence of unifying the derivatising agent (MTBSTFA) and adopting the standard derivatising conditions (1 h at 60 ºC).

Furthermore, an important outcome of the 2nd interlaboratory study was that the GC-MS protocol proved superior for the analysis of IB, KP and NP in matrices with higher complexity, i.e. wastewater and river water. This was due to the detector response in GC-MS not being affected by matrix suppression as in the case of LC-MS. Alternatively, the determination of DF was not shown to be particularly consistent, regardless of an analytical protocol used. Considering its chemical structure, DF differs from other NSAIDs (Figure 2), since it involves a secondary amine group and two chlorine atoms. Therefore, a different behaviour of this compound during the sample preparation and analysis may be anticipated. A solution to improve the determination of DF may be to use a more strongly related internal standard, e.g. isotopically labelled DF. Secondly, with the supplementary testing of extraction efficiency we showed a ten-folds higher recovery of DF for the GC-MS protocol, when pretreatment involves acidifying to pH 2 – 3. It may therefore be proposed that both analytical protocols need to be adopted to improve determination of DF before they reach the level of application in routine laboratories throughout Europe.

The underlying research in the 1st round was to follow the actual stability of NSAIDs during the exercise. While a decay of NSAIDs was expected in wastewater and river water, due to biodegradation or physico-chemical interactions with the sample matrix, no such effects were observed and a satisfactory stability was proved. Furthermore, the 2nd exercise addressed also filtration and compared the influence of different filter material categories on the analysis of NSAIDs. The filtration step could potentially have affected the analysis in two ways. First, depending on the analyte polarity and filter material, the analytes can adsorb to a filter. Secondly, by removing the organic matter present in the matrix, filtration may be one way to reduce the ion suppression effect and improve the LC-MS performance. In contrast to our expectations, the results of the statistical testing showed that filtration does not affect the analysis.

The results of both interlaboratory studies are presented in two papers:

- First interlaboratory exercise on non-steroidal anti-inflammatory drugs analysis in environmental samples (Talanta, 2008)
- Second interlaboratory exercise on non-steroidal anti-inflammatory drugs analysis in environmental samples (sent for publication to Talanta, 2009)

3.1.1 Scientific paper: “Determination of non-steroidal anti-inflammatory drug (NSAIDs) residues in water samples”

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Determination of non-steroidal anti-inflammatory drug (NSAIDs) residues in water samples

Tina Kosjek[a], Ester Heath[b,*], Aleš Krbavčič[a]

[a]*Faculty of Pharmacy, Aškerčeva 7, 1000 Ljubljana, Slovenia*
[b]*Jožef Stefan Institute, Department of Environmental Sciences, Jamova 39, 1000 Ljubljana, Slovenia*

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Abstract

Pharmacologically active substances used to treat human and animal illnesses can enter the aquatic environment via effluents from wastewater treatment plants or in the case of veterinary drugs directly through liquid manure discharge. Some of these substances enter the environment either as the parent compound or as active/inactive metabolites. Due to their pharmacological activity, their determination and understanding their behavior and fate in the environment are important.

The scope of this paper was to develop an analytical procedure to determine common pharmaceutical residues in wastewaters. Pharmacologically active substances were chosen according to their wide spread application in Slovenia and Central Europe and are members of analgesics, e.g., non-steroidal anti-inflammatory drugs: ibuprofen, naproxen, ketoprofen and diclofenac. Selected compounds were isolated from synthetic water using a novel SPE sorbent Strata™ X. Due to the non-volatile nature of these compounds they were first silylised prior to gas chromatographic-mass spectrometric detection. The developed procedure was tested with synthetic wastewaters and their extraction efficiency (>84%) and method limits of detection (2–6 ng L^{-1}) were determined. Our procedure has been adopted and optimised for "real" water samples and applied to eleven drinking and ten river water samples from Slovenia. The results showed no traces of NSAIDs in all potable water samples and low-range contamination (ng L^{-1}) of Slovene rivers. These results show that NSAIDs contamination of Slovene waters is comparable with published results of water contamination in Central Europe.

Keywords: Pharmaceutical residues; NSAIDs; Water; Contamination; Solid phase extraction; Gas chromatography-mass spectrometry

1. Introduction

In recent years increasing attention has been directed toward the discharge, presence and potential effects of pharmaceuticals in the environment. Thousands of tons of pharmacologically active substances are used yearly to treat or prevent illnesses, or to help people face the stresses of modern life. The discharge of therapeutic agents from production facilities, hospitals and private household effluent as well as improper disposal of unused drugs pose a burden on the environment (Christensen, 1998). Pharmaceuticals are released into the environment either as the parent compound or as active/inactive metabolites. Thus, often it is not only the parent compound which should be the subject for a risk assessment but also the active metabolites (Christensen, 1998; Halling-Sørensen et al., 1998). Concentrations of pharmaceutical residues measured in water may give rise to human exposure in the ng per day range, which is at least three to four orders of magnitude lower than that required to produce a pharmacological effect. Risks arising from acute exposure can therefore be regarded as unlikely. However, possible effects of life-long exposures have still to be determined (Christensen, 1998).

Pharmaceuticals have been selected or designed due to and because of their biological activity. In respect to their purpose they should be considered as suspicious environmental contaminants (Christensen, 1998). Furthermore, they often have low biodegradability, and can accumulate,

* Corresponding author. Tel.: +386 1 477 35 84; fax: +386 1 423 54 00.
E-mail address: ester.heath@ijs.si (E. Heath).

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reaching detectable and biologically active amounts (Zuccato et al., 2000). Quantitative evaluation of the fate of pharmaceuticals in the aquatic environment, proper risk assessment and improvement of the efficiency of sewage treatment plants need sensitive and reliable analytical methods.

There are no data regarding pollution with pharmaceutical residues in Slovenia. Therefore, the aim of our study was to develop an analytical procedure, which allows the quantification of pharmaceuticals in water at the ng L^{-1} level. By analysing tap, well and river samples from around Slovenia, we hope to gauge the extent of pharmaceutical residues in Slovene waters. Model compounds were selected among the pharmaceuticals, which predominate in the analyses of environmental samples, as well as on the lists compiled from prescription data. Most of these pharmaceuticals belong to the class of analgesics (non-steroidal anti-inflammatory drugs, NSAIDs), antibiotics, antihypertensives, antiasthmatics, diuretics and psycholeptics (Kümmerer, 2001a). For this reason, the following four pharmaceuticals from the class of NSAIDs were chosen as model compounds: ibuprofen, naproxen, diclofenac and ketoprofen. The four investigated drugs belong to a group of the most commonly prescribed drugs between non-steroidal anti-inflammatory drugs. Data from annual reports (Oražem and Pečar-Čad, 2000, 2001, 2002) show a quantity of the drugs dispensed by prescriptions from health-centres. However, these data underestimate the total use of pharmaceuticals in Slovenia, because drugs dispensed over-the-counter and those spent in hospitals also contribute to the total. The quantities of annually prescribed pharmaceuticals have been published (Oražem and Pečar-Čad, 2000, 2001, 2002). The quantity of naproxen, together with other NSAID representatives, is the most outstanding in the group of investigated drugs and is reported to be between 1.9 and 2.6 tons/year. Furthermore, naproxen is eliminated partly unmetabolised (60%) and is persistent in the environment. Naproxen is therefore expected to pose the biggest load (among the four investigated drugs) on the Slovenian aquatic environment.

Pharmaceutical residues are usually present in environmental water samples in trace levels. The most common sample isolation and pre-concentration technique is solid-phase extraction (SPE) (Rodríguez et al., 2003) where as well as isolation and pre-concentration, the matrix-solvent (water) is exchanged with a more volatile organic solvent suitable for gas chromatography (GC). Due to low vapour pressure, gas chromatographic separations of selected NSAIDs can be performed only after derivatisation of the native compounds to less polar species (Rodríguez et al., 2003). This involves converting the carboxylic group present on these drugs to the methyl ester derivative using diazomethane (Rodríguez et al., 2003; Öllers et al., 2001; Ternes, 2001; Poole, 1991). The yield of the reaction is usually high, however, because of high toxicity and low stability of diazomethane, alternatives have been proposed. Koutsouba et al. (2003) and Sacher et al. (2001) derivatise the carboxylic group using pentafluorobenzyl bromide with triethylamine as a catalyst. The most widely used alternatives to diazomethane are alkylsilyl reagents (Poole, 1991), namely *N*-methyl-*N*-(tert.-butyldimethysilyl) trifluoroacetamide (Rodríguez et al., 2003) or *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) (Heath, 1998).

An analytical procedure for the determination of NSAIDs in water, based on solid phase extraction (SPE) with a new, patent-pending sample preparation sorbent Strata™ X, followed by derivatisation with MSTFA and GC-MSD analysis was developed and tested on synthetic and authentic well, tap and river water samples.

2. Experimental

2.1. Chemicals

Sigma-Aldrich Company Ltd (Gillingham, GB) supplied all the drugs under investigation (ibuprofen, diclofenac, naproxen and ketoprofen) and the derivatisation agent MSTFA (*N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide). Mecoprop (2-(4-chloro-2-methylphenoxy) propanoic acid) was used as an internal standard and was obtained from Labor. Dr. Ehrenstorfer-Schäfers (Ausburg, Germany). Methanol (MeOH), toluene and 37% hydrochloric acid (HCl) were of analytical grade and were provided by Merck (Darmstadt, Germany).

2.2. Synthetic water

Water solution of pharmaceutical compounds (1 mg of each studied compound in 500 mL of distilled water) was spiked with 1 mg of the internal standard mecoprop. The spiked solution was diluted to concentrations from 0.2 mg L^{-1} to 0.02 mg L^{-1}.

2.3. Environmental samples

Water samples were collected in June–July and September 2004. In total, well water samples from two sites, eleven tap water and river water samples from nine locations were collected. River water samples (1L) were collected at a depth of 0.25 to 1.0 m from the riverbank. Each 1 L of sample was filtered (0.45-μm filter, Sartorius, Goettingen, Germany), acidified to pH 2.6 to enhance trapping of the acidic compounds on the solid-phase extraction (SPE) sorbent and stored at 4 °C prior to solid-phase extraction SPE.

2.4. Solid phase extraction

Commercially available 3 mL SPE cartridges with 60 mg of Strata™ X (surface modified styrene-divinylbenzene polymer) sorbent (Phenomenex®, Torrance, ZDA), were used. SPE was performed using 12-fold vacuum extraction

box (Supelco, Bellefonte, USA). The SPE cartridges were first conditioned with 1.5 mL MeOH and 1.5 mL matrix-solvent (aq. HCl with pH 2.6). Extraction volumes were 500 mL in case of synthetic water samples and 1000 mL for the actual water samples. Extraction was performed under vacuum at a flow rate of 1–2 mL min^{-1}. After the enrichment step, the cartridge was dried for 1 min in vacuum (approx. −16 mm Hg). The analytes were eluted with three fractions of 0.5-mL elution solvent (MeOH) and the eluant was collected in 1.5-mL glass vial and dried under a stream of nitrogen. The residues were dissolved in 0.5 mL (synthetic water) and 0.1 mL (water samples) of toluene and derivatised by adding 70 μL (synthetic water) or 30 μL (water samples) of MSTFA. The samples were reacted in the dark on a shaker (Veb MLW Labortechnic, Ilmenau, Germany) for 12 h.

2.5. Gas chromatography–mass spectrometry

Derivatised drugs were determined by GC-MSD on an instrument HP 6890 (Hewlett-Packard, Waldbron, Germany) fitted with a 30 m×0.25 mm×0.25 μm Hewlett-Packard HP-5 MS capillary column. Carrier gas was helium, with a flow rate held at constant velocity of 37 cm s^{-1}. Injection was performed in the splitless mode at an injection temperature of 250 °C. Injection volume was 1 μL. The GC oven was programmed as follows: 2 min at 100 °C, first ramp at 4 °C min^{-1} to 180 °C, second ramp at 10 °C min^{-1} to 230 °C (held for 20 min) and then, ramped at 20 °C min^{-1} to 270 °C and held at this temperature for 7 min. Mass spectra were obtained in the electron impact mode (70 eV), detection was in mass range 50–500 m.u. (full-scan) with the transfer line temperature set at 280 °C.

3. Results and discussion

3.1. SPE

Breakthrough of the selected SPE sorbent was investigated using a synthetic wastewater containing approx. 0.1 mg of each of the test compounds and 500 mL of the samples were passed through two cartridges connected sequentially. After the enrichment step, the cartridges were then analysed separately. Since the analytes were not detected in the eluant from the second cartridge, it was proven that the applied cartridge dimension (60 mg/3 mL) was adequate for the quantitative adsorption of the investigated drugs.

The recommended wash and elution volume (Phenomenex® users guide) for the 60 mg/3 mL Strata™ X cartridge is at least 1.2 mL. Therefore, the analytes were eluted from the cartridge with 3 fractions of 0.5 mL MeOH and the elution was performed twice (2×1.5 mL) using the same cartridge. Since there was no trace of analytes in the second portion of eluant we conclude that 1.5 mL of the elution solvent was a sufficient volume for the quantitative elution of the tested analytes.

3.2. GC-MS

The trimethylsilyl (TMS) derivatives of target compounds, ibuprofen, mecoprop (internal standard), naproxen, ketoprofen and diclofenac (Fig. 1), were determined by capillary gas chromatography with mass spectrometric detection in EI mode of operation.

Typical total ion chromatogram of selected pharmaceuticals and internal standard is shown in Fig. 2.

The chemical structure of all investigated compounds after derivatisation (TMS-esters) was proved with mass spectrometric detection. Table 1 shows the retention times (t_R) of the derivatised compounds, molecular weight of pure compounds and their derivatised compounds, mass-to-charge ratio of typical fragments and their intensity.

Fig. 3 is an example mass spectrum of trimethylsilyl ester of ibuprofen. Its molecular ion is seen at m/z=278. Fragment ion at m/z=263 represents the fragmentation of methyl group from molecular ion ($[M_{TMS}–CH_3]^+$), while m/z=205 shows trimethylsilyl group fragmentation ($[M_{TMS}–SiMe_3]^+$). Ion at m/z=160 is developed with sequential fragmentation of trimethylsilyl and carboxyl groups. The

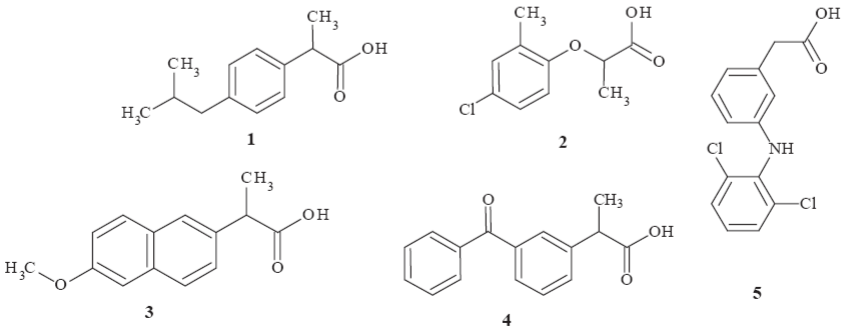

Fig. 1. Chemical structures of investigated compounds: ibuprofen (1), mecoprop (internal standard [2]), naproxen (3), ketoprofen (4) and diclofenac (5).

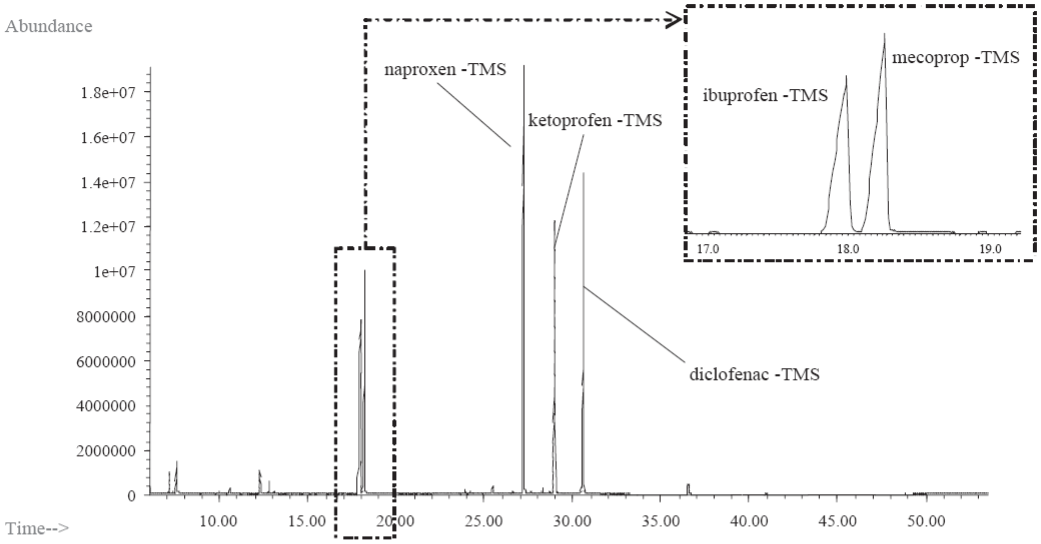

Fig. 2. Total ion chromatogram of the selected compounds after derivatisation.

base ion is present at m/z=73 and is typical for MSTFA derivatisation (Heath, 1998).

3.3. Extraction efficiency

Extraction efficiency ($\%_{EXTR}$) was calculated for each compound (ibuprofen, ketoprofen, diclofenac, naproxen and internal standard mecoprop) as a ratio of a mean of peak areas of extracted and derivatised compound ($A_{SPE+DER}$) to a mean of peak areas of derivatised compound without previous SPE (A_{DER}) performed (Eq. (1)). 2–6 parallel samples were used for this purpose (Table 2).

$$\%_{EXTR} = A_{SPE+DER}/A_{DER} \quad (1)$$

Water solutions of pharmaceuticals at two concentrations (0.02 mg L^{-1} and 0.20 mg L^{-1}) were spiked with internal standard and processed as described in the Experimental section. Toluene solutions of pharmaceuticals with the internal standard at two concentrations (0.02 mg mL^{-1} and 0.20 mg mL^{-1}) were derivatised as described herein. Extraction efficiencies ($\%_{EXTR.}$) for all pharmaceuticals and internal standard are given in Table 2 and range from 91 to 103% at the higher concentration and from 84 to 104% at the lower concentration for all compounds except diclofenac. An extraction efficiency of 157% and high deviation ($\delta/2$) was observed for diclofenac, which was probably due to disintegration of its TMS ester during GC-MSD analysis.

Table 1
Retention time (t_R) of derivatised compounds, molecular weight (M_X), molecular weight of TMS ester ($M_{X\text{-TMS}}$), mass-to-charge ratio (m/z) of typical fragments and their relative intensity

Analyte–TMS	Ret. time t_R/min	M_X	$M_{X\text{-TMS}}$	m/z	Fragment structure and their relative intensity
Ibuprofen-TMS	17.97	206	278	73	-Si$(CH_3)_3$,100 %
				160	30 %
				278	$[M_{IP\text{-}TMS}]^+$,10 %
				263	$[M_{IP\text{-}TMS}\text{-}CH_3]^+$,25 %
Naproxen-TMS	27.21	230	302	185	100%
				73	-Si$(CH_3)_3$,55 %
				302	$[M_{NP\text{-}TMS}]^+$,45 %
				287	$[M_{NP\text{-}TMS}\text{-}CH_3]^+$,25 %
Ketoprofen-TMS	28.99	254	326	282	100 %
				73	-Si$(CH_3)_3$,80 %
				311	$[M_{KP\text{-}TMS}\text{-}CH_3]^+$,20 %
Diclofenac-TMS	30.54	296	368	214	100%
				73	-Si$(CH_3)_3$,35 %
				367	$[M_{DF\text{-}TMS}\text{-}H]^+$,25 %
				352	$[M_{DF\text{-}TMS}\text{-}H\text{-}CH_3]^+$, 10 %
Mecoprop-TMS (internal standard)	18.23	214	286	73	-Si$(CH_3)_3$,100 %
				286	$[M_{MP\text{-}TMS}]^+$,50 %
				271	$[M_{MP\text{-}TMS}\text{-}CH_3]^+$,5 %

3.4. Derivatisation efficiency

Since derivatised standards of selected compounds were commercially unavailable, it was not possible to calculate the derivatisation efficiency. However, since after derivatisation no underivatised compounds were found to be present when analysed by GC-MS, derivatisation was assumed complete.

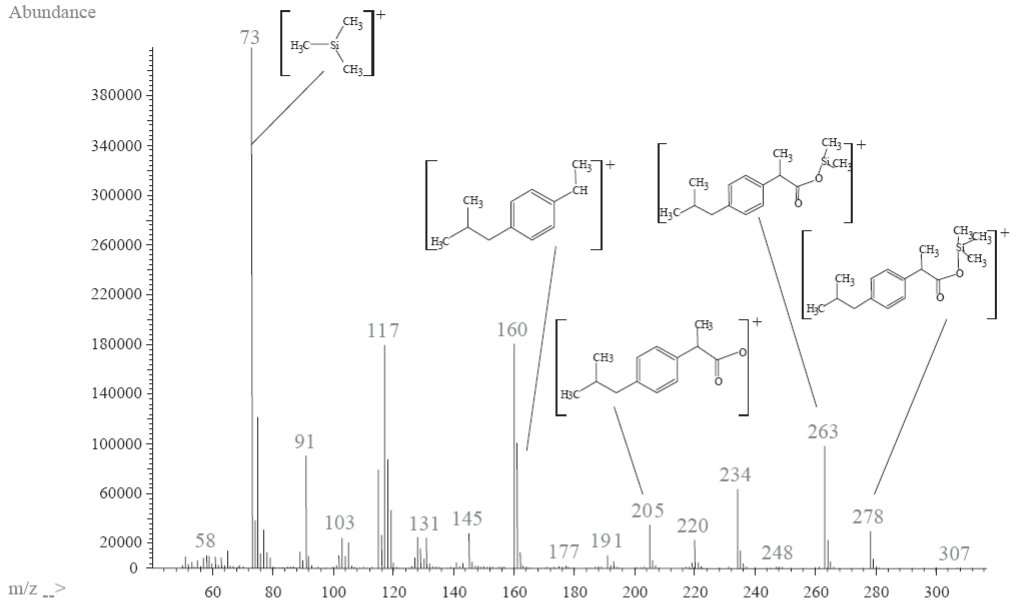

Fig. 3. Mass spectrum of ibuprofen-TMS.

3.5. Linearity

The linearity of the analytical method was tested using pre-derivatised standard mixtures containing mecoprop as internal standard (0.20 mg mL^{-1}), and the test compounds in concentrations between 0.02 and 0.25 mg mL^{-1}. With the exception of diclofenac (r^2=0.990) correlation coefficients higher than 0.996 were obtained for all the compounds under investigation. The TMS ester of diclofenac was proved to be unstabile under the applied conditions. At the same time as lower diclofenac-TMS quantities, an additional peak appears in total ion chromatogram at t_R 28.7 min. From analogy between the mass spectra of diclofenac-TMS and the mass spectra of the unknown peak we conclude that the latter originates from the breakdown of diclofenac-TMS.

Table 2
Extraction efficiencies ($\%_{EXTR}$) for the investigated drugs and internal standard

Compound	$c_{\text{before DER}}$/ mg mL^{-1}	$n_{SPE+DER}$	n_{DER}	$\%_{EXTR}$	$\pm\delta/2$
Ibuprofen	0.0331	4	2	84.2	0.671
	0.296	6	6	91.9	0.336
Mecoprop	0.0214	4	2	91.5	0.399
	0.191	6	6	96.1	0.157
Naproxen	0.0188	4	2	104.1	0.129
	0.114	2	4	102.1	0.057
Ketoprofen	0.0320	4	2	99.3	0.017
	0.286	6	6	103.4	0.158
Diclofenac	0.0254	4	2	103.7	0.088
	0.227	6	6	157.0	22.053

Abbreviations: $c_{\text{before DER}}$, concentration of the selected compound in toluene prior to derivatisation; $n_{SPE+DER}$, number of extracted and derivatised samples; n_{DER}, number of derivatised samples; $\pm\delta/2$, deviation from $\%_{EXTR}$ calculated as:

$$\delta/2 = 1/2\left[\left(\text{mean}A_{SPE+DER} + s_p/\text{mean}A_{DER} + s_p\right) - \left(\text{mean}A_{SPE+DER} - s_p/\text{mean}A_{DER} - s_p\right)\right]$$

$$s_p^2 = \left(\left[\left(n_{SPE+DER} - 1\right)s_1^2 + \left(n_{DER} - 1\right)s_2^2\right]/\left[n_{SPE+DER} + n_{DER} - 2\right]\right);$$

s_p^2—pooled variance; mean$A_{SPE+DER}$: mean peak area of extracted and derivatised sample with standard deviation s_1; meanA_{DER}: mean peak area of derivatised sample with standard deviation s_2.

3.6. Detection limits

Instrumental limits of detection (ILDs) were determined by selecting the lowest concentration of the spiked sample that produces a chromatographic peak having an area under curve equal to three times the standard deviation of the baseline noise of the blank sample (Knoll, 1985). The ILDs in full-scan acquisition mode are in μg L^{-1} (Table 3) but can

Table 3
ILDs of TMS derivatives of target compounds in toluene obtained with full-scan acquisition mode and MLDs of the investigated drugs in distilled, deionised water

Pharmaceutical compound	Ibuprofen	Naproxen	Ketoprofen	Diclofenac
ILD/μg L^{-1}	20.3*	56.1*	20.9*	31.5*
MLD/ng L^{-1}	1.96	5.55	2.12	3.06

* TMS-esters of pharmaceutical compounds.

be reduced further by using selected ion monitoring (SIM) mode.

Method limits of detection (MLDs) were calculated from ILDs taking into account the concentration factors from SPE procedure. For this calculation, a 100% extraction efficiency was presumed and the results are shown in Table 3.

Comparison of the ILD and MLD (Table 3) shows that approx. 1000 times concentration of the compounds in a sample is achieved. However, the concentration factor and therefor, to a certain extent, MLDs can be further improved by increasing the amount of water extracted, by reducing the volume of toluene used for dissolution of the dry extract or by reducing the derivatising agent volume. The proposed suggestions are under investigation.

The minimal volume of surface water needed for isolation using our analytical procedure was calculated on the basis of ILD (Table 3). For calculation purposes we assumed that the Slovene environment is polluted with pharmaceutical residues in the same range as the rest of Central Europe, including Germany (Kümmerer, 2001b) where the data were taken from. Calculations were made for two model compounds: ibuprofen and diclofenac according to Eq. (2):

$$V_x = 1/c_x(V_K * \mathrm{MLD} * M_x/M_{X-\mathrm{TMS}}) \quad (2)$$

where V_x represents the volume of water sample, c_x is the lowest concentration of the compound determined in literature (Kümmerer, 2001b), V_K is the volume of the solution before GC-MSD analysis (0,13 mL), MLD is method limit of detection, M_x is molecular mass of compound and $M_{X\text{-TMS}}$ is molecular mass of trimethylsilylised molecule.

The results of our calculations showed that the minimal volume for detecting ibuprofen is expected to be from 7 to 39 mL of surface water sample. In case of diclofenac the minimal volumes were from 7 to 659 mL of surface water sample. These results show that the extraction volume (1000 mL) of the surface water sample should allow detection of selected NSAIDs in the low ng L^{-1} range.

3.7. Determination of the selected pharmaceuticals in environmental samples

Nine tap, two well and ten river water samples from Slovenia were examined using the procedure described in the methodology. Some of river samples were sampled twice (June–July 2004 and September 2004). There were no traces of the pharmaceuticals under study in the tap and well water samples, while 11 out of 16 river samples contained naproxen (17–80 ng L^{-1}) and diclofenac (9–49 ng L^{-1}), and one sample showed a concentration approximately four to five times higher than the rest of tested compounds: naproxen: 313 ng L^{-1} and diclofenac: 282 ng L^{-1}. This sample was taken in a river downstream of a pharmaceutical factory. According to fragmentation pattern, ketoprofen was detected in 8 and ibuprofen in 1 out of 16 of the river samples, but because interferences were present with the same retention time, their quantification could not be determined in SCAN mode. However, these limitations will be addressed by modifying the chromatographic separation and using SIM. Sampling of river waters that showed higher concentrations of selected contaminants was repeated at the same sampling sites 2 months after the initial sampling (September 2004). From the results (Table 4) it is seen that the concentrations of the tested NSAIDs were lower in the September samples. The sample that contained the highest amount of naproxen and diclofenac sampled in June (Krka river sample downstream) where approximately five times lower in case of autumn sampling. The reason might be the nature of sampling (not continuous sampling) and/or the time of sampling, i.e. second sampling was performed when river flow was higher resulting in greater dilution. In the future sampling will be repeated using a greater number of sampling points and continuous sampling. Also, sediment sampling will be taken into account.

Table 4
Determined concentrations of the selected NSAIDs

Sample	Date of sampling	Concentration (ng L^{-1})		
		Naproxen	Diclofenac	Ketoprofen
KRKA 1	Jul–04	*	–	–
	Sep–04	–	–	*
KRKA 2	Jul–04	313	282	*
	Sep–04	60	49	*
LJUBLJANICA 1	Jul–04	–	–	–
	Sep–04	<MLD	–	–
LJUBLJANICA 2	Jul–04	73	*	–
	Sep–04	<MLD	–	*
SAVA	Jul–04	80	9	*
	Sep–04	*	*	–
MURA	Jul–04	49	41	*
DRAVA 1	Jul–04	46	26	*
	Sep–04	<MLD	–	–
DRAVA 2	Jul–04	24	32	*
	Sep–04	42	–	–
PŠATA	Jul–04	17	*	–

* Compound was detected, but quantification could not be determined in SCAN mode.

4. Conclusions

In the presented work an analytical procedure for determination of pharmaceutical residues in water samples was developed. The qualitative determination of the selected compounds (naproxen, ketoprofen, ibuprofen, diclofenac) included development and optimisation of following analytical steps: SPE, derivatisation and GC-MSD analysis. Under optimal working conditions (flow, solvent volume, cartridge dimension, derivatisation conditions) isolation of selected compounds from water samples with efficiencies >84% was achieved. For all four tested compounds, ILDs were determined (20–56 μg L^{-1}) and MLDs for optimised method were calculated (2–6 ng L^{-1}).

Finally, the contamination of NSAIDs in representative samples of Slovene waters (two well, eleven tap and nine river water samples) was determined. According to our results the contamination of Slovene waters is comparable with the published literature for Central Europe (Kümmerer, 2001b). However, to determine the extent of pharmaceutical residue contamination in natural Slovene waters, we intend a more comprehensive study taking into account a greater number of sampling points, continuous water sampling and sediment samples.

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3.1.2 Scientific paper: “First interlaboratory exercise on non-steroidal anti-inflammatory drugs analysis in environmental samples”

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First interlaboratory exercise on non-steroidal anti-inflammatory drugs analysis in environmental samples

M. Farré[a], M. Petrovic[a,b], M. Gros[a], T. Kosjek[c], E. Martinez[a], E. Heath[c], P. Osvald[d], R. Loos[e], K. Le Menach[f], H. Budzinski[f], F. De Alencastro[g], J. Müller[h], T. Knepper[h], G. Fink[i], T.A. Ternes[i], E. Zuccato[j], P. Kormali[k], O. Gans[l], R. Rodil[m], J.B. Quintana[m], F. Pastori[n], A. Gentili[n], D. Barceló[a,*]

[a] Department of Environmental Chemistry, IIQAB-CSIC, C/Jordi Girona 18-26, 08034 Barcelona, Spain
[b] Institucio Catalana de Recerca i Estudis Avanzats (ICREA), Barcelona, Spain
[c] Jozef Stefan Institute, Ljubljana, Slovenia
[d] Environmental Institute, Kos, Slovak Republic
[e] Institute for Environment and Sustainability, JRC, Ispra, Italy
[f] CNRS, LPTC, Université Bordeaux 1, Talence, France
[g] ENAC-ISTE-Central Environmental Laboratory EPF Lausanne, Switzerland
[h] Europa Fachhochschule Fresenius, University of Applied Science, Idstein, Germany
[i] Federal Institute of Hydrology (BFG), Koblenz, Germany
[j] Mario Negri Institute for Pharmacological Research, Milan, Italy
[k] Pesticide Residues Laboratory, General Chemical State Laboratory, Athens, Greece
[l] Umweltbundesamt GmbH, Abt. Umweltwirksame Stoffe und Metaboliten, Wien, Austria
[m] IUMA, University Institute of Environment University of A Coruña, A Coruña Spain
[n] Universita "La Sapienza" di Roma, Italy

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ABSTRACT

Comparability of monitoring data are essential for any meaningful assessment and for the management of environmental risks of emerging pollutants. The reliability and comparability of data at European level is often limited, because analytical methods for emerging pollutants are often not fully validated, not harmonized or not suitable for all relevant matrices.

This paper describes a collaborative interlaboratory exercise for the analysis of non-steroidal anti-inflammatory drugs (NSAIDs) residues in freshwater and wastewater, held in the framework of the EU project "Network of reference laboratories for monitoring of emerging environmental pollutants" (NORMAN). The NSAID compounds selected in this study were ketoprofen, naproxen, ibuprofen and diclofenac.

Thirteen laboratories distributed along nine European Countries (Austria, France, Germany, Greece, Italy, Slovak Republic, Slovenia, Spain, and Switzerland) took part in this exercise, 126 samples were analyzed and a total number of 473 values in duplicate were collected.

Samples selected in this study include environmental water (river water and waste water) and artificial water (fortified environmental and distilled water) with different ranges of complexity.

Two analytical methods were proposed by the organiser; one is based on the use of solid phase extraction (SPE) followed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) and the second one is based on SPE followed by gas-chromatography–mass spectrometry (GC–MS), however, in the first round some different approaches were also admitted.

The main goals of this interlaboratory comparison were to evaluate the available analytical schemes for NSAID analysis in natural waters, to evaluate the repeatability (r) and reproducibility (R) between participating laboratories, and to evaluate the influence of the analytical method and sample matrices on the results.

1. Introduction

Human and veterinary pharmaceutical compounds are a source of increasing environmental concern as they are used in large quantities and their physical and chemical properties make them likely

* Corresponding author. Tel.: +34 93 400 61 00; fax: +34 93 204 59 04.
E-mail address: dbcqam@cid.csic.es (D. Barceló).

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to be transported into hydrologic systems, where their effects on human health and aquatic ecosystems are mostly unknown.

Pharmaceuticals are in general, after intake and absorption into humans or animal blood system, a subject of metabolic degradation processes. However, significant fractions of the original substances are excreted in un-metabolized form or as active metabolites via urine or faeces to be emitted into raw sewage, which may or may not be treated [1,2]. Yet, some pharmaceuticals are not degraded in waste treatment plants and enter the environment in their original form [3,4]. In addition to metabolic excretion, disposal by flushing of unused or expired medication and drug-containing waste from manufacturing facilities can also contribute to environmental contamination [2]. Flushing unused medicines down the drain appears to be of minor importance, while patient excretion following therapy is widely considered to be the primary pathway to the environment [5]. Even posthumously, the drugs administered in the closing phases of our lives likely leach into cemeteries and groundwater [6].

Different studies carried out in Europe [7,8], Canada [9], and USA [10] during the last decade show that one of the most common group of pharmaceuticals found in wastewater, river water and even in drinking water are non-steroidal anti-inflammatory drugs (NSAIDs) representatives, because they are commonly used in treatment of mild to moderate pain, and chronically in treatment of rheumatic diseases. Moreover, some of them are available as non-prescription pharmaceuticals. A number of reservoirs tapped for drinking water have been monitored along the Lergue River in Southern France, where pharmaceuticals and other wastewater-related dominant contaminants such as paracetamol, diclofenac, and carbamazepine were found [11]. Other examples have been reported; Zuccato et al. reported the presence of clofibric acid and diazepam in treated drinking water [3], in Italy; Heberer et al. [12] have reported the presence of diclofenac in the drinking water of Berlin; Loraine and Pettigrove identified and quantified ibuprofen (0.93 μg/L) and ibuprofen methyl ester (4.95 μg/L) in treated water [13].

Due to that, several methods have been developed for the determination of NSAIDs and their metabolites in water in the lower ng/L range using solid phase extraction (SPE) coupled to gas chromatography–mass spectrometry (GC–MS) based methods [14]. However, due to the elevated polarity and acidic nature of NSAIDs, with pK_a values between 4 and 4.5, liquid chromatography–mass spectrometry (LC–MS) and LC–tandem MS have experienced an impressive progress during the last years, both in terms of technology development and application, avoiding the derivatization step required by GC–MS methods. Recently, both groups of analytical techniques, i.e. LC–MS/MS and GC–MS have been used for the trace analysis of acidic drugs in environmental samples [15].

However, there is need for harmonization and validation of analytical methods for NSAID residue analysis. According to new EU recommendations, validation of analytical methods should be conducted in step-by-step processes with different levels of verification, and the achievements in each step should be evaluated by interlaboratory studies. In the present work, we performed the first level of verification, and the quality of the existing methods of analysis was evaluated, and a selection of more suitable methods to be validated in a second step was carried out. In this sense, this interlaboratory study was organised by the Environmental Chemistry Department, IIQAB-CSIC in Barcelona (Spain) under the frame of NORMAN EU project. The study was performed using either LC–MS/MS or GC/MS, both of which employed SPE as purification and concentration step.

During the exercise, three series of three samples each were analyzed. Every batch of samples consisted of three natural wastewater samples, three fortified river samples and three fortified deionised water samples. In total, every laboratory received nine samples for analysis. The samples were prepared by the Environmental Chemistry Department, IIQAB-CSIC in Barcelona, Spain.

In this round, 14 participations were carried out in 13 laboratories in Austria, France, Germany, Greece, Italy, Slovak Republic, Slovenia, Spain, and Switzerland.

The main goals of this study were to compare the results using different analytical methods, to evaluate the quality of individual approaches, to evaluate the accuracy and quality parameters in different aqueous matrices and to assess variations between different approaches (LC–MS/MS, GC/MS) and different laboratories.

2. Experimental

2.1. Experimental design, sample collection and handling

A total number of 126 samples corresponding to 14 participations were distributed in 13 laboratories (one laboratory participate twice, a LC–MS/MS based method and also using a method based on GC/MS). Three series of samples were analyzed in three batches, and every batch was composed by an effluent sample of a wastewater treatment plant (WWTP), a fortified Ebro river water and a fortified deionised water. Table 1 shows sample codification. The environmental samples were selected according to their expected NSAID concentration and matrix complexity, and a wastewater effluent sample from a WWTP near Barcelona (Spain) fitted well these criteria. Ebro river samples were collected near the area of Amposta (Barcelona, Spain). In all cases, samples were collected in Pyrex borosilicate glass containers, previously rinsed with tap and high-purity water. The samples were transported at 4 °C to the laboratory in Barcelona. In order to minimize the sources of variation, all samples were collected, transported, homogenised and prepared at the same time in a central laboratory (Environmental Chemistry Department, IIQAB-CSIC, Barcelona).

After sampling, the water samples were filtered through 2.7 and 0.45 μm glass micro-fibre filters to remove suspended matter and homogenised in a polyethylene bucket. The samples were transported refrigerated, and each participant received approximately 1.1 L of each sample. Participants were requested to measure and note the temperature of each sample at reception, in order to check that all samples were received in similar conditions for all participants. Then, all laboratories were requested to keep the samples under freezing conditions until the extraction of each batch. Water samples were allowed to reach room temperature and were spiked with surrogate standard. Samples were then extracted and analyzed during the same dates by the participants and the total duration of the exercise was 4 months.

In order to maintain the anonymous character of all participants, an identification number was provided to every laboratory. This number was requested to be used later for the presentation of results.

Table 1
Sample codes table

Batch 1	A1 (fortified river water)	B1 (wastewater)	C1 (fortified MilliQ water)
Batch 2	A2 (wastewater)	B2 (fortified MilliQ water)	C2 (fortified river water)
Batch 3	A3 (fortified MilliQ water)	B3 (fortified river water)	C3 (wastewater)

Table 2
List of participants in alphabetical order and the main characteristics of the analytical methods involved in the exercise

Participant	Pre-treatment	Extraction	Extract reconstitution	Chromatography	Column	Derivatization	Reference
CNRS, LPTC, Université Bordeaux 1,Talence, France	Neutral pH	Oasis HLB (60 mg, 3 mL), Waters	Ethyl acetate	GC–MS	Capillary column 30 m, 0.25 μm film thickness	MTBSTFA (MSTFA)	[23]
Environmental Institute, Kos, Slovak Republic	Neutral pH	Oasis HLB (60 mg, 3 mL), Waters	Ethyl acetate	GC–MS	Capillary column 30 m, 0.25 μm film thickness	MTBSTFA (MSTFA)	[22]
EPF Lausanne, Switzerland	Acidification pH 2	ENVI-18 reverse phase	Toluene	GC–MS	RTX-capillary column 60 m, 0.25 μm film thickness	Pentafluorobenzyl bromide	[18]
Europa Fachhochschule Fresenius, University of Applied Science, Idstein, Germany	Acidification pH 2	Oasis MCX 3cc (60 mg), Waters	Hexane	GC–MS	Capillary column 30 m, 0.25 μm film thickness	Diazomethane	[18]
Federal Institute of Hydrology (BFG), Koblenz, Germany	Acidification pH 2	Oasis MCX 3cc (60 mg), Waters	100 μg methanol + 400 μg water, 0.1 M formic acid	LC-ESI (−) MS/MS	Zorbax Eclipse XDB-C8	–	[19]
General Chemical State Laboratory (Athens, Greece)	Acidification pH 3	SPE Oasis HLB 6cc, 200 mg, Waters	Water 0.3% formic acid	LC-ESI (+) MS/MS	Xterra® Waters	–	[20]
IIQAB-CSIC, Barcelona, Spain	Neutral pH	Oasis HLB (60 mg, 3 mL), Waters	Methanol:water (25:75, v/v)	LC-ESI (−) MS/MS	Purosphere Star RP-18 endcapped column	–	[7]
Institute for Environment and Sustainability, JRC, Ispra, Italy	Neutral pH	Oasis HLB (60 mg, 3 mL), Waters	Methanol:water (25:75, v/v)	LC-ESI (−) MS/MS	Purosphere Star RP-18 endcapped column	–	[7]
IUMA, University Institute of Environment University of A Coruña, Spain	Neutral pH	Oasis HLB (60 mg, 3 mL), Waters	Ethyl acetate	GC–MS	Capillary column 30 m, 0.25 μm film thickness	MTBSTFA	[21]
Jozef Stefan Institute, Ljubljana, Slovenia		Oasis HLB (60 mg, 3 mL), Waters	Ethyl acetate	GC–MS	Capillary column 30 m, 0.25 μm film thickness	MTBSTFA	[22]
Mario Negri Institute for Pharmacological Research, Milan, Italy	Acidification pH 3	Oasis MCX 3cc (60 mg), Waters	Methanol, 2% NH_4^+, 0.2% NaOH in methanol	LC-ESI (−) MS/MS	Zorbax Eclipse XDB-C8	–	[23]
Umweltbundesamt GmbH, Abt. Umweltwirksame Stoffe und Metaboliten, Wien, Austria	Neutral pH	Oasis HLB (60 mg, 3 mL), Waters	Methanol:water (25:75, v/v)	LC-ESI (−) MS/MS	Purosphere Star RP-18 endcapped column	–	[21]
Umweltbundesamt GmbH, Abt. Umweltwirksame Stoffe und Metaboliten, Wien, Austria	Acidification pH 2	Oasis HLB (60 mg, 3 mL), Waters	Ethyl acetate	GC–MS	Capillary column 30 m, 0.25 μm film thickness	MTBSTFA	[22]
Universita "La Sapienza" di Roma, Italy	Acidification pH 3	Oasis HLB (500 mg), Waters	Methanol:water (25:75, v/v)	LC-ESI (−) MS/MS	Alltima C18 (4.6 × 250 mm; 5 μm)	–	[24]

Table 3
Statistical values corrected after the outlier exclusion for each compound in the different types of water

Group	No. of accepted results	Mean	Standard deviation	Standard error of mean	Median	Minimum value	Maximum value	95% Confidence interval		No. of outliers
								From	To	
Ketoprofen										
Wastewater										
Batch 1	13	678.77	282.23	78.27	800.00	25	1090	508.21	849.33	0
Batch 2	12	601.08	235.24	67.91	620.95	112	897	451.61	750.54	1
Batch 3	13	642.55	303.61	84.21	662.00	144	1200	459.06	826.03	1
Fortified river water (fortification 290 ng/L)										
Batch 1	13	238.73	121.08	33.58	200	73	461	165.56	311.90	0
Batch 2	13	297.92	134.13	37.20	260	62	538	216.85	378.98	0
Batch 3	12	241.39	92.317	26.65	230	106.2	420	182.74	300.05	1
Fortified MilliQ water (fortification 83 ng/L)										
Batch 1	12	164.24	99.52	28.73	133.5	64	365	101	227.47	0
Batch 2	11	101.71	40.52	12.22	100.0	60	208.8	74.49	128.93	1
Batch 3	11	97.35	33.96	10.24	91.00	37	151	74.54	120.17	0
Naproxen										
Wastewater										
Batch 1	12	913.18	744.55	214.93	848.00	18	2140	440.11	1386.3	1
Batch 2	13	858.17	635.68	176.31	887.00	77	1790	474	1242.3	0
Batch 3	13	818.54	603.27	167.32	930.00	77	1800	153.95	1183.1	0
Fortified river water (fortification 1124 ng/L)										
Batch 1	13	1109.0	845.51	234.50	1430.0	53	24.37	598.01	1620	0
Batch 2	12	1088.9	746.59	215.52	1047.5	308	2446	614.51	15.63	1
Batch 3	13	1066.6	764.33	211.99	1230.0	53.5	2325	604.69	1528	0
Fortified MilliQ water (fortification 266 ng/L)										
Batch 1	11	174.32	96.214	29.010	200.00	51	330	109.68	238.95	2
Batch 2	12	158.83	96.254	27.786	165.00	33.5	327	97.68	219.99	1
Batch 3	12	201.96	106.87	30.850	225.00	24	369	134.06	269.86	1
Ibuprofen										
Wastewater										
Batch 1	13	1834.2	640.75	177.71	1980.0	233	2680	1446.9	2221.4	1
Batch 2	14	1777.6	589.47	157.54	1715.5	645	2951.5	1437.3	2117.9	0
Batch 3	13	1918.3	385.03	106.79	1904.0	1124	1685.5	1685.6	2151.0	1
Fortified river water (fortification 675 ng/L)										
Batch 1	14	536.04	278.79	74.510	508.75	22	1067.1	375.1	699.0	0
Batch 2	13	523.73	156.46	43.395	484.00	310	875.0	429.2	618.3	1
Batch 3	13	542.74	190.58	52.859	603.00	278	811.1	427.6	657.9	1
Fortified MilliQ water (fortification 225 ng/L)										
Batch 1	13	260.46	139.51	38.693	215.00	66	621	176.15	344.8	1
Batch 2	13	253.15	102.87	28.532	246.00	165	547	190.97	315.3	1
Batch 3	13	206.12	45.461	12.609	203.00	96	265	178.65	233.6	1
Diclofenac										
Wastewater										
Batch 1	14	1482.2	784.75	209.73	1545.0	92	2557	1029.2	1935.2	0
Batch 2	14	1476.4	919.93	245.86	1564.5	177	2920	945.29	2007.4	0
Batch 3	14	1503.8	982.03	262.46	1457.0	177	3343	936.84	2070.7	0
Fortified river water (fortification 398 ng/L)										
Batch 1	11	400.36	198.83	59.951	340.00	133	767	266.79	533.93	1
Batch 2	11	408.27	145.78	43.955	457.00	129	609	310.34	506.21	1
Batch 3	12	392.88	157.37	45.428	400.00	154	705	292.89	492.86	2
Fortified MilliQ water (fortification 200 ng/L)										
Batch 1	13	205.71	109.18	30.280	193.00	61	483.2	139.73	271.69	1
Batch 2	14	167.43	79.237	21.177	156.00	61.4	284	121.69	213.17	0
Batch 3	14	150.51	71.030	19.700	148.00	33	320	107.58	193.43	1

All samples included in the study were delivered to each participating laboratory at 4 °C. An aliquot of each sample was kept in the laboratory in Barcelona, in order to perform an additional study and check whether the samples were stable when kept under unfavourable conditions. For this test, a set of samples was extracted immediately after sampling, i.e. within 24 h after sampling. Another two sets of samples were kept refrigerated and were extracted and analyzed 1 week and 10 days after sampling, respectively. Two more sets of samples were stored at room temperature and were extracted and analyzed also within 1 week, and 10 days after sampling. The results for the different sets extracted after different periods of time and under various conditions were compared with those extracted immediately after sampling (24 h). In all cases, no significant differences in NSAID concentrations were quantified and the stability of samples was confirmed in all situations. Finally, the homogeneity of the samples was checked and

confirmed by analyzing three randomly selected samples for each type. The NSAID concentrations were compared between the samples and no significant differences were found, with the coefficients of variation lower than 10%, in all cases.

2.2. Chemicals

Ibuprofen, naproxen, ketoprofen, and diclofenac used were of the highest purity available (>99%), and were kindly supplied by Jescuder (Rubí, Spain).

Deuterated d3 ibuprofen was used as the internal standard. Individual stock standard solutions used for spiked samples were prepared on a weight basis in methanol and stored at −20 °C.

2.3. Statistical parameters

For each series, the mean value (X), the standard deviation (σ), variance (σ^2), upper warning limit (UWL) value, lower warning limit (LWL) value, number of outlier values, repeatability (r), reproducibility (R) and coefficient of variation (CoV) were calculated.

The formulae used to calculate the UWLs, and the LWLs as were UWL = $(X+2\sigma)$ and LWL = $(X-2\sigma)$, respectively.

As acceptance criteria for each result were used, the Z-score function according with the IUPAC [16,17] following the AOAC, and ISO directives.

The Z-values where calculated according to

$$Z = \frac{X_{\text{lab}} - X}{\sigma}$$

where X_{lab} is the result for a laboratory, X the mean value of all laboratories values, and σ the standard deviation in the correspondent population (taking into account the results obtained for the different laboratories in a series for a type of sample).

The results whose Z-value was over 3 were directly excluded. For the results whose Z-score values were a number between 2 and 3 the Dixon test was applied with a 5% of significance level.

In order to follow the stability of samples along the test, it was necessary to study whether the mean concentrations difference between series were significant. Therefore, the analysis of variance (ANOVA) was applied to the results. First of all, it was necessary to prove whether the groups of data followed Gaussian distributions and if the differences between standard deviations of the groups were significant to select a parametric or non-parametric procedure. The normality test of the Kolmogorov and Smirnov method was applied, while the Bartlett's test was performed to establish if the differences among the standard deviations were significant. Finally, was selected the Kruskal–Wallis test, a non-parametric ANOVA.

The measurement of precision of each laboratory to repeat the measurements on a sample at different intervals, reproducibility

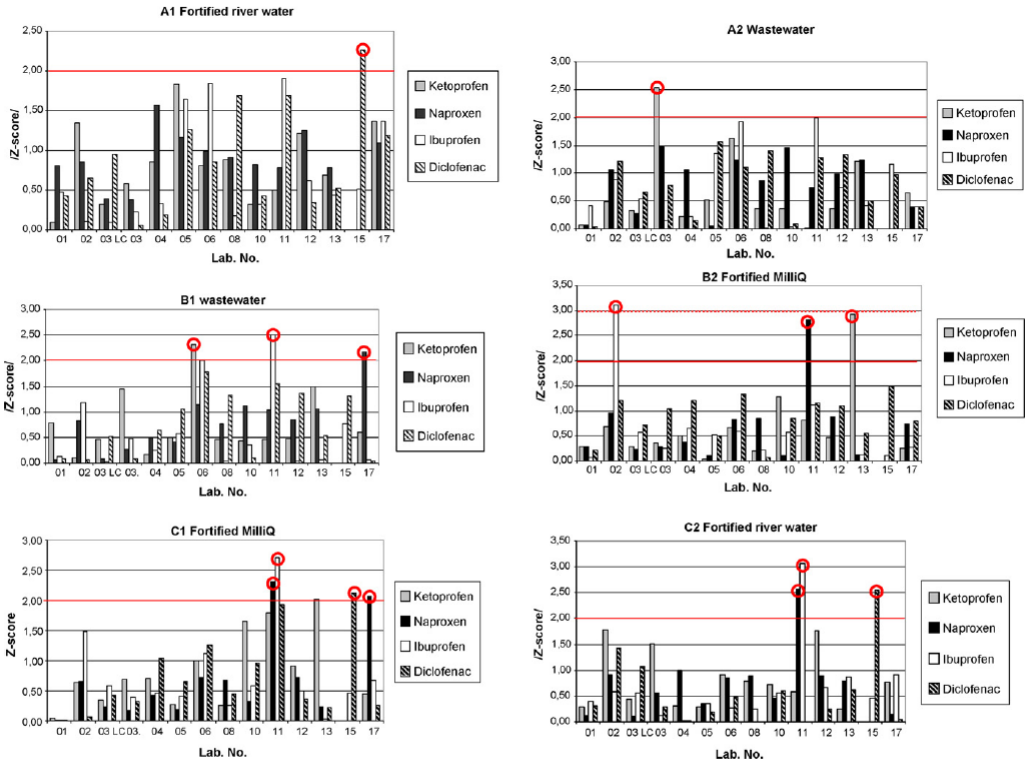

Fig. 1. Z-score absolute values.

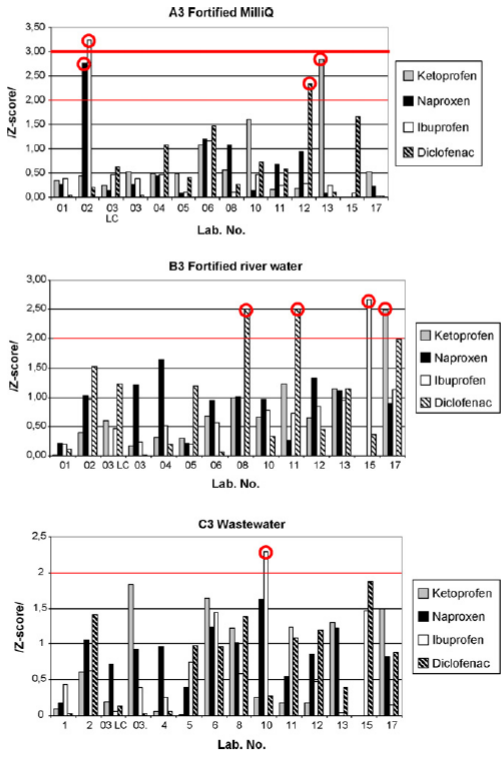

Fig. 1. (*Continued*).

(R) was calculated in terms of dispersion (D) as

$$D = R^{-1} = \frac{\Sigma r_{\text{lab}}}{N} = \frac{\Sigma(2 \times 2^{1/2})\sigma_{\text{lab}}}{N}$$

where $r^{-1}{}_{\text{lab}} = \sum(2 \times 2^{1/2})\sigma_{\text{lab}}$, N is the number of samples (only results for stable samples were included), and σ_{lab} is the standard deviation between results from the same laboratory on a stable sample at different intervals.

The hierarchical cluster analysis was based on the *similarity* matrices. The *similarity* measures were the *squared Euclidean* distances between the results obtained for a compound. The average linkage method was used as clustering approach.

2.4. Analytical methods

In the first round of this Interlaboratory exercise on the determination of NSAID residues in environmental water samples, a set of rules was proposed for the pre-concentration, extraction, and analysis of the samples. Analytical techniques utilized were based on liquid, or gas chromatography. Table 2 lists the participant laboratories in alphabetical order, as well as the main characteristics of the analytical methods used by each of them.

2.4.1. Extraction

Polymeric Oasis HLB 60 mg/3 mL (Waters) cartridges were proposed for off-line SPE extraction.

2.4.1.1. SPE protocol for LC–MS/MS analysis. Conditioning was carried out using 5 mL of methanol followed by 5 mL of deionised water (HPLC grade) at a flow rate of 1 mL/min. The samples (500 mL of ground and river water, and 200 mL of WWTP effluent) at neutral pH were allowed to percolate through the cartridges at a flow rate of 10 mL/min. Then the cartridges were rinsed with 5 mL of HPLC-grade water and dried under vacuum to remove excess of water for 15–20 min. Finally, the cartridges were eluted with 2×4 mL of methanol at 1 mL/min, evaporated under nitrogen stream, and reconstituted with 1 mL methanol–water (25:75, v/v).

2.4.1.2. SPE protocol for GC–MS analysis. Cartridges were conditioned using 3 mL of ethyl acetate followed by 3 mL of methanol and 3 mL of deionised water (HPLC grade) at a flow rate of 1 mL/min. The samples (500 mL of ground and river water, and 200 mL of WWTP effluent) were allowed to percolate through the cartridges at a flow rate of 10 mL/min, rinsed, and dried under vacuum to remove excess of water for 15–20 min. Finally, the cartridges were eluted with 3×1 mL of ethyl acetate and concentrated to 1 mL under a stream of nitrogen.

An off-line SPE method based on the extraction of the pre-filtered samples at neutral pH using polymeric cartridges Oasis HLB (60 mg, 3 mL) was proposed by the organisation based on next steps: condition of the SPE cartridges with 5 mL of methanol and 5 mL of deionised water (HPLC grade) at a flow rate of 1 mL/min, followed of the percolation of water samples (500 mL of ground and

river water, and 200 mL of WWTP effluent) through the cartridges at a flow rate of 10 mL/min, rinsing with 5 mL of HPLC-grade water, and drying under vacuum for 15–20 min, to remove excess of water. Finally, cartridges elution with 2 × 4 mL of methanol at 1 mL/min, evaporated under nitrogen stream and reconstitution with 1 mL methanol–water (25:75, v/v) for LC–MS/MS analysis, and with 1 mL of ethyl acetate for GC–MS. However, for the first round of the interlaboratories the participants were allowed to modify the proposed method (acidification, cartridge selection, temperature programme and elution conditions). During the Second round of the interlaboratory study, variations in the whole analytical procedures will not be allowed.

All the methods included in this exercise were based on the use of polymeric cartridges. Eight of the participants applied optimized methods that included acidification of the sample during extraction. The reconstitution of the sample extracts was also performed in different manners either according to the mobile phases in the case of LC or according to the derivatization procedure in the case of GC. Details of the various procedures for sample treatment are summarized in Table 2.

2.4.2. LC–ESI-tandem MS analysis

The LC analyses were performed using a RP-18 column. The method proposed outliner the analysis to be performed under negative ion conditions. The mobile phase was methanol as eluent A and MilliQ water as eluent B. Other mobile phases were also admitted. Two SRM transitions for each compound were acquired where possible; one was used for identification and one for quantification.

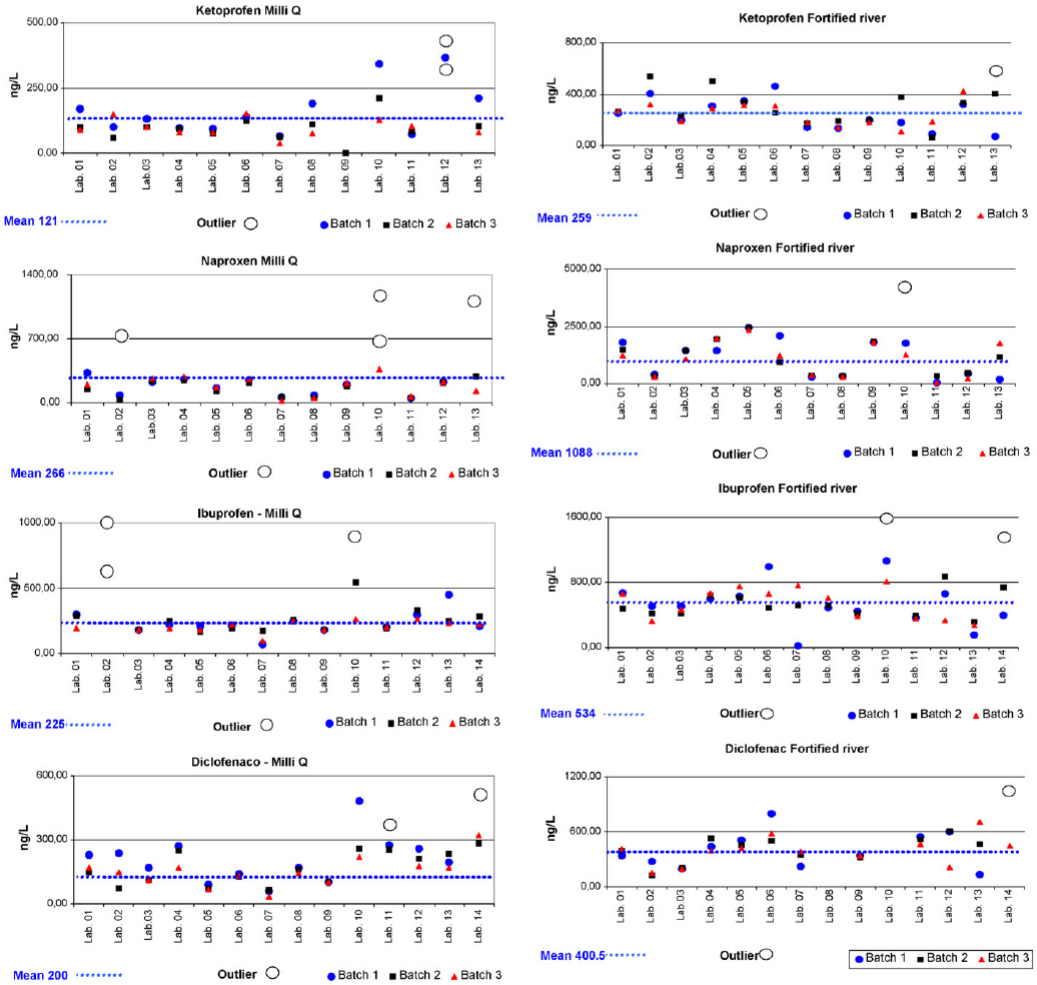

Fig. 2. Results obtained for each participant for naproxen, ibuprofen, diclofenac and ketoprofen expressed in ng/L in the different samples, and mean value of results (blue line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

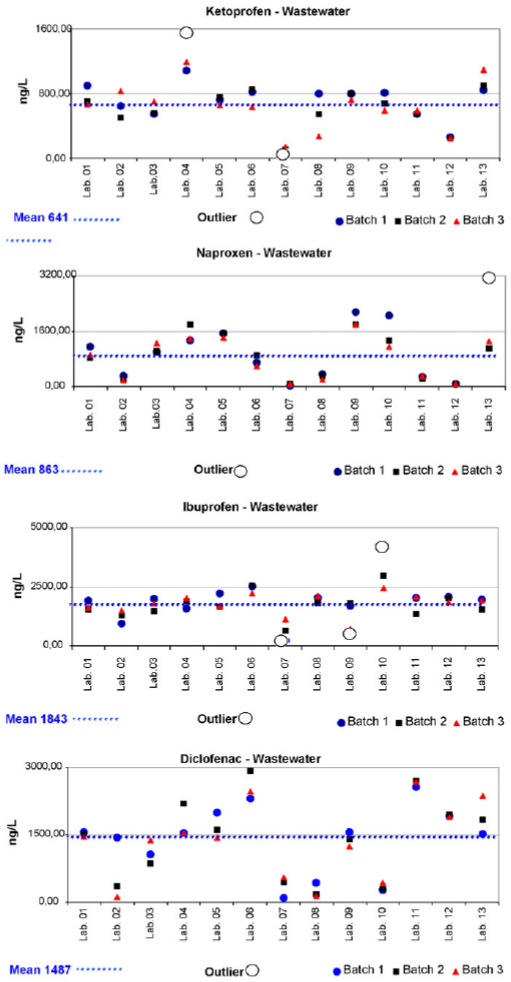

Fig. 2. (*Cntinued*).

Further chromatography and spectrometric conditions were optimized by the participants according to the available equipments and skills.

2.4.3. Gas chromatography–mass spectrometry

The method suggested by the organiser was derivatization with *N*-methyl-*N*-trimethylsilyl-trifluoracetamide (MTBSTFA) for 1 h at 60 °C. For GC–MS analysis, 1 μL of sample was injected in a split-less mode, at 250 °C; The GC oven was programmed as follows: 2 min at 65 °C, first ramp at rate 30°/min to 180°, rate 5°/min to 300 (hold 12 min). The target ions chosen were ibuprofen: *m*/*z* 263, naproxen: *m*/*z* 287, ketoprofen: *m*/*z* 311, diclofenac: *m*/*z* 352 and 354.

Most of the participants using GC followed this method, although the capillary columns were purchased from different suppliers and differences especially in the oven programme were introduced according to the different equipments available in the different laboratories.

3. Results

A total number of 14 participations took part of this study: 7 participations using a method based on GC–MS, and 7 participations using LC–MS/MS.

A total number of 486 results were collected. The mean values, standard deviations (σ), variances (σ^2), standard error of mean, median and upper and lower warning limits (UWL and LWL) between results from the participant laboratory at different intervals were calculated.

Z-score values and the Dixon test were used to calculate the outliers within the results. The calculation gave 24 outliers (4.9% of the

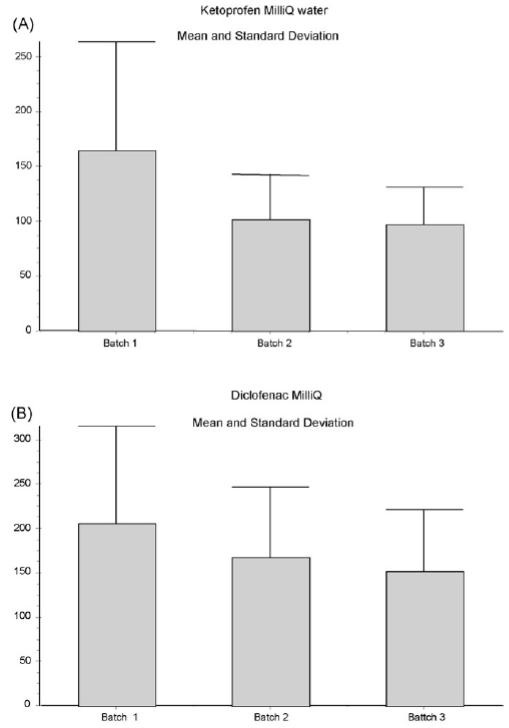

Fig. 3. Representation of the mean values and their standard deviations obtained for ketoprofen and diclofenac during the different intervals of time (Batch 1, Batch 2, Batch 3), for fortified MilliQ water.

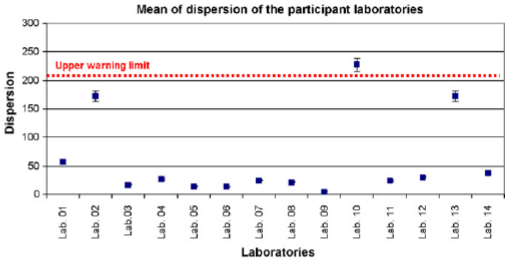

Fig. 4. Reproducibility (R) expressed as the inverse of dispersion (D). Dispersion mean values for the participants analyzing the selected compounds along the exercise are represented. Dotted line means the dispersion upper warning limit.

total number of results). In the group of results obtained by GC–MS 15 outliers were obtained from 216 results (a 6.9% of total results). In the case where LC–MS/MS was utilized nine outliers (corresponding to 3.3%) were obtained. Fig. 1 presents the absolute values of Z-score. According to the range of concentrations measured during the exercise, the complexity of the wastewater sample and the differences of the analytical methods used, the number of outliers obtained in this exercise can be considered low. The fact that more outliers were obtained by GC was expected due to the additional derivatization step in the sample preparation.

The distribution of outlier values for the different compounds resulted in similar percentages. The sample matrix with the higher number of outliers was deionised water. On the other hand, wastewater had the highest concentrations of the analytes. Since this was the most complex sample matrix, higher levels of variability between participants were obtained.

The coefficients of variation were high, as it was expected, because the selected compounds are emerging pollutants and different analytical methods were compared.

The outlier values were excluded of the final data treatment and the statistical parameters (mean values (X_i), standard deviation (σ_i), variance (σ_2), coefficient of variation (CoV) were recalculated. Table 3 shows the corrected statistical values (after outlier exclusion) obtained for each compound in the different types of samples along the exercise.

Results for the different samples expressed in ng/L and mean values are plotted in Fig. 2.

In order to establish the stability of samples along this intercomparison test, differences between variances obtained for every type of sample at different intervals were evaluated. In all cases the distribution of results followed a Gaussian distribution.

In two cases, ketoprofen and diclofenac in deionised water, the results from Bartlett's test suggested that the differences among standard deviation were significant along the exercise. Fig. 3 represents the mean values and the standard deviations along the

Table 4
Repeatability and reproducibility (R) expressed through r^{-1} and dispersion (D) values of each laboratory for ketoprofen, naproxen, ibuprofen and diclofenac in wastewater (A) and river water (B)

Laboratory codes, chromatographic method	Ketoprofen	Naproxen	Ibuprofen	Diclofenac
(A)				
01, LC	356	488	546	113
02, GC	455	186	805	1978
03, LC	237	386	747	727
04, GC	220	704	645	1086
05, GC	133	233	898	792
06, LC	335	436	565	900
07, LC	174	96	1261	684
08, GC	752	217	495	469
09, GC	131	572	200	453
10, LC	311	1366	1018	253
11, LC	66	94	1166	226
12, LC	14	0	307	85
13, GC	368	412	621	1218
14, LC	0	0	309	1492
D	254	371	684	748
(B)				
01, LC	28	795	314	888
02, GC	308	143	257	726
03, LC	75	613	128	361
04, GC	328	891	99	281
05, GC	49	191	211	597
06, LC	298	1704	733	2074
07, LC	59	149	1065	3013
08, GC	92	61	169	478
09, GC	33	65	97	273
10, LC	397	997	512	1448
11, LC	178	462	47	133
12, LC	153	368	773	2188
13, GC	656	2242	235	664
14, LC			700	1980
D	204	668	381	1079

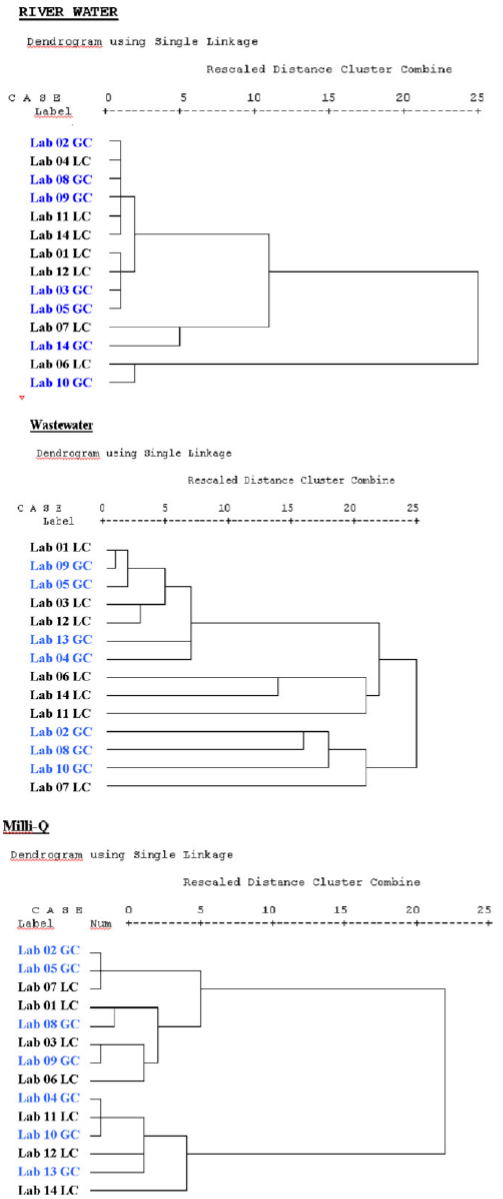

Fig. 5. Cluster analysis representation. Two-dimensional dendograms using the single linkage method for the three types of samples: (A) river; (B) wastewater; (C) MilliQ.

exercise for these compounds in MilliQ water. As can be seen, in these cases an important decrease in the measured concentrations was produced after the first batch of analysis, whereas good stability was obtained for wastewater and river water.

Repeatability data for river water and wastewater are summarized in Table 4A and B. Reproducibility may be expressed quantitatively in terms of the dispersion characteristics of the results. Fig. 4 shows the dispersion mean values for the participants analyzing the selected compounds along the exercise are represented. The dotted line represents the UWL, over which the reproducibility of a participant is considered to be significantly lower in comparison to the others. Therefore, it can be concluded that overall the reproducibility was quite low.

The possible interrelation between results was studied using the hierarchical cluster analysis. So, as all the results for each type of water were studied together and the results for each participant were grouped, first according the Average Linkage Cluster method, and second according to the Nearest Neighbour Cluster method. In both cases, the measured interval between results was the squared Euclidean distance. In Fig. 5 dendograms corresponding to the cluster analyses using the single linkage method are represented.

Both cluster analyses concluded that the results of the participating laboratories are independent of the analytical method used for the analysis of the samples (LC or GC). In addition, no relation was obtained between the results and the temperature of the samples at reception in the range of temperatures studied.

4. Conclusions

The number of participants that initiated this interlaboratory exercise was 17, and the final number of participants was 13 (77%). The final number of results collected was 486 and 24 values were outliers (4.9%) and discarded.

The number of outlier values by liquid chromatography was 9 (3.3% of results), whereas the number of outlier using GC–MS was superior than 15 (6.9%), probably due to an additional step in the sample preparation (derivatization).

Four laboratories obtained the higher number of outlier values and can be associated to samples handling and calculation errors. The sample with the highest number of outliers was the fortified deionised water, possibly because of the lowest concentration applied in the sample.

The second sample having a high number of outlier values was wastewater, most probably due to the complexity of the matrix. For this matrix higher levels of variability were also observed.

The stability of the samples was followed along the exercise by means of the ANOVA. In all cases the ANOVA test showed good stability for the NSAIDs selected in this study, and the variations among means were not significantly greater than expected by chance, with the exception of ketoprofen and diclofenac in MilliQ water.

A generally good agreement was obtained between the concentrations of fortification and the mean values reported by the participants. However, the precision of individual participants was quite low along the exercise, although only one participant showed a significantly low level of reproducibility. In order to minimize sources of variation in future validation steps, a protocol for sample treatment should be unified, and it should be clearly stated how to pre-treat the samples prior to analysis, e.g. how to defreeze de samples.

The hierarchical cluster analysis showed that no relation can be found between the results and if the chromatographic method is based on GC or LC.

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3.1.3 Scientific paper: “Second interlaboratory exercise on non-steroidal anti-inflammatory drugs analysis in environmental samples”

Submitted to Talanta, May 2009.

Second Interlaboratory Exercise on Non-Steroidal Anti-Inflammatory Drug Analysis in Environmental Aqueous Samples

E. Heath[*1], T. Kosjek[1], M. Farre[2], J. B. Quintana[3,4], L.F. de Alencastro[5], S. Castiglioni[6], O. Gans[7], K. Langford[8], R. Loos[9], J. Radjenović[2], L. Mainero Rocca[10], H. Budzinski[11]D. Tsipi[12], M. Petrovic[2,13], D. Barcelo[2]

[1]Jožef Stefan Institute, Ljubljana, Slovenia

[2]Department of Environmental Chemistry IDAEA-CSIC, C/ Jordi Girona 18-26, 08034 Barcelona, Spain

[3]IUMA - University Institute of Environment, University of A Coruña, A Coruña, Spain

[4]IIAA – Institute of Food Analysis and Research, Department of Analytical Chemistry, Nutrition and Food Sciences, University of Santiago de Compostela. Santiago de Compostela, Spain

[5]Central Environmental Laboratory (GR-CEL), Ecole Polytechnique Fédérale de Lausanne (EPFL) - 1015-Lausanne, Switzerland

[6]Department of Environmental Health Sciences "Mario Negri" Institute for Pharmacological Research, Via La Masa 19, 20156 Milan, Italy.

[7]Umweltbundesamt GmbH, Abt. Organische Analysen, Wien, Austria.

[8]NIVA, Norwegian Institute for Water Research, 0349, Oslo, Norway.

[9]European Commission (EC), Joint Research Centre (JRC), Institute for Environment and Sustainability, Ispra, Italy nstitute for Environment and Sustainability, JRC, Ispra, Italy

[10]Universita "La Sapienza" di Roma, Italy

[11]CNRS, LPTC - Université Bordeaux 1, Talence – France

[12]Pesticide Residues Laboratory, General Chemical State Laboratory, Athens, Greece

[13]Institucio Catalana de Recerca i Estudis Avanzats (ICREA), Barcelona, Spain

[*] Correspondence to: Ester Heath, Jožef Stefan Institute, Departmet of Environemntal Sciences, Jamova 39, Ljubljana, Slovenia; E-mail: ester.heath@ijs.si; Fax: +38612519385

Abstract

Several interlaboratory exercises were organised within the framework of European FP6 project NORMAN. Among others, non-steroidal anti-inflammatory drugs were investigated in different aqueous samples in two sequential ring studies. The aim of both studies was to evaluate the state-of-art in Europe and to determine possible sources of variation, while also attempting to diminish them. In the present paper we discuss the results of the 2nd Interlaboratory study, while the results of 1st round were presented before. The main scope of the 1st exercise organised within NORMAN project was to assess the laboratory proficiency regardless of the analytical method applied, to evaluate the stability of the target compounds during sample storage, and to define possible sources of variation during sample shipment, storage and analysis. In the 2nd round we primarily aimed to diminish these sources of variation by applying two predetermined analytical protocols based on liquid chromatography – mass spectrometry or gas chromatography – mass spectrometry. The two analytical protocols were compared in terms of their ability to determine individual analytes in matrices of different complexity, i.e. tap water, river water and wastewater. Furthermore, the 2nd exercise addressed also the filtration and compared the influence of different filter material categories on the analysis of non-steroidal anti-inflammatory drugs.

Results presented herein evaluate laboratory performance using z-score, bias, proximity and Youden plots. Overall, the laboratory performances were found to be satisfactory for determining NSAIDs in aqueous samples. The two analytical protocols, LC-MS and GC-MS, are assessed according to their sensitivity and measurement uncertainty, where the GC-MS proved superior for the analysis of ibuprofen, ketoprofen and naproxen in matrices with higher complexity. Finally, neither the filtration itself, nor the filter materials were shown to significantly affect the determination of NSAIDs.

Keywords: Non-steroidal anti-inflammatory drugs (NSAIDs), Ibuprofen, Naproxen, Ketoprofen, Diclofenac, Interlaboratory

1 Introduction

Pharmaceutically active substances are a class of emerging contaminants that have raised concern in recent years. Even though the amounts of pharmaceuticals and their bioactive metabolites being introduced into environment are likely to be low, their continuous input lead to high long term presence in the environment and may have chronic effects on aquatic and terrestrial organisms. Among all pharmaceutical compounds widespread and polar drugs, such as acidic non-steroidal anti-inflammatory drugs (NSAIDs), deserve particular attention. This is due to their physico-chemical properties: high water solubility, acidic pKa, low sorption properties and often poor degradability, which allow them to pass through all man-made treatments and natural filtration steps and enter surface water, groundwater and drinking waters [1,2]. The analytical methodologies for determining NSAIDs are still evolving and are applied at the level of individual laboratories. However, there is a need for harmonisation and validation of analytical methods for NSAIDs residue analysis. In the absence of standard reference materials, the main steps of analytical procedures should be evaluated by interlaboratory studies, a common practice in different research areas [3,4,5,6,7,8,9,10,11,12] and a corner stone of quality assurance [13]. The results of the analysis allow comparisons to be made between, and information to be obtained about, the laboratories, methods, or the test materials. The laboratories may come from within one organisation or may encompass different laboratories across the world. The quality value of the measurand may be known, or the object of the study may be to arrive at a consensus value. Common to all interlaboratory trials is one organisation that takes responsibility for sourcing the material, distributing it to the participating laboratories, collecting and processing of the data, and finally publishing the report [13].

Within the framework of EU FP6 project NORMAN two sequential interlaboratory studies were set-up to determine Ibuprofen, Ketoprofen, Naproxen and Diclofenac residues in various aqueous samples. The first level of verification and the quality of existing analytical procedures was evaluated by the Department of Environmental Chemistry, IDAEA-CSIC, Barcelona, Spain [8]. Here aquatic samples of differing complexity were prepared, distributed and analysed according to individual laboratory practices. The study was performed using either LC-MS/MS or GC/MS methods developed by the individual participant laboratories. All methods employed SPE as a purification and concentration step. The objectives of this 1st Interlaboratory Exercise was to determine NSAIDs in different samples in various European laboratories and to evaluate the main sources of variation in the results and to ascertain any

significant differences between results obtained by LC and GC, for samples with different matrices, and to check the stability of the samples. The results of that study revealed the following:

1) No significant difference between GC or LC based methods.
2) No relation exists between the submitted results and the temperature at reception, but temperature was still recognised as an important source of variation.
3) The number of outliers was linked to the number of steps in analytical procedure and the complexity of the method.
4) A good agreement was obtained between the concentrations of fortification and the mean values reported by the participants.
5) The precision of individual participants was low along the exercise suggesting the need for a protocol to unify sample treatment including handling, how to defrost the samples and chemical analysis in order to minimize sources of variation in the second interlaboratory exercise.

Based on these findings, protocols for sample pre-treatment and two harmonised protocols for GC and LC separation were prescribed. The second ring exercise was performed by the "Jožef Stefan" Institute, Ljubljana, Slovenia in collaboration with IDAEA-CSIC in Barcelona, Spain. The objectives of this study were to address weaknesses arising from the first interlaboratory exercise including the influence of temperature during sample shipment, effect of sample filtration, influence of complexity of the matrix, storage of frozen cartridges instead of samples and to evaluate overall variation in results arising from LC or GC pre-defined analytical procedures.

2 Materials and Methods

2.1 Experimental design, sample collection and handling

Samples were prepared by the Department of Environmental Sciences, "Jožef Stefan" Institute in collaboration with IDAEA-CSIC. Samples of wastewater treatment plant effluent (WWTP Rubi, Barcelona, Spain) and river water (Llobregat, Spain) were filtered through 2.7 µm and 0.5 µm glass micro-fibre filters. Deionised water was not filtered. Afterwards,

samples were homogenized, spiked (Table 1) and sub-sampled for homogeneity and stability testing. The samples (900 mL) were transferred into 1 L polyethylene bottles and frozen. The frozen samples were then shipped on dry-ice to the participating laboratories. A total number of 117 samples were sent to 13 participants in 12 laboratories, distributed among 9 European countries: Austria, France, Greece, Italy, Norway, Slovakia, Slovenia, Spain and Switzerland. The samples reached the participant laboratories within 24 to 72 hrs in a frozen state. Laboratory codes 1 – 13 were used to ensure anonymity. Separately, 1.5 mL of a standard NSAID mixture in methanol was sent separately at ambient temperature.

Three batches of samples were prepared for each laboratory, where each batch consisted of 3 samples prepared from wastewater (batch A), river water (batch B) and tap water (batch C) (Table 1).

Table 1: Sample matrices and encoding

2.2 Chemicals

Ibuprofen, Naproxen, Ketoprofen, and Diclofenac were supplied by Jescuder (Rubí, Spain). The purity of the standards was confirmed by LC-MS (UPLC-QTOF, Waters Corp., Milford, MA, USA) and GC-MS (Hewlett Packard, Waldbron, Germany), and the chromatographic response matched that of authentic standards purchased from Sigma Aldrich (≥ 98 % purity, St. Louis, MO, USA). The internal standard was deuterated Ibuprofen-d3 obtained by participants themselves. *N*-Methyl-*N*-[tert-butyldimethyl-silyl]trifluoroacetamide (MTBSTFA, provided by participants) was used as a derivatising agent in the GC-MS analytical procedure.

2.3 Analytical methods

As already explained, the 1st Interlaboratory exercise [8] did not make any special requirements on the analytical procedures for determining NSAIDs. In contrast, for this round of the Interlaboratory study the participants were asked to follow the analytical protocols based on gas chromatography - mass spectrometry, GC-MS, or liquid chromatography – mass spectrometry, LC-MS, available in the participant laboratories. Both analytical procedures involved concentration and clean-up steps using an off-line solid phase extraction (SPE) with polymeric Oasis HLB 60 mg / 3 mL (Waters) cartridges. Solid phase extraction was followed by LC-MS/MS or GC-MS analysis, where the latter involved an additional

derivatisation step using MTBSTFA. Prior to the SPE, participants were asked to perform an additional filtration step on the sample series 1 and 2 (Table 1): A1, A2, B1, B2, C1 and C2, where the internal standard was added post filtration. The extraction volumes were 400 mL for river water and tap water samples (Batches B and C) and 200 mL for wastewater (Batch A). No adjustment of the sample pH was made prior to the extraction.

LC-MS/MS analytical protocol

The conditioning of the SPE cartridges was carried out using 5 mL of methanol, followed by 5 mL of ultra-pure water (HPLC grade). The samples were allowed to percolate through the cartridges at a flow rate of 5 mL min^{-1}. After enrichment the cartridges were rinsed with 5mL of HPLC grade water and then dried under vacuum (15–20 min) to remove excess water. Finally, the cartridges were eluted with 8 mL of methanol (2 × 4 mL), evaporated under a nitrogen and reconstituted with 1 mL of methanol : water (25:75, v/v). The LC-MS/MS analyses were performed in negative ion mode using an RP-18 column for the chromatographic separation. The mobile phases were methanol with 5 mM NH_4 acetate and water with 5 mM NH_4 acetate. In the tandem MS operation, two multiple-reaction monitoring (MRM) transitions (identification and quantification ion) were acquired for each compound, whenever possible.

GC-MS analytical protocol

In the GC-MS analytical protocol the cartridges were conditioned with ethylacetate (3 mL), methanol (3 mL) and finally rinsed with ultra-pure water (3 mL). Likewise the LC-MS/MS, procedure followed the enrichment, rinsing and drying step, after which the cartridges were eluted with 2 × 1 mL of ethylacetate. Prior to GC-MS analysis the samples were derivatised with MTBSTFA at 60°C for 1h. Separation was preformed using (30 m × 0.25 mm × 0.25 μm) capillary column with 95% methyl / 5% phenyl polysiloxane stationary phase for chromatographic separation. The GC oven was programmed as follows: 2 min at 65 ºC, then ramped at 30 ºC / min to 180 ºC, further ramped at 5 ºC / min to 300 ºC, and finally held for 12 min. The target ions used for quantification were *m/z* 263 for ibuprofen, *m/z* 287 for naproxen, *m/z* 311 for ketoprofen and *m/z* 352 and 354 for diclofenac.

2.4 Statistical parameters

The homogeneity of sample preparation was statistically evaluated by a χ^2 test, using the Equation 1:

$$\chi^2 = \sum \frac{(O_i - E_i)^2}{E_i} \qquad \textit{Equation 1}$$

where O_i is a mean concentration of two parallels in each sample and E_i the mean concentration of each batch containing 10 samples.

As an acceptance criterion for each result the z-score value was calculated using Equation 2:

$$z = \frac{x_{lab} - x_0}{\sigma_0} \qquad \textit{Equation 2}$$

where x_{lab} is a laboratory mean (median), x_0 a mean (median) concentration for the initial dataset and σ_0 is the corresponding standard deviation. Both, the classical approach using mean value and the robust approach using the median were employed in calculations of z-scores. A z-score higher than 3.0 [14] indicates an unsatisfactory performance and such result was automatically excluded from the further data processing as an outlier. For values between $2.0 \leq |z| \leq 3.0$ [7], that is suspect outliers, the Dixon discordance test [15] was applied at a 5 % significance level.

After excluding any outliers, the corrected mean (X), standard deviation (σ), coefficient of variation (CV), standard error of mean (σ_M), median (M), minimum (Min) and maximum value (Max) were calculated for each series of results and the normality was confirmed by Lilliefor's Test. In addition, laboratory biases (D) were estimated for each result (or average of results) reported by each participant. According to ISO/DIS 13528:2002(E) [16] the biases were classified into three categories $|D \geq 3.0\ \sigma|$ indicating an "action signal", $|2.0\ \sigma \leq D < 3.0\ \sigma|$ considered as a "warning signal" and $-2.0\ \sigma < D < 2.0\ \sigma$ indicating "acceptable value". The outlier results were excluded from the calculation of D.

Proximity to the mean Pr(*X*) was calculated as:

$$\Pr(X) = \frac{1}{n} \times \sum \frac{|x_{lab} - X|}{X} \qquad \textit{Equation 3}$$

where n is the sample size, D the laboratory bias and X the corrected mean after the outlier exclusion. Similarly, the 'proximity to the median' Pr(M) was calculated, as shown in Equation 4.

$$\Pr(M) = \frac{1}{n} \times \sum \frac{|x_{lab} - M|}{M} \qquad \textit{Equation 4}$$

To evaluate the effect of filtration on determination on NSAIDs in the different matrices three statistical tests were used. An F-test at 5 % significance level was used for comparison of the variances between filtered (series '2') and unfiltered (series '3') sample series within each batch [17]. In addition, a paired t-test was applied to compare unfiltered and filtered mean values within each laboratory. In case of ibuprofen in tap water samples (batch C, series, 1, 2 and 3) three variances were compared with ANOVA.

3 Results and Discussion

3.1 Sample preparation

To minimise the variation between the samples, the matrices were collected, prefiltered, spiked, homogenised, divided-up and frozen within 24 h. To assure and to confirm the quality of sample preparation, the homogeneity of mixing was tested for each batch. Thus the homogenised batches (A, B and C, series 2 and 3, Table 1), were spiked and then sub sampled. According to ISO/DIS 13528 [16] ten samples per batch were taken randomly from different layers in the polyethylene container. The samples were then analysed in parallels and the homogeneity was statistically evaluated using the χ^2 test. The homogeneity was confirmed in all cases at the 95 % confidence level.

Given that the stability of samples during shipment and storage was one of the goals of the 1st NORMAN Interlaboratory exercise [8], and was confirmed for all analytes, the authors felt that there was no need to perform similar tests in the 2nd round. Furthermore, testing the stability of compounds in the aqueous media was an irrelevant issue for 2nd round as participants were asked to perform the SPE extraction short upon sample receipt (48 h), while the analyses themselves were allowed to be carried out within 3 months from extraction. Instead, the stability of NSAIDs in frozen cartridges was tested within three months after the sample extraction, where no decrease in the analyte content was observed within the studied period of time.

3.2 Laboratory proficiency testing

A total number of 108 samples were analysed in this exercise by 12 participants from 11 different institutions. Seven LC and five GC laboratories took part in the exercise and submitted 773 results including parallel and < LOD values. Among these, 428 values were pooled out for subsequent data mining process, starting with the determination of outliers. The z-score calculation, which was performed by classical and robust approach and the Dixon test, yielded 15 (3.5 %) or 18 (4.2 %) outliers, respectively. Figure 1 shows the absolute z-score values according to the classical approach for each laboratory.

Figure 1: Absolute z-score values for each of the participant laboratories (Lab ID 1-13) calculated using classical approach. The outliers are marked with circles. Dotted line: z = 2; solid line: z = 3.

The highest number of outliers was determined for tap water, which is a consequence of the lower concentration levels, agreeing with the results of the 1st Interlaboratory exercise [8]. By the use of the robust approach, the total number of outliers increased for three, which were, as shown in Table 2, all on account of ibuprofen in tap water using the LC-MS/MS analytical procedure. Out of 249 results obtained by LC-MS/MS, 12 (4.8 %) or 15 (6.0 %) were excluded as outliers using the classical and robust approach, respectively. The number of excluded GC results was considerably lower compared to the LC method, i.e. 3 out of 179 (1.7 %), regardless of which outlier-testing approach was used. Out of the total of twelve participants, the outlier results were reported by 5 (or 6 using the robust approach) laboratories, which generally experienced difficulties in determining only a single compound (e.g. Naproxen in laboratory 7 and Ketoprofen in laboratory 13), while, on the other hand, they showed a satisfactorily method performance for determining the remaining analytes (Figure 1).

Table 2: Calculated number (the percentage of total is stated in the brackets) of outlier values according to the sample matrix, analyte and analytical method. The outliers were calculated according to the classical and robust approach.

The percentage of the outlier values reported in the 2nd round was considerably lower compared to the 1st round [8], which may be attributed to the diminishing the weaknesses recognised in the 1st Interlaboratory exercise, i.e. sample shipment to the participant laboratories and/or pre-determination of the analytical protocols. Unifying the analytical protocols revealed a particularly evident improvement in case of GC based analytical protocol, where the number of outliers was up to 5 folds lower in 2nd round (3 outliers out of

428 processed results), when compared to 1st round (15 outliers out of 486 results). Such outcome is in agreement with our expectations since GC analytical protocols employed in the 1st exercise were rather heterogeneous, i.e. up to 3 different sorbent materials for SPE, 3 different elution solvents and 4 different derivatisation reagents were used [8].

After outlier exclusion the mean, standard deviation, coefficient of variation, standard error of mean, median, minimum and maximum value were calculated for each NSAID in each of the 9 samples. As shown in Table 3, the sample series '2' and '3' were spiked with the same level of the tested compounds, while spiking of the Ibuprofen in tap water was equal in all three series: '1', '2' and '3'. An excellent agreement was obtained between the concentrations measured in tap water and the actual spiking levels (Table 3). In general, the mean and median concentrations matched closely and followed the spiked concentrations. However, in wastewater and river water, the concentration response is, as a rule, smaller than the actual concentration increase due to spiking. Clearly, this phenomenon can issue from the suppression effect of the matrix compounds, which interfere with the LC and GC analysis. This effect is principally emphasized in the atmospheric pressure ionisation methods (API), which are generally used in LC, and has been several times reported [18,19,20,21,22,23]. Thus, it was shown that, in batch B the LC samples yielded a considerably lower response to the spiked concentration in comparison to the GC samples, which were in general only slightly affected. This effect corresponds to complexity of applied river water sample (Batch B), which in our case contained a lot of particulate matter and was sampled during the dry season downstream from the city of Barcelona. In addition, it was extracted in twice higher volume (400 mL) as wastewater (200 mL) and thus the extracts presumably involved more organic matter than wastewater. Further, an unusually high concentration of Ibuprofen (approx. 7.5 $\mu g\ L^{-1}$, Table 3) was determined in the natural river water sample, what is more likely a result of the sampling conditions (grab sample, extremely low water level and flow at sampling time), than a picture of Llobregat pollution.

Table 3: Summary of the corrected statistical parameters after the outlier exclusion: mean (X), standard deviation (σ), coefficient of variation (CV), standard error of mean (σ_M), median (M), minimum (Min) and maximum (Max).

Alternatively, high coefficients of variation (CV, Table 3) were observed particularly in tap water for all four analytes, while Diclofenac revealed high variability also in the remaining two matrices. As further discussed, this indicates that the highest uncertainty was found in the

determination of Diclofenac, without respect to the analytical method used. Additionally, the smallest number of outliers observed for this compound is also attributed to the high coefficient of variation. Indeed, as shown in Figure 2, the CV increases for the lowest concentration samples (tap water), but remains lower for Ibuprofen, probably as a consequence of the fact that the internal standard is Ibuprofen-d3. Therefore, a more suitable internal standard (e.g. isotopically labelled diclofenac) may improve method performance for Diclofenac.

Figure 2: CV plotted against the mean concentrations of tested compounds determined in wastewater, river water and tap water samples.

A comparison of the CV values (Figure 3) in both complex matrices, i.e. river water and wastewater, resulted in considerably higher CV in the samples analysed by LC-MS procedure, which again implies that the ion suppression affected the LC analysis. On the other hand, the GC-MS provided a high certainty in the analysis of Ibuprofen, Ketoprofen and Naproxen in both matrices, whereas, the determination of Diclofenac did not prove particularly consistent, regardless of the analytical procedure.

Figure 3: CV derived for LC-MS and GC-MS procedure. Top: wastewater; bottom: river water.

The ISO/DIS 13528 [16] classification of the laboratory biases resulted in the complete absence of "action signals" and one or less "warning signals" per series of results, which indicated that the calculated mean (X) and standard deviation (σ), with the underlying normal distribution, were good approximates for the true mean and the standard deviation values. The Pr(X) and Pr(M) values were derived for each analyte determined by the participating laboratories. These parameters describe a general capability of a laboratory to determine an analyte without respect to the tested matrix. Pr(X) and Pr(M) are plotted in Figure 4 a and b, respectively, where the outlier values are included in order to illustrate a full performance of laboratories [7]. Comparison of both figures reveals that Figure 4b, where Pr(M) is plotted for each laboratory, more significantly shows the differences in laboratory performance regarding each test compound. In addition, on the x-axes the analytical protocol is marked, which, in contrast with the results from the first exercise, shows a relatively good performance of GC laboratories and may be the consequence of method harmonisation, as explained before.

Figure 4: a) Bar-chart showing the Pr(X) values for each analyte measured by participating laboratories. b) Pr(M) results. The analytical procedure is indicated bellow laboratory ID.

Figure 4 also shows that one laboratory in particular has a high Pr(X) and Pr(M) values for three compounds, while the six laboratories (3, 4, 8, 10, 11 and 12) showed excellent method performance for all four analytes.

As C1 and C2 were split-level samples with respect to the concentration of ibuprofen (Table 3), a Youden graph was plotted from the reported results for C1 samples (x-axes) against those reported for C2 samples (y-axes, Figure 5). The median values for both samples are also plotted (dotted lines: x = 55 ng L^{-1}, y = 46 ng L^{-1}), where their intersection point is accepted as the most probable value [7]. The results show an excellent agreement with the spiking level for ibuprofen in the batch C, i.e. 50 ng L^{-1}.

Figure 5: Two-sample Youden plot for ibuprofen in C1 and C2 samples. The borderline represents 95 % around the origin of the plot.

Further, the three isolated points positioned in the upper right quadrant of the Youden plot (Figure 3) illustrate that the reported results were too high indicating a systematic error in these three laboratories [7,13]. Youden plots were plotted for all tested compounds in at least two series of samples. Since all the plots reveal similar outcomes only one representative plot is shown.

3.3 Filtration test

Filtration as a step in sample preparation process may cause two additional effects. First, depending on the analyte polarity and filter material, the analytes can adsorb to a filter, and consequently the concentrations determined in final samples are lower than the actual concentrations before filtration. On the other hand, by removing the organic matter present in matrix, the filtration may be a way to reduce the ion suppression effect and thus improve the LC-MS analysis.

In order to evaluate the effect of filtration the sample series '2' and '3' in each batch (A, B, C) were prepared in parallel. As indicated in Table 3, the participants were asked to filter samples in series '2' prior to SPE, while sample in series '3' were extracted without the pre-filtration. By statistical testing of the reported values it was shown that the samples were drawn from the same population, suggesting that the filtration had no effect on the analysis. As illustrated in Table 4, only in case of Naproxen in tap water a significant difference in variances was observed. However, this was not confirmed with the 'paired t-test' on sample

means and therefore it is concluded that filtration did not cause the difference in two-sample variances.

Table 4: Results on testing the effect of filtration

As the filter material was not predetermined in the analytical protocols at least four different types of materials were used in different laboratories: glass fibre, nitrocellulose membrane, nylon membrane, cellulose acetate and non-specified membrane filters. Among the twelve participating laboratories, seven used glass microfibre filters, while the remaining five used membrane filters. In order to test the influence of the filter material, F- test was applied to compare the variances between glass-fibre and membrane filtering in all the filtered samples (A1, A2, B1, B2, C1 and C2). It was proven that at a 95 % confidence level that the filter material had no impact on the analysis of NSAIDs.

By showing that filtration does not impact determination of the compounds that is no sorption on the filters was observed and the matrix effect was not reduced, the series of samples 2 and 3 (and all three series in case of Ibuprofen in tap water) were made possible to be compared on measures of precision. Obtained results are in agreement with published literature [24].

4 Conclusions

Twelve participants from eleven different European research institutes and universities took part in a 2nd Interlaboratory Exercise on the determination of selected NSAIDs in aqueous matrices. The 1st NORMAN Interlaboratory Exercise was a test round focusing on the stability of compounds during sample storage, whereas the 2nd round was based on two predetermined analytical protocols (LC-MS/MS and GC-MS). Further, the 2nd round specifically addressed the filtration and eliminated the weaknesses recognised in the 1st round. Thus, in contrast to the 1st round, the samples were shipped on dry ice and were extracted on arrival at the participating laboratories.

On the basis of the 1st and 2nd Interlaboratory Exercise we conclude that shipping samples on dry ice, as well as using a standard laboratory protocol contributed towards a reduced number of outliers and improved laboratory performance, particularly for GC analysis. Thus, the

distribution of the outliers between the GC and LC protocols is contrary to the results of the 1st round of the NORMAN Interlaboratory Exercise. However, as the outliers were distributed among only 5 participants this suggests that the performance of a single laboratory had a large impact on the final number of outliers. Another aim of the 2nd round was to test, whether the pre-filtration affected the determination of the analytes in the tested matrices. The results of the test implied that the filtration itself as well as filter material, did not affect the analysis of selected NSAIDs in none of the three tested matrices.

Within the 2nd round the two analytical protocols, LC-MS and GC-MS, are assessed according to their sensitivity and measurement uncertainty. On the basis of the results which included 7 LC based and 5 GC based results, GC-MS analytical procedure was proved superior for the analysis of ibuprofen, ketoprofen and naproxen in matrices with higher complexity. Higher uncertainty was found in the determination of diclofenac, without respect to the analytical method used. To verify the outcomes of this interlaboratory exercise an option would be to involve the higher number of participants.

Importantly, the results of the 2nd Interlaboratory Exercise are not directly comparable with the 1st Interlaboratory Exercise, especially not in terms of repeatability and reproducibility of results. The main reason is that in the 1st Inerlaboratory Exercise different matrices were spiked with the same amounts of analytes in order to confirm stability, while in the 2nd Interlaboratory round spiking and/or treatment of samples differed.

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Figure(s)

Figure 1

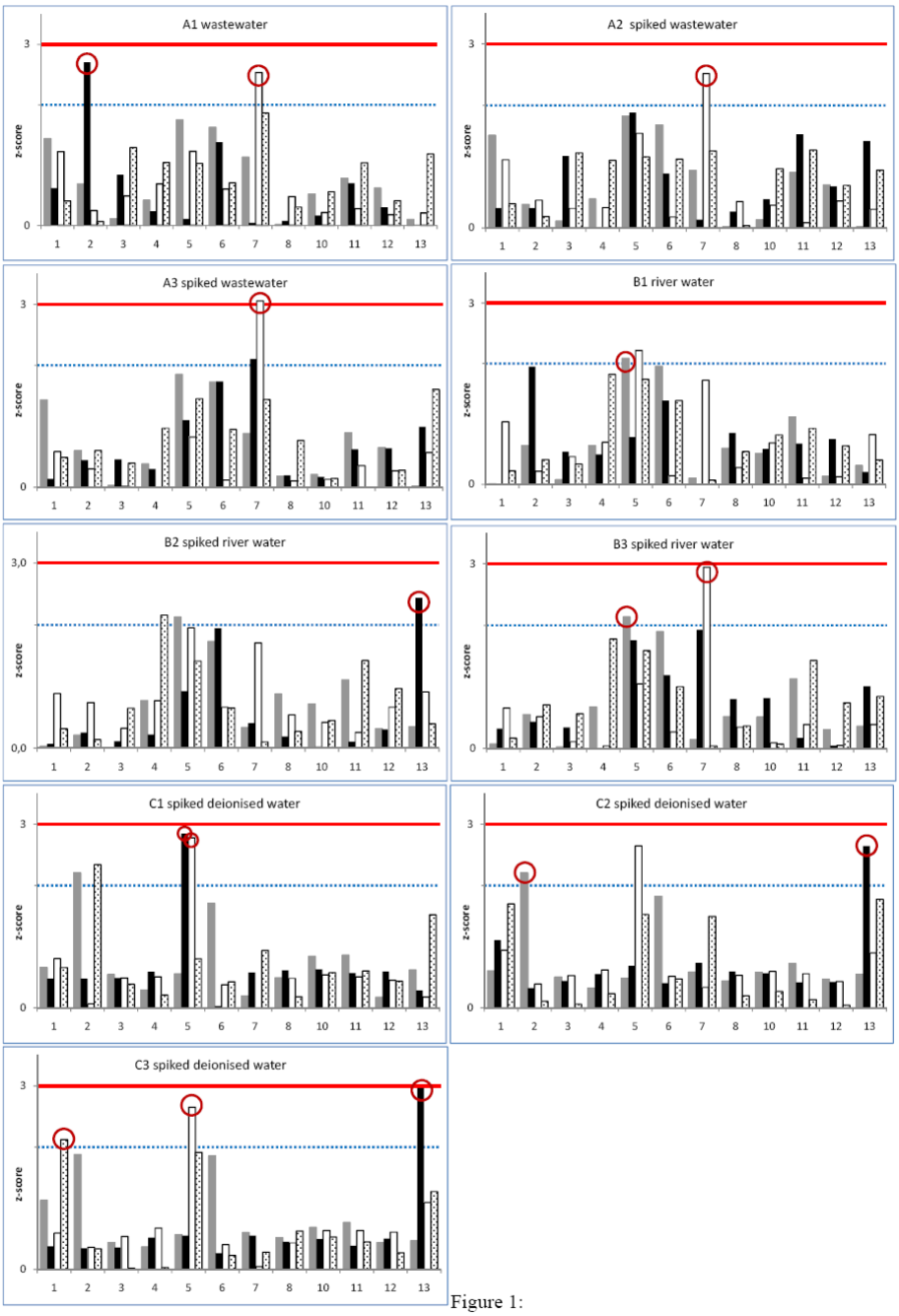
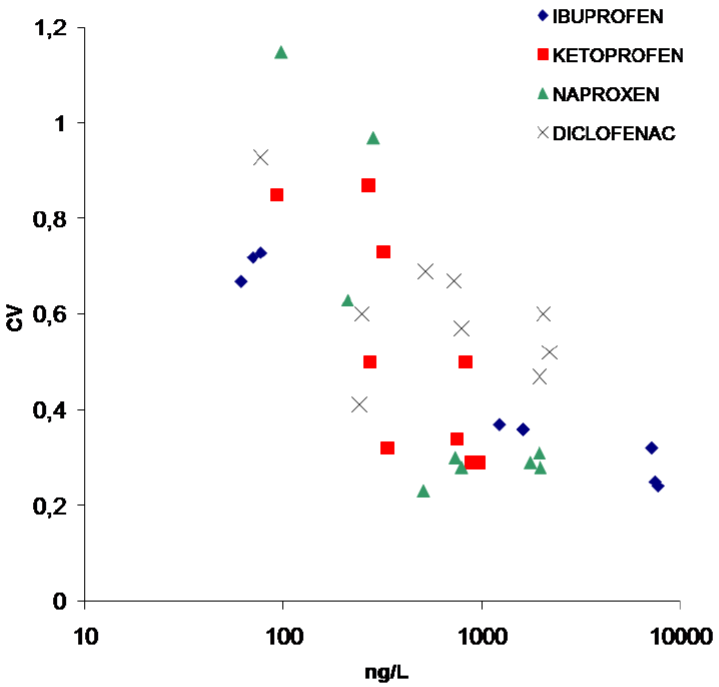


Figure 1:

Figure 2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NAPROXEN
DICLOFENAC
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0,4
0,2
0
CV
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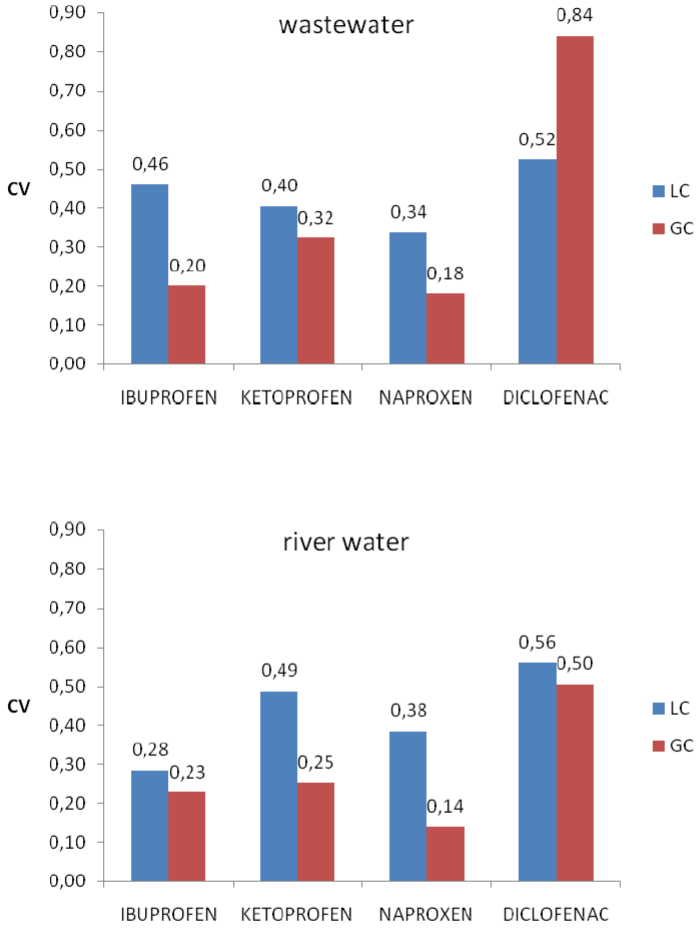
1000
10000
ng/L

Figure 3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0,30
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0,10
0,00
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0,20
0,40
0,32
0,34
0,18
0,52
0,84
IBUPROFEN
KETOPROFEN
NAPROXEN
DICLOFENAC
LC
GC
river water
CV
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0,23
0,49
0,25
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IBUPROFEN
KETOPROFEN
NAPROXEN
DICLOFENAC
LC
GC

Figure 4

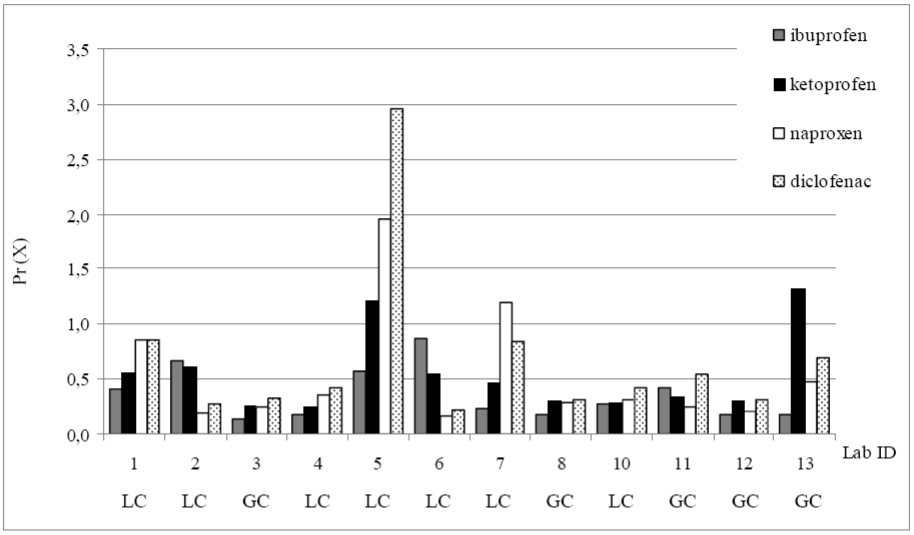


Figure 4a: Pr(X)

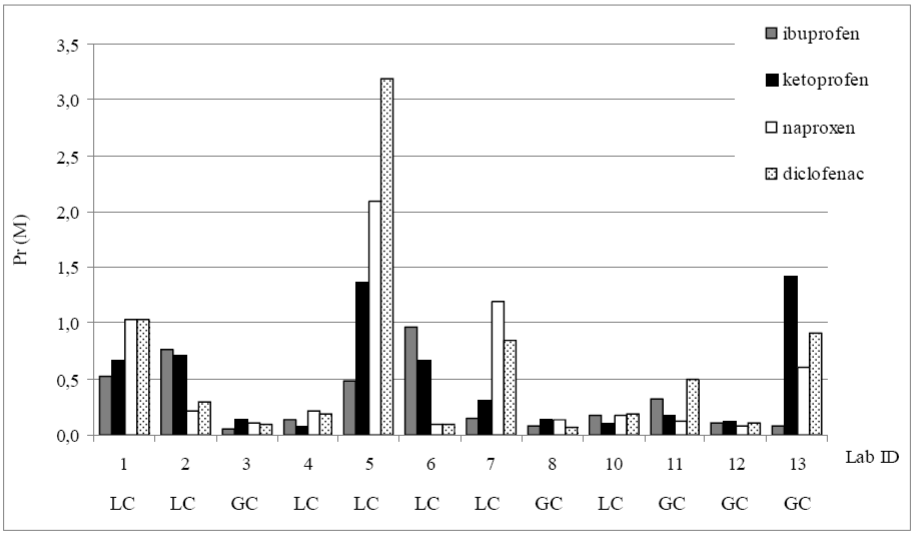
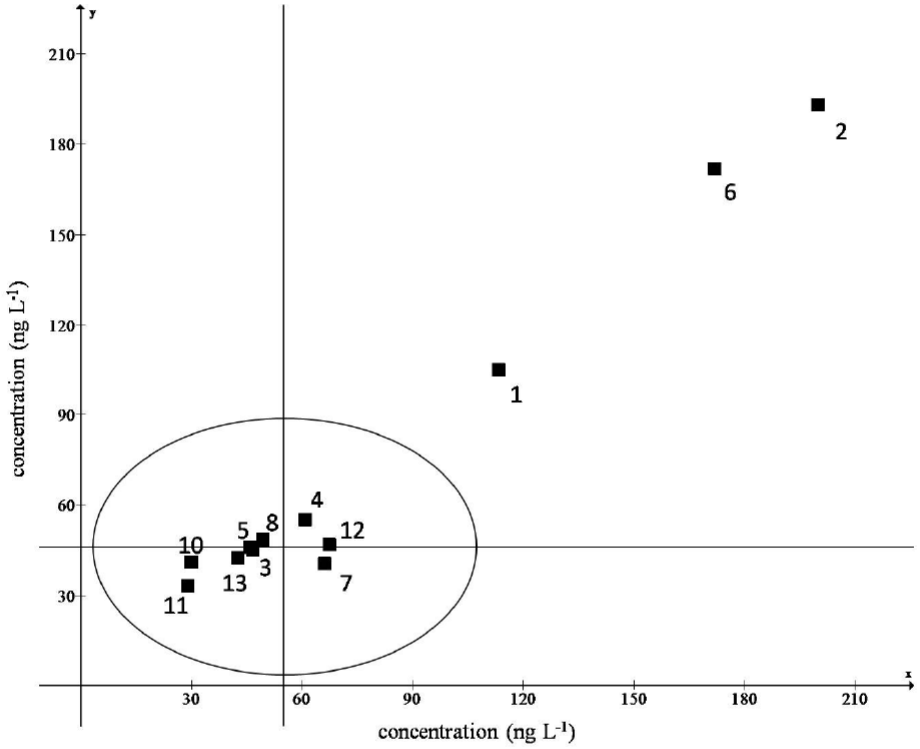


Figure 4b: Pr(M)

Figure 5


y
x
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180
150
120
90
60
30
30
60
90
120
150
180
210
concentration (ng L-1)
concentration (ng L-1)
1
2
3
4
5
6
7
8
10
11
12
13

Table 1:

SAMPLE CODE and MATRIX		
Batch A	**Batch B**	**Batch C**
A1 Natural wastewater	B1 Natural river water	C1 Spiked tap water
A2 Spiked wastewater	B2 Spiked river water	C2 Spiked tap water
A3 Spiked wastewater	B3 Spiked river water	C3 Spiked tap water

Table 2:

Outliers / sample matrix	**Classical approach**		**Robust approach**
wastewater	4 (0.9 %)		4 (0.9 %)
river water	4 (0.9 %)		4 (0.9 %)
tap water	7 (1.6 %)	**+3**	10 (2.3 %)
Outliers / analyte			
ibuprofen	3 (0.7 %)	**+3**	6 (1.4 %)
ketoprofen	5 (1.2 %)		5 (1.2 %)
naproxen	6 (1.4 %)		6 (1.4 %)
diclofenac	1 (0.2 %)		1 (0.2 %)
Outliers / analytical method			
LC-MS/MS	12 (2.8 %)	**+3**	15 (3.5 %)
GC-MS	3 (0.7 %)		3 (0.7 %)

Table 3:

Sample	Matrix	Spiking level (ng/L)	Filtration	No. acc. results	X	σ	CV	σ_M	M	Min	Max	No. outliers
IBUPROFEN (ng/L)												
A1		-	YES	12	1238	460	0,37	133	1265	433	1987	0
A2	wastewater	416	YES	12	1622	577	0,36	167	1668	570	2588	0
A3		416	NO	12	1620	586	0,36	169	1669	537	2633	0
B1		-	YES	11	7545	1853	0,25	559	7351	4500	11684	1
B2	river water	416	YES	12	7250	2302	0,32	665	7537	2358	11235	0
B3		416	NO	11	7791	1864	0,24	332	7663	4600	11891	1
C1		50	YES	12	77	56	0,73	16	55	29	200	0
C2	tap water	50	YES	11	61	41	0,67	12	46	33	172	1
C3		50	NO	12	70	50	0,72	14	47	31	571	0
KETOPROFEN (ng/L)												
A1		-	YES	11	334	108	0,32	33	350	111	520	1
A2	wastewater	790	YES	12	967	284	0,29	82	985	434	1400	0
A3		790	NO	12	830	416	0,50	120	905	107	1705	0
B1		-	YES	10	269	234	0,87	74	147	69	725	0
B2	river water	790	YES	11	754	259	0,34	78	812	91	997	1
B3		790	NO	12	886	261	0,29	75	893	428	1389	0
C1		47	YES	11	93	79	0,85	24	40	30	217	1
C2	tap water	205	YES	11	319	231	0,73	70	248	123	854	1
C3		205	NO	11	273	136	0,50	41	230	170	571	1
NAPROXEN (ng/L)												
A1		-	YES	11	507	115	0,23	35	510	325	675	1
A2	wastewater	412	YES	11	791	224	0,28	67	808	332	1022	1
A3		412	NO	11	737	220	0,30	66	742	317	1030	1
B1		-	YES	12	1754	516	0,29	149	1825	609	2646	0
B2	river water	412	YES	12	1956	608	0,31	175	1976	771	2993	0
B3		412	NO	11	1978	563	0,28	170	1977	852	2925	1
C1		45	YES	10	97	111	1,15	35	46	26	388	1
C2	tap water	120	YES	12	283	276	0,97	80	154	113	1014	0
C3		120	NO	11	210	132	0,63	40	167	111	516	1
DICLOFENAC (ng/L)												
A1		-	YES	12	521	357	0,69	103	586	59	1186	0
A2	wastewater	523	YES	12	730	487	0,67	141	693	110	1341	0
A3		523	NO	11	796	452	0,57	136	860	71	1444	0
B1		-	YES	12	1959	924	0,47	267	1887	352	3640	0
B2	river water	523	YES	12	2054	1234	0,60	356	2030	300	4715	0
B3		523	NO	12	2216	1152	0,52	332	2284	386	4262	0
C1		63	YES	12	77	71	0,93	21	48	10	243	0
C2	tap water	220	YES	12	250	149	0,60	43	245	22	515	0
C3		220	NO	11	244	101	0,41	30	233	21	433	1

Table 4:

	test significance		
tested samples	**F-test**	**t-test**	**ANOVA**
IBUPROFEN			
A2 / A3	NO	NO	
B2 / B3	NO	NO	
C1 / C2 / C3			NO
KETOPROFEN			
A2 / A3	NO	NO	
B2 / B3	NO	NO	
C2 / C3	NO	NO	
NAPROXEN			
A2 / A3	NO	NO	
B2 / B3	NO	NO	
C2 / C3	YES	NO	
DICLOFENAC			
A2 / A3	NO	NO	
B2 / B3	NO	NO	
C2 / C3	NO	NO	

3.2 Removal and effects of pharmaceuticals in water treatment

The experiments described under this chapter, and, in part, also in the two subsequent ones, involved setting up a series of laboratory-scale bioreactors with activated sludge wastewater treatment technology. The following are the objectives of the bioreactor operation and involve studying the following:

- pharmaceutical cycling during activated sludge treatment,
- the formation of biodegradation products for their later identification and toxicity assessment,
- the biodegradation pathways of pharmaceuticals,
- the effect of bioreactor operational conditions (HRT, nutrient addition, aeration) on the removal of pharmaceuticals and the production of biotransformation products,
- the possibility of sequential coupling of activated sludge treatment with AOP to improve the removal of pharmaceuticals and their TPs, and
- the effects of pharmaceuticals on the microbial community structure in the activated sludge.

The laboratory scale bioreactors were operated in parallel, where the operational conditions were adopted depending on the objectives of individual experiments. The pharmaceuticals or TPs were added into the bioreactor inflow, except for the control bioreactor, which was fed solely with the artificial wastewater, without the addition of test compounds. Primarily, the removal of the selected pharmaceuticals (NSAIDs and CLA) by the activated sludge treatment was evaluated and compared with the removal of these compounds in real municipal WWTPs (data are collected in Figure 3). The removal of readily biodegradable pharmaceuticals (IB, KP and NP) was slightly better (5 - 20 %) than that reported by several authors [4,16,17,19,47,52,121,122,123] for actual activated sludge WWTPs. The superior removal of these three compounds in the bioreactors can be explained by the acclimatization of the activated sludge to the substrate pharmaceuticals, as well as the relatively long HRT of 48 hrs, in comparison to actual WWTPs which normally apply a HRT of 15 hrs. In contrast to IB, KP and NP, the elimination of DF and CLA was found to be relatively poor, agreeing with data presented in Figure 3. As reported by Kimura et al. [126] CLA and DF were neither sufficiently removed by conventional active sludge process, nor by MBR, which was attributed to the presence of chlorine in their chemical structures [126]. Further, the elimination of CBZ, which was studied in the frame of later experiments [218], was within the range of the elimination rates determined for actual WWTPs (Figure 3).

The efficiency of wastewater treatment largely depends on the bacterial diversity present in a WWTP [234]. Therefore, a fundamental understanding of the structure of the microbial community and stability as well as its response to different contaminants in the wastewater is needed for stable and efficient WWTP operation. To our knowledge, there is no study addressing the influence of pharmaceutical residues in wastewater on the bacterial community present in activated sludge. Therefore, the changes in bacterial communities in activated sludge that were caused by long-term exposure to NSAIDs and CLA were investigated. The studies based on the analyses of T-RFLP profiles and clone libraries, and compared the activated sludge subjected to the presence of pharmaceuticals with activated sludge from a control bioreactor without the addition of pharmaceuticals. NSAIDs and CLA concentrations of 50 μg L^{-1} caused a shift in the structure of activated sludge bacterial communities and reduced the microbial diversity. In the reactors operated with higher concentrations of pharmaceuticals, i.e. at 200 and 500 μg L^{-1}, greater structural divergence was observed. Further, the inability to detect *Nitrospira* in the reactor with the addition of pharmaceuticals suggests an important effect on bacteria, which play a key role in the second stage of nitrification in a WWTP. Even though the concentrations of pharmaceuticals that caused effects on microbial community structure were higher than those usually determined in municipal WWTP (Table 5), the results of this research may apply, when considering the treatment in hospital or industrial plants. Nevertheless, because there exist no regulatory requirements to monitor the emissions of pharmaceuticals from WWTPs, their concentrations are only sporadically being determined, and mostly on municipal WWTPs, while hardly any data reported the occurrence of pharmaceuticals in manufacturing or hospital plants.

The results of the experiments described above are presented in two papers:

- Removal of pharmaceutical residues in a pilot wastewater treatment plant (Analytical and Bioanalytical Chemistry, 2007)
- Influence of pharmaceutical residues on the structure of activated sludge bacterial communities in

wastewater treatment bioreactors (Water Research, 2008)

3.2.1 Scientific paper: “Removal of pharmaceutical residues in a pilot wastewater treatment plant”

Anal Bioanal Chem (2007) 387:1379–1387
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ORIGINAL PAPER

Removal of pharmaceutical residues in a pilot wastewater treatment plant

Tina Kosjek · Ester Heath · Boris Kompare

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Abstract Concern is growing over the contamination of the environment with pharmaceutical residues, among which non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most abundant groups. Their widespread appearance in the aquatic environment is because of their high consumption and their incomplete removal during wastewater treatment. Because effective operation of wastewater-treatment plants is important for minimising the release of xenobiotic compounds, for example pharmaceutical products, into the aquatic environment, our study focuses on removal of commonly used NSAIDs (ibuprofen, naproxen, ketoprofen, diclofenac) and clofibric acid in a specially designed small-scale pilot wastewater treatment plant (PWWTP). This study shows that, except for diclofenac, steady-rate removal of NSAIDs over a two-year monitoring period has been achieved. Elimination of the compounds in the PWWTP was ≥87% for ibuprofen, naproxen and ketoprofen but only 49–59% for diclofenac. We also studied clofibric acid. Results after one month of operation revealed 30% elimination with no sign of adaptation by the biomass. Also described are degradation products of diclofenac, which we were able to identify because of the similarity of their mass spectra with those in the NIST library and by comparing the retention times of different compounds. Although the structures of these compounds were confirmed with a high probability (99%), we still need to compare the fragmentation of authentic compounds with degradation products formed under our experimental conditions. Degradation products of ibuprofen, naproxen, ketoprofen, and clofibric acid were found but these must be identified by use of high-resolution mass spectrometry and analysis of authentic compounds.

Keywords Pilot wastewater treatment plant · Ibuprofen · Ketoprofen · Naproxen · Diclofenac · Degradation products

T. Kosjek
Haslauer d.o.o.,
Zabukovica 87, 3302 Griže, Slovenia

T. Kosjek · E. Heath (✉)
Department of Environmental Sciences, Jožef Stefan Institute,
Jamova 39,
1000 Ljubljana, Slovenia
e-mail: ester.heath@ijs.si

T. Kosjek · B. Kompare
Institute of Sanitary Engineering,
Faculty of Civil and Geodetic Engineering,
University of Ljubljana,
Hajdrihova 28,
1001 Ljubljana, Slovenia

Introduction

Pharmaceutical products are, by design, biologically active substances, and although amounts released to the environment are low, their continuous input may lead to chronic low level exposure and accumulation with potential negative effects on man and the environment. Further, possible bioaccumulation in the food web and chronic toxicity because of synergistic or additive effects should be considered when assessing the risks arising from their presence in the environment [1, 2].

The main sources of pharmaceutical residues in the environment are human medicine, the pharmaceutical industry, and veterinary medicine. Discharge of therapeutic agents in effluents from production facilities, hospitals, and private households, improper disposal of unused drugs, and direct discharge of veterinary medicines, lead to contamination of surface water, ground water, and, eventually, drinking water, where they pose a potential danger to

humans [3]. Pharmaceutical products are released into the environment either as the parent compound or as active/inactive metabolites. Often, therefore, not only the parent compounds should be the subject of risk assessment but also their active metabolites [2, 3].

Commonly used pharmaceuticals have been detected in wastewater, surface water, and even in drinking water [4–11]. The factors affecting their occurrence in the environment are their overall consumption and the fate of individual compound in the human or animal body, in wastewater treatment plants, and within the aquatic environment itself. Thus, besides consumption, information on the biodegradability, metabolic pathways, conjugation and de-conjugation, sorption, and persistence are needed to predict their occurrence and fate in the environment [12]. Further, by recognizing the possibilities of their (bio) degradation in real wastewater treatment plants (WWTP) it is possible to limit or prevent their entrance into the environment, and consequently, reduce the risk arising from their presence in the environment.

The objective of our study was to establish and/or improve possible technology for removal of pharmaceutical product residues in WWTP. To achieve this we designed a pilot wastewater treatment plant (PWWTP) using activated sludge obtained from a Slovenian municipal WWTP. In the PWWTP we studied the removal and degradation of pharmaceutical products using an analytical procedure developed previously in our laboratory [4]. The compounds involved in our study were acidic pharmaceuticals (Fig. 1)—four nonsteroidal anti-inflammatory drugs (NSAIDs: ibuprofen, ketoprofen, diclofenac, and naproxen) and clofibric acid (CLA), an active metabolite of clofibrate, a drug used as a blood-lipid regulator. The model compounds were selected on the basis of their widespread use in Slovenia [13–15] and Central Europe [9, 16], their reported ecotoxicity [17–19], and their persistence in WWTP or in the aquatic environment [20–23].

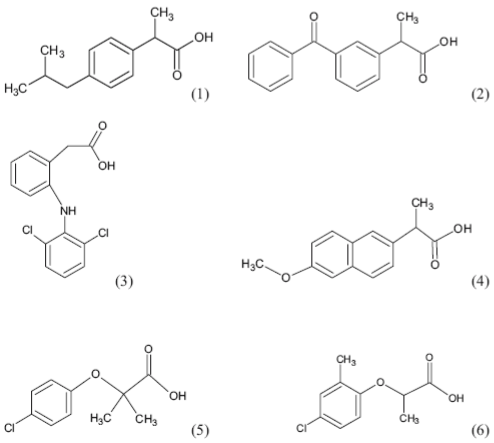


Fig. 1 Chemical structures of the compounds investigated: *1*, ibuprofen; *2*, ketoprofen; *3*, diclofenac; *4*, naproxen; *5*, clofibric acid; and *6*, mecoprop (internal standard)

Our study also focused on detection of the drug metabolites or degradation products which can be generated when a pharmaceutical compound is digested in the human/animal organism or, later, when the drug or metabolite is a substrate in several biotic or abiotic processes in the environment, including treatment in WWTPs.

Experimental

Chemicals

Sigma–Aldrich (Gillingham, UK) supplied all the drugs investigated (ibuprofen (CAS 15687-27-1) diclofenac sodium salt (CAS 15307-79-6), naproxen (CAS 22204-53-1), ketoprofen (CAS 22071-15-4), and clofibric acid (CAS 882-09-7)) and the derivatisation agent MSTFA (*N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide). Mecoprop (2-(4-chloro-2-methylphenoxy) propanoic acid), which was added as an internal standard before solid phase extraction to compensate for possible losses during sample preparation and analysis, was obtained from Labor. Dr. Ehrenstorfer-Schäfers (Ausburg, Germany). Methanol (MeOH), toluene, and 37% hydrochloric acid (HCl) were of analytical grade and were obtained from Merck (Darmstadt, Germany).

Artificial waste water was prepared by dissolving a nutrient-mineral composition in tap water. The chemicals used were: yeast extract (130 mg L^{-1}), casein peptone (130 mg L^{-1}), meat extract (130 mg L^{-1}), CH_3COONH_4 (317 mg L^{-1}), NH_4Cl (40 mg L^{-1}), K_2HPO_4 (24 mg L^{-1}), KH_2PO_4 (8 mg L^{-1}), $CaCO_3$ (100 mg L^{-1}), $MgCO_3$ (100 mg L^{-1}), NaCl (40 mg L^{-1}), and $FeSO_4.7H_2O$ (5 mg L^{-1}). CH_3COONH_4, NH_4Cl, KH_2PO_4, $MgCO_3$, and $FeSO_4.7H_2O$ were obtained from Merck, casein peptone, meat extract, and yeast extract from Fluka Chemie, K_2HPO_4 from Kemika (Zagreb, Croatia), $CaCO_3$ from Carlo Erba (Milano, Italy), and NaCl from Riedel-de Haën (Seelze-Hannover, Germany).

Pilot wastewater treatment plant configuration

To reduce costs the reactors were designed to be as small as possible but with dimensions that enabled installation of equipment (aerators, pumps) and maintenance (cleaning, repair). Standard aquarium aerators, i.e. a membrane pump with a porous stone, were used as aerators and a standard

aquarium centrifugal mixing pump was adopted for the recycle and return sludge pump. Because of the dimensions of the pumps and to allow maintenance, the total reactor volume was 4.7 L, of which the reactor wetted volume was 4.0 L. From the food to biomass ratio (*F*/*M*) and from the residence times common in municipal wastewater plants, we calculated a minimum retention time of 12 h and a maximum of 48 h (2 days). For the sake of simplicity and for the first series of experiments we selected a daily feed rate of 2.0 L per day, with a residence time of 48 h. The designed PWWTP consisted of three parallel reactors. Two reactors, R1 and R2, operated under continuous input of high (R1, 0.05 mg L^{-1}) and low (R2, 0.005 mg L^{-1}) concentrations of the selected NSAIDs and CLA. R0 served as a control in our PWWTP and was therefore analyzed with the same frequency as R1 and R2; R0 was also used to validate the analytical method. An additional reason for using the R0 model reactor in PWWTP was to follow biomass adaptation during exposure to NSAIDs at two different concentrations (in R1 and R2). For this purpose a separate study (including electron microscopy, PCR, ...) is being conducted and will be reported separately.

The efficiency of elimination in R1 and R2 was determined from the difference between the influent and effluent concentrations of the compounds. The rate of removal of a single compound was determined by use of Eq. (1), where % *el.eff.* represents the elimination efficiency (%), $c_{\text{X-INF}}$ is the concentration of a compound in the influent, and $c_{\text{X-EFF}}$ is the concentration of the same compound in the effluent.

$$\%\,el.eff. = \frac{(c_{\text{X-INF}} - c_{\text{X-EFF}})}{c_{\text{X-INF}}} \times 100 \qquad (1)$$

The reactors were operated at an average temperature of 22 °C (±5%) and at pH 7.7 (±2%) (influent) and pH 7.3 (±5%) (effluent).

Figure 2 shows the layout of the reactors in the PWWTP. Each reactor consisted of a selector, an anaerobic chamber, an aerobic chamber, and a sedimentation basin (Fig. 3). For PWWTP start-up we used activated sludge from a Slovenian municipal WWTP, which we kept active throughout operation of the PWWTP. During two years of operation we also studied the microbiological composition and adaptation of the biomass. The results will be presented separately.

Pharmaceutical test solutions

Two reactors (R1 and R2) operated under continuous input of NSAID and CLA (R1, 0.05 mg L^{-1}; R2, 0.005 mg L^{-1}) and R0 served as a control. Removal of the NSAIDs in the reactors was followed for two years. CLA was added in the

Fig. 2 Experimental set-up of the three pilot WWTP reactors (R0, control; R1 and R2, 0.05 and 0.005 mg L^{-1}, respectively, of the pharmaceutical mixture)

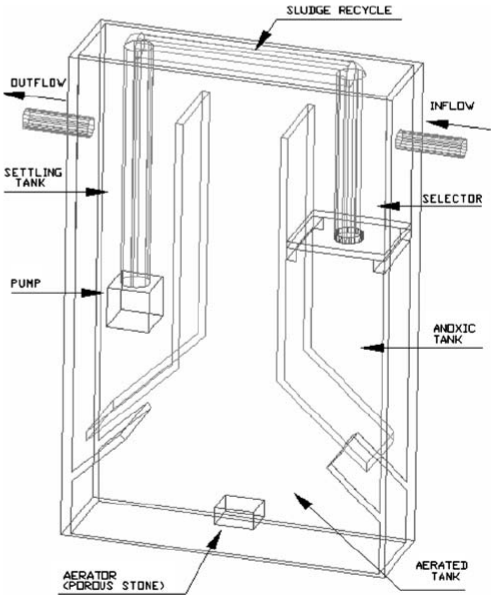


Fig. 3 Three-dimensional schematic diagram of the pilot reactor showing inflow, selector, anoxic/anaerobic tank, aerated tank, settler with sludge return to aerated tank, and sludge recycle

last month only. A stock solution of a mixture of pharmaceutical compounds (0.25 mg mL^{-1} of each of ibuprofen, ketoprofen, naproxen, diclofenac, and clofibric acid) was prepared in methanol and 1 mL of the stock (0.25 mg mL^{-1}) solution was diluted to 5 L with nutrient-mineral medium and used as the R1 reactor solution with a daily feed rate of 2.0 L. Similarly, for reactor R2 0.1 mL of the stock solution was diluted to 5 L of the mineral-nutrient medium. The effect of methanol added (a total of 1 mL or 0.1 mL per 5 L medium) was neglected. The control reactor (R0) was supplied with artificial wastewater without addition of the pharmaceutical products.

Sampling

Each PWWTP influent (influents R0, R1, and R2) and effluent (effluents R0, R1, and R2) was sampled every seven days to determine the amounts of the compounds removed and the continuity of reactor operation for the first 6 months of continuous operation of the PWWTP. After this period sampling was performed on a monthly basis. Each 0.2 L sample was filtered (0.45 μm filter; Sartorius, Goettingen, Germany), to remove suspended matter, acidified to pH 2.6 with 37% HCl (to inhibit further biological degradation and to enhance trapping of the acidic compounds on the solid phase extraction (SPE) adsorbent), and stored at 4 °C until solid phase extraction (SPE).

Solid phase extraction

SPE was performed to concentrate the sample and change the matrix, and thus prepare it for further analysis, and for sample clean-up. Commercially available 3 mL SPE cartridges containing 60 mg Strata X 33 μm (surface modified styrene–divinylbenzene polymer) adsorbent (Phenomenex, Torrance, ZDA), were used. SPE was performed using a 12-fold vacuum extraction box (Supelco, Bellefonte, USA). The SPE cartridges were first conditioned with 1.5 mL MeOH and then washed with 1.5 mL aqueous HCl solution at pH 2.6 [4]. This was followed by sample loading at a flow rate of 2 mL min^{-1}. After the enrichment step the cartridge was dried for 1 min under vacuum (approx. ~0.5 bar). The analytes were eluted with MeOH (3×0.5 mL). The eluent was collected in a 1.5 mL glass vial and evaporated to dryness under a stream of nitrogen. The residues were dissolved in 0.5 mL toluene (influents) or 0.2 mL of toluene (effluents) and derivatised by adding 30 μL of derivatising agent MSTFA [24]. The samples were first placed for 5 min on a shaker (Veb MLW Labortechnic, Ilmenau, Germany) and then left in an oven (Sterimatic ST-11, Instrumentaria, Zagreb, Croatia) at 60 °C for 1 h to complete the derivatisation.

Gas chromatography–mass spectrometry

The derivatised drugs were determined by GC–MSD on an HP 6890 instrument (Hewlett–Packard, Waldbron, Germany) fitted with a 30 m×0.25 mm×0.25 μm HP-5 MS capillary column (Hewlett–Packard). The carrier gas was helium at a constant velocity of 37 cm s^{-1}. Injection was performed in the splitless mode at an injection temperature of 250 °C. The injection volume was 1 μL. The GC oven temperature was maintained at 100 °C for 2 min, then programmed at 4° min^{-1} to 180 °C, then at 10° min^{-1} to 230 °C (held for 5 min), and then at 20° min^{-1} to 270 °C, which was held for 7 min. Mass spectra were obtained in electron-impact mode (electron energy 70 eV). Detection was performed in full-scan mode in the mass range 50–500 m.u. (the transfer line temperature 280 °C) for study of (bio)degradation products and in SIM mode with characteristic ions for each compound for quantitative analysis of NSAID and clofibric acid residues [4].

Performance of the analytical procedure

The analytical method used for determination of nonsteroidal anti-inflammatory drugs is described in detail elsewhere [4]. The method was later optimised and adopted for use with wastewater. To shorten the time required for sample preparation and analysis, reduced sample volumes (200 mL) were used and the derivatisation time was reduced from 12 h at room temperature to 1 h at 60 °C. Further, to improve the detection limit, effluent extracts were dissolved in a smaller volume (200 μL) of toluene.

For SPE recovery studies, R0 effluent was spiked with a standard solution of the analytes at a concentration of 20 μg L^{-1}. Separately; extracts of the water matrices were also spiked at the same concentrations. Average recovery for three replicates was: ibuprofen 93±8%, naproxen 104±4%, ketoprofen 114±6%, and diclofenac 93±5%. Recovery of clofibric acid was not tested.

Limits of detection (LOD) were determined as three times the standard deviation of the blank (R0) and were 3 to 114 ng L^{-1} for effluent and 3 to 34 ng L^{-1} for influent. Limits of quantification (LOQ; 10 times the standard deviation of blank) were from 11 to 380 ng L^{-1} for effluent and 9 to 114 ng L^{-1} for influent.

Because of their similar chemical structures the same analytical procedure [4] was suitable for determination of the NSAIDs and for CLA, a compound which was incorporated later in the study. All model compounds were free acids (Fig. 1), as also was the internal standard (mecoprop, CAS 93-65-2, Fig. 1) used for quantitative determination. Achiral clofibric acid and the enantiomeric herbicide mecoprop are structural isomers which can occur together in environmental waters. The compounds were, however, successfully sepa-

rated and quantified by GC–MSD, by use of the method described above. The EI mass spectra of their trimethylsilyl esters contained significant ionic fragments with the same *m/z* ratios, but the abundances were different for the two compounds. The compounds also had different retention times.

We performed two experiments to ensure mecoprop was not present in our PWWTP. Influent from the WWTP that provided the activated sludge is regularly checked in our laboratory for organic contaminants including mecoprop. Also, R0 influent and effluent were carefully monitored for the presence of mecoprop; results were negative for every test performed. We therefore accepted that mecoprop was not present in the activated sludge used. To monitor surface and real wastewater, however, we have already replaced mecoprop with ibuprofen-D3 and so to reduce costs mecoprop is used in the PWWTP only.

Results and discussion

Elimination of selected pharmaceuticals in PWWTP

The efficiency of elimination of NSAIDs and CLA in the PWWTP was calculated by use of Eq. (1) and compared for R1 and R2. The results (Table 1) show removal of ibuprofen, ketoprofen, and naproxen is high and constant (>87%) after continuous operation for 2 years. In contrast, efficiency of elimination of diclofenac was smaller (49–59%), with a larger standard deviation.

Comparison of removal in the two reactors fed with different concentrations of pharmaceutical products revealed elimination efficiency was higher in reactor R1. The effluent concentration of every compound was, furthermore, comparable for reactors R1 and R2, which means the rate of removal was high at the higher concentration but stopped at the same minimum concentration (between 0.1 and 0.4 μg L^{-1}) in both reactors. From this we can conclude that abiotic/biotic processes are capable of removing a substance to a minimum concentration despite different initial NSAID concentrations. One possible explanation is that diffusion of the NSAIDs ceases at a particular low concentration, possibly because of insufficient contact between microbes and the nutrient–mineral medium containing dissolved NSAIDs, preventing further penetration into the cells. Another possibility is that the settling biomass is disturbed by the aquarium pump, causing some of the biomass to escape through the outflow in the effluent. The sampled biomass may contain adsorbed or absorbed NSAIDs which, after acidification and filtration, return to the water phase and are determined as non-eliminated NSAID. Further research will be needed to confirm this hypothesis.

Table 1 Average removal of the selected compounds, with standard deviations, in reactors R1 and R2 (n=31 for R1 and 29 for R2)

Pharmaceutical	Reactor	% el.eff	Standard deviation (%)
Ibuprofen	R1	90.8	13
	R2	91.5	6
Naproxen	R1	93.6	8
	R2	86.6	11
Ketoprofen	R1	91.1	10
	R2	89.6	7
Diclofenac	R1	59.3	25
	R2	49.0	32

As hydraulic retention time (HRT) could greatly affect biodegradation, it was selected to be as long as possible, because of the consumption of artificially prepared food, i.e. lower food consumption means longer HRT. The largest HRT was designed to be 48 h, which means a feed of 2.0 L day^{-1}, although any shorter HRT may be set. Ordinary medical dosing apparatus was used for infusion (Fig. 2); this enabled small manipulation for regulation of HRT. During the design phase the sludge residence time (SRT) was foreseen to be 15–25 days. In the beginning, when the sludge was suspended in the form of flock, it was easy to maintain this SRT. Later, when the sludge developed a more filamentous and attached form, we stopped directing the sludge to waste to achieve the designed SRT. Instead, natural die-off of the sludge biomass was washed out with the outflow. From the concentration of suspended sludge at the outflow it is estimated the effective SRT was more than 100 days.

Besides biodegradation, this study also suggests other removal mechanisms, for example abiotic degradation or adsorption by activated sludge and/or on the reactor walls. At the end of the experiment, the reactor walls, tubes and other surfaces in contact with the analytes, including the activated sludge, will be extracted and analysed for adsorbed analytes. The examined compounds are polar, however; we thus expect them to be highly mobile in water and expect losses because of adsorption to be insignificant. Temperature could also be a factor affecting the rate of (bio) degradation, including abiotic thermal degradation. Because no substantial fluctuations in temperature were recorded during the first year of operation, however, this was not considered. As already mentioned, CLA was included in our study only recently, so preliminary rates of removal only are presented in this paper. Average elimination of clofibric acid 9 days after its introduction was 29%. Clofibric acid is known to be highly resistant to biodegradation. According to the literature [12, 20, 25], substantially greater removal than that stated above is not expected, even after a greater biomass adaptation time. A biomass adaptation time of several weeks to months is needed to

achieve stable CLA removal, however. If the elimination of CLA is not significantly higher after this time, additional treatment methods (ozonation, UV, or H_2O_2 treatment, nanomembrane filtering, ...) will be used to increase elimination of this compound in the PWWTP.

During six months of PWWTP operation visible biomass adaptation occurred. An initial study with electron microscopy revealed that attached fungal cultures and relatively small numbers of bacteria and protozoans adapted to the conditions in our PPWTP. Also, no significant differences were observed between the reactors, even though, at the very least, differences were expected between R0 (control) and the other two reactors (R1 and R2) that were exposed to the NSAIDs. Further investigation of the biomass with a polymerase chain reaction (PCR) are in progress, however, and will be reported separately.

As mentioned above, removal of diclofenac was less efficient than removal of the other three NSAIDs (ibuprofen, ketoprofen, naproxen). A high standard deviation was obtained for the average rate of elimination of this compound (Table 1), indicating its removal was unstable. This behaviour may be a consequence of the different chemical structures of the compounds (Fig. 1). All the NSAIDs are arylalkanoic acids but only diclofenac contains two chlorine atoms and an NH bridge in the hydrocarbon structure. Greater resistance of diclofenac to biodegradation has also been reported in the literature [25]. In addition, photodegradation of diclofenac [22, 26, 27] and oxidation by ozone and H_2O_2 [28] have been reported to be successful.

Identification of NSAID degradation products

In the total-ion chromatograms (TIC) obtained from influent and effluent samples we observed chromatographic peaks possibly originating from NSAID degradation. Figure 4 shows a total ion chromatogram obtained from an effluent sample. The TMS (trimethylsilyl) derivatives of the NSAIDs are labelled, as also are ibuprofen, naproxen, ketoprofen, and diclofenac degradation products.

On the basis of similarity between mass spectra (mass fragments and their abundance) we identified and explained the origin of the newly generated compounds. In determination of the chemical structures of degradation products the match with data from the NIST (National Institute of Standards and Technology) library and the match of retention times of a single chromatographically separated compound detected in different samples were both considered. Figure 5 shows the mass spectrum of the suggested degradation product of diclofenac, 1-(2,6-dichlorophenyl)-1,3-dihydro-2*H*-indol-2-one (CAS: 015362-40-0), with the characteristic fragment ions identified. Comparison of the mass spectrum of this degradation product with that of the parent compound (Fig. 6) shows that the first mass spectrum lacks the *m*/*z* 73 fragment ion, which originates from trimethylsilyl derivatives [24], and therefore implies that this compound does not contain a free carboxyl or hydroxyl group. According to the mass spectra the compound was most possibly generated from diclofenac by elimination of water and formation of an amide bond.

Other degradation products were studied in a similar manner. The compounds identified included 2-((2,6-dichlorophenyl)amino)benzyl alcohol and its methyl ether, which are also degradation products of diclofenac. The chemical structures of these compounds are illustrated in Fig. 7. A chemical structure for a degradation product of naproxen is also suggested, (6-methoxy-2-naphthyl)ethanol; this was identified in the same way as the degradation products of diclofenac.

In addition to the degradation products identified (Fig. 7) we suggested origins of several compounds, e.g. derivatives of ibuprofen, ketoprofen, clofibric acid, naproxen, and diclofenac, as indicated in Fig. 4. The degradation products are shown in this paper as evidence of incomplete

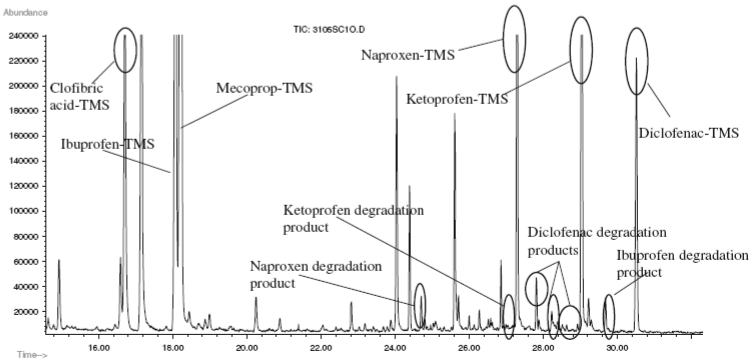


Fig. 4 Total-ion chromatogram obtained from PWWTP effluent (mecoprop was used as internal standard)

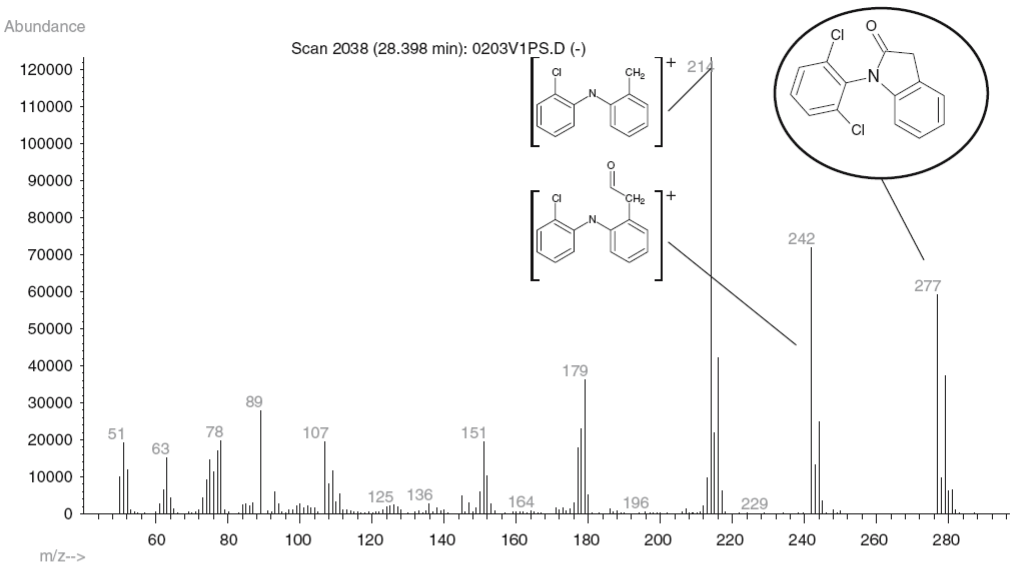


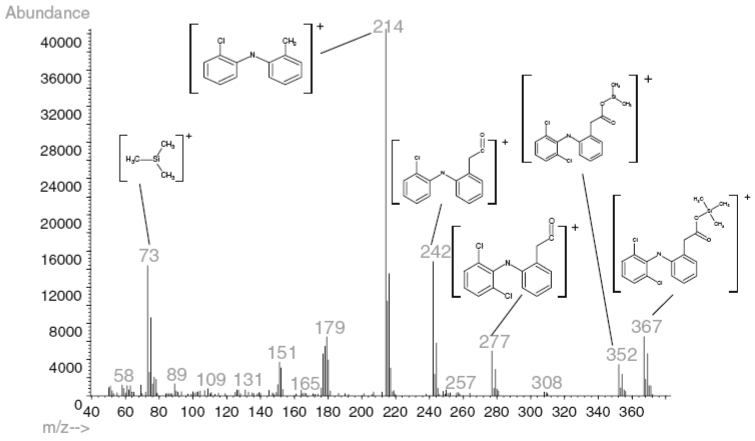
Fig. 5 Mass spectrum obtained from diclofenac degradation product (2,6-dichlorophenyl)-1,3-dihydro-2H-indol-2-one

elimination of the pharmaceutical products. Another study on the degradation products alone will be reported separately.

To determine the exact chemical structures of the degradation products, however, and to confirm their structures, high-resolution mass spectrometry will be used. The only commercially available standard is (2,6-dichlorophenyl)-1,3-dihydro-2*H*-indol-2-one (CAS: 15362-40-0), the structure of which had already been confirmed with 99% probability by use of the NIST Library. Although the structure of the compound was confirmed by finding a match in the NIST Library we still need to compare fragmentation of authentic standards and the degradation products. The degradation products, which are not commercially available, will be synthesised to confirm their identity and enable their quantitative determination.

Most of the degradation products detected seem to have resulted from the aerobic conditions in the reactor. Anoxic conditions (dissolved O_2 <1 mg L^{-1}) were not achieved with this design of PWWTP because the return flow was

Fig. 6 Mass spectrum obtained from diclofenac trimethylsilyl ester



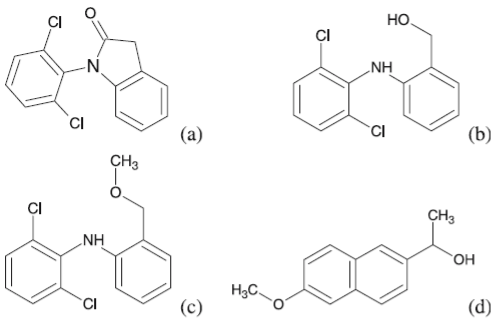


Fig. 7 Chemical structures of the degradation products of diclofenac (**a**) (2,6-dichlorophenyl)-1,3-dihydro-2*H*-indol-2-one, (**b**) 2-((2,6-dichlorophenyl)amino)benzyl alcohol, and (**c**) 2-((2,6-dichlorophenyl)amino)benzyl alcohol methyl ether, and of the degradation product of naproxen, (5-methoxy-2-naphthyl)ethan-1-ol (**d**)

too strong and brought too much dissolved O_2 into the anoxic/anaerobic segment. In the future the PWWTP will be redesigned to enable anoxic/anaerobic conditions. We expect an active anoxic (or anaerobic) reactor chamber to contribute to greater and more stable degradation of the chlorinated NSAID diclofenac.

Conclusions

Removal of selected pharmaceutical products in a pilot wastewater treatment plant has been studied. The compounds were selected because of their widespread use in Slovenia and Central Europe and their reported toxicity and persistence in wastewater treatment plants or the aquatic environment. Four of the model compounds, diclofenac, ibuprofen, naproxen, and ketoprofen, were nonsteroidal anti-inflammatory drugs (NSAIDs) and the fifth was an active metabolite of a blood-lipid-reducing agent (clofibric acid). The analytical method used for determination of the compounds in water samples included solid phase extraction, derivatisation to trimethylsilyl esters, and determination by GC–MS. The analytical method enabled to determine the removal of the NSAIDs and to follow degradation product formation in a PWWTP.

Elimination of ibuprofen, ketoprofen, and naproxen in the PWWTP was high (>87%) and constant whereas elimination of diclofenac was lower (49–59%), in agreement with literature reports. Clofibric acid, which was recently included in our study, was also poorly eliminated in the PWWTP. So far, only overall elimination has been determined; the exact mechanism of removal (biodegradation, degradation, and adsorption on activated sludge and/or the reactor walls) will be part of future studies.

Similarities between mass spectra and data in the NIST library of mass spectra were used to identify (2,6-dichlorophenyl)-1,3-dihydro-2*H*-indol-2-one, 2-((2,6-dichlorophenyl)amino)benzyl alcohol, and 2-((2,6-dichlorophenyl)amino)benzyl alcohol methyl ether as degradation products of diclofenac. A degradation product of naproxen, (5-methoxy-2-naphthyl)ethan-1-ol, was also identified with high probability and other peaks that may originate from the pharmaceutical products were also found (Fig. 4); we are, however, less certain of their structures and therefore only mention these in the manuscript. Their structure and origin will be studied in detail in the future and the structures of all degradation products will be confirmed by use of high-resolution mass spectrometry and degradation product standards will be purchased or synthesised for this purpose.

Our future research will also encompass application of additional WWTP technology (ozone/H_2O_2, photolytic and UV degradation, membrane filtration, anoxic and anaerobic conditions, etc...) to improve elimination efficiency. The main objective of our study, however, is to discover effective PWWTP conditions for elimination of the compounds selected which could be applied in a municipal WWTP. To accomplish this, we will examine other pharmaceutical compounds (e.g. carbamazepine, estrogenic compounds) in a similar manner. Comparative ecotoxicity and genotoxicity testing of the parent compounds and their degradation products will be included, in addition to toxicity testing of the PWWTP influent and effluent, to enable better assessment of the risk of WWTP effluents and pharmaceutical residues in the environment.

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3.2.2 Scientific paper: “Influence of pharmaceutical residues on the structure of activated sludge bacterial communities in wastewater treatment bioreactors”

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Influence of pharmaceutical residues on the structure of activated sludge bacterial communities in wastewater treatment bioreactors

Barbara Kraigher[a], *Tina Kosjek*[b,c,d], *Ester Heath*[b], *Boris Kompare*[d], *Ines Mandic-Mulec*[a,*]

[a]*Chair of Microbiology, Department of Food Science and Technology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia*
[b]*Department of Environmental Sciences, Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia*
[c]*Haslauer d.o.o., Zabukovica 87, 3302 Grižе, Slovenia*
[d]*Institute of Sanitary Engineering, Faculty of Civil and Geodetic Engineering, University of Ljubljana, Hajdrihova 28, 1000 Ljubljana, Slovenia*

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ABSTRACT

Concern is growing over contamination of the environment with pharmaceuticals because of their widespread use and incomplete removal during wastewater treatment, where microorganisms drive the key processes. The influence of pharmaceuticals on bacterial community structure in activated sludge was assessed in small-scale wastewater treatment bioreactors containing different concentrations (5, 50, 200 and 500 $\mu g L^{-1}$) of several commonly used pharmaceuticals (ibuprofen, naproxen, ketoprofen, diclofenac and clofibric acid). T-RFLP analyses of the bacterial 16S rRNA genes indicated a minor but consistent shift in the bacterial community structure in the bioreactor R50 supplied with pharmaceuticals at a concentration of 50 $\mu g L^{-1}$, compared to the control reactor R0, which was operated without addition of pharmaceuticals. In the reactors operated with higher concentrations of pharmaceuticals, a greater structural divergence was observed. Bacterial community composition was further investigated by preparation of two clone libraries of bacterial 16S rRNA genes from reactors R0 and R50. Most clones in both libraries belonged to the Betaproteobacteria, among which *Thauera*, *Sphaerotilus*, *Ideonella* and *Acidovorax*-related spp. dominated. Nitrite-oxidizing bacteria of the genus *Nitrospira* sp., which are key organisms for the second stage of nitrification in wastewater treatment plants, were found only in the clone library of the reactor without pharmaceuticals. In addition, diversity indices were calculated for the two clone libraries, indicating a reduced diversity of activated sludge bacterial community in the reactor supplied with 50 $\mu g L^{-1}$ of each of selected pharmaceuticals.

1. Introduction

'New emerging contaminants' have recently gained much public and scientific attention and represent an important issue for environmental pollution. They are manufactured continually, applied widely and released into the environment, thus contaminating groundwater and surface water bodies; however, their burden on the environment has not been comprehensively assessed yet. Among 'new emerging contaminants', pharmaceuticals are particularly interesting due to their pharmacological activity and consumption at rates of tons per year (Kasprzyk-Hordern et al., 2008; Daughton and

* *Corresponding author*. Tel.: +386 1 423 3388; fax: +386 1 257 3390.
E-mail address: ines.mandic@bf.uni-lj.si (I. Mandic-Mulec).
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Ternes, 1999). Moreover, it is expected that their worldwide production will increase, due to developing health care systems and higher life expectancies in industrial countries. Substantial amounts of pharmaceuticals can reach the environment, either through direct discharge into the water bodies or due to the inefficient elimination in wastewater treatment plants (WWTPs). As a result, numerous pharmaceuticals have been detected in surface waters, groundwater, wastewater and even in drinking water, in concentrations ranging from ng L^{-1} to several μg L^{-1} (Carballa et al., 2008; Gómez et al., 2007; Ashton et al., 2004; Farré et al., 2001; Daughton and Ternes, 1999).

Biological wastewater treatment is one of the most important biotechnological processes, in which microorganisms play a key role in removal of organic contaminants. However, biological WWTPs are primarily designed to remove easily degradable organic substances, whereas degradation efficiency of complex organic compounds is usually less efficient. Thus, several studies have demonstrated that some pharmaceuticals are efficiently eliminated (ibuprofen, naproxen and ketoprofen), while others are resistant to biodegradation (diclofenac and clofibric acid) (Kosjek et al., 2007; Quintana et al., 2005; Ashton et al., 2004). Various removal mechanisms (e.g. sorption, biodegradation and abiotic degradation) may contribute to the total elimination of organic contaminants; yet, for polar acidic pharmaceuticals, microbial degradation is believed to be the most important removal process in activated sludge wastewater treatment (Quintana et al., 2005). A broad bacterial consortium is required to achieve the desired biological conversions and the performance of wastewater treatment largely depends on the bacterial diversity present (Saikaly et al., 2005). Therefore, a fundamental understanding of the microbial community structure and stability as well as its response to different chemicals entering the wastewater, are desirable for stable and efficient WWTP operation.

The aim of this study was to evaluate the influence of commonly used pharmaceuticals on the structure of activated sludge bacterial communities in small-scale pilot wastewater treatment reactors. The compounds involved in the study were polar acidic pharmaceuticals: four non-steroidal anti-inflammatory drugs (NSAIDs: ibuprofen, naproxen, ketoprofen, and diclofenac) and clofibric acid (CLA), an active metabolite of blood-lipid regulators. Terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA gene sequences was used to characterize and compare the community structure of the bioreactors. Furthermore, to facilitate a more detailed, sequence-based identification of the bacterial species, and to investigate the effect of pharmaceuticals on bacterial community composition, two 16S rRNA gene clone libraries were constructed from the sludge samples of two bioreactors, with and without pharmaceuticals.

2. Materials and methods

2.1. Bioreactor description and sampling of activated sludge

Bioreactors with wetted volume of 4.0 L were fed by artificial wastewater at a hydraulic retention time of 48 h and at a sludge retention time of over 100 days after acclimatization of biomass. In order to follow biomass adaptation under exposure to two different concentrations of pharmaceuticals, two reactors, R5 and R50, were operated under continuous input of high (R50, 50 μg L^{-1}) and low (R5, 5 μg L^{-1}) concentrations of each of the selected pharmaceuticals: ibuprofen, naproxen, ketoprofen, diclofenac, clofibric acid (all provided by Sigma–Aldrich, St. Louis, MO, USA). The third reactor (R0) served as a control and was supplied with artificial wastewater without pharmaceuticals. Activated sludge from a Slovenian municipal WWTP was used for the reactors start-up. Reactors were operated continuously for two years without changing conditions to allow adaptation of the activated sludge community, at an average temperature of 22 °C (±5%) and at pH 7.7 (±2%) (influent) and 7.3 (±5%) (effluent). Oxygen concentration was maintained at 7.9 mg L^{-1} (±5%). Total solids concentration was app. 6 g L^{-1} (±20%) with F/M ratio of 0.03 (±20%). NH_4^+ in the influent and in the effluent was measured using continuous flow analyzer (FlowSys Alliance Instruments, Salzburg, Austria) and indicated a constant nitrification performance eliminating on average 95% (±5%) of the NH_4^+ from the influent. Removal of ibuprofen, ketoprofen and naproxen was high and constant (>87%), while elimination efficiencies of diclofenac and clofibric acid were <60% and <30%, respectively. A detailed description of the reactors configuration, operation and removal of pharmaceuticals is given in Kosjek et al. (2007).

Activated sludge samples were collected from the three reactors after two years of adaptation of the biomass to the conditions in the reactors. Comparison of the community structure in the seeding sludge from Slovenian WWTP to the adapted sludge in the reactors showed a divergence to app. 66% similarity, as reflected from T-RFLP profiles (unpublished), which was attributed to the new conditions in the bioreactors and to artificial wastewater composition. Sampling of activated sludge in the reactors was performed three times, at monthly intervals. For each sampling, three 10 mL samples were collected from each reactor, in order to assess heterogeneity within the reactor, and transferred into sterile plastic tubes and stored at 4 °C until DNA extraction on the same day (or stored at −80 °C for later analyses). Additionally, three more reactors (R50P, R200 and R500) were set up using activated sludge from reactor R50 as inoculum. These reactors were operated under similar conditions to R0, R5 and R50, except for the concentrations of the pharmaceuticals, which were supplied at 50, 200 and 500 μg L^{-1} for R50P, R200 and R500, respectively.

2.2. Analysis of bacterial community structure

Terminal restriction fragment length polymorphism (T-RFLP) analysis was used to compare activated sludge bacterial community structures. DNA was extracted from 1-mL subsamples (three independent DNA isolations were obtained for each sample) using the UltraClean soil DNA isolation kit (MoBio Solano Beach, CA, USA) according to the manufacturer's instructions. Isolated chromosomal DNA (concentration of 50–100 ng μL^{-1}) was checked on an 1% agarose gel, compared to the Gene Ruler DNA Ladder Mix (Fermentas, Litva) to estimate the size and concentration,

and used as a template for PCR with 16S rRNA gene primers 27f labelled with 6-FAM (6-carboxyfluorescein) at the 5′ end and 927r (Heuer and Smalla, 1997) following the protocol as described in Kraigher et al. (2006), except that 30 cycles instead of 35 were applied for PCR. Approximately 200 ng of fluorescently labelled PCR amplification reactions were digested with the restriction enzyme *Hae*III (MBI Fermentas, Litva) in a 30-μL reaction and subsequently purified by ethanol precipitation. Restriction enzyme *Hae*III is widely used for T-RFLP profiling of 16S rRNA genes in the complex samples such as soil or activated sludge as its restriction sites are often present in the sequences and it is expected to provide a high resolution. Analysis of terminal restriction fragment (T-RF) sizes and quantities was performed on an ABI PRISM 310 DNA sequencer (Applied Biosystems Inc., USA) as described in Kraigher et al. (2006). Profiles were generated using Genescan analysis software (ABI). T-RFs with peak heights of less than 50 fluorescence units and T-RFs that were less than 50 bp long were excluded from the analyses. Comparisons of T-RFLP profiles of triplicate DNA isolations per sample were performed as described in Kraigher et al. (2006).

2.3. Preparation of 16S rRNA gene clone libraries and sequence analysis

Two clone libraries were constructed from the control reactor R0 and the reactor R50. Three independent DNA preparations from each of the two reactors were pooled and used as a template for PCR amplifications of 16S rRNA genes PCR was performed with forward primer 27f and reverse primer R1401 (Nübel et al., 1996) with the thermocycling conditions used for T-RFLP. Products from four independent PCR amplifications (each made with three pooled DNA preparations) were pooled, purified with Qiaquick PCR Gel Extraction Kit (QIAGEN, Stanford, CA, USA), and cloned into pGEM®-T Easy Vector (Promega, Madison, WI, USA) according to the manufacturer's protocol, after which competent high efficiency JM109 *E. coli* cells (Promega, Madison, WI, USA) were transformed and plated on LB (Luria-Bertani) plates supplied with ampicillin, IPTG and X-gal. White colonies were screened for inserts of the expected size (about 1380 bp) using the vector primers SP6 and T7 (Promega, Madison, WI, USA). From each clone library, 96 clones were selected for sequencing by Macrogen Inc. (Seoul, Korea). Plasmids were isolated from colonies and single extension 16S rRNA gene sequencing was performed by applying primer 27f. The sequences obtained were manually proofread and corrected, if necessary, with Chromas Version 2.3. Potential chimeric sequences were detected by the Chimera Check program version 2.7 of the Ribosomal Database Project (RDP) (Maidak et al., 2001) and by the Bellerophon program (Huber et al., 2004). Additionally, sequences were manually split into two parts and putative chimeras were determined by comparing phylogenetic trees constructed from the two sets of sequences and removed from further analyses. The sequences were then compared with available database sequences using the Basic Local Alignment Search Tool (BLAST) and the RDP for initial phylogenetic assignment. GenBank sequences most similar to clone sequences were downloaded and included in phylogenetic tree reconstruction using neighbour-joining method with 500 bootstrap replicates and the Kimura-2-parameter evolutionary model within the MEGA version 3.1 (Kumar et al., 2004). In addition, *in silico* T-RFLP of sequences from clone libraries was performed with the T-DisctinctiEnz program (http://www.biorcgld.org/tools/restriction/t_DistinctiEnz.pl) and the lengths of *in silico* T-RFs of each clone were included in the phylogenetic trees. Sequences from the two clone libraries were deposited in the GenBank database under accession numbers EU499447 to EU499598.

2.4. Calculation of diversity indices

Diversity indices were calculated from sequence data based on the number of OTUs (operational taxonomic units). An OTU was defined as a 16S rRNA gene sequence group in which sequences differ by 3% or less (McCaig et al., 1999). The richness (S) of OTUs was obtained from the total number of distinct OTUs in each library or distinct T-RFs in each profile. The Shannon diversity index (H) (Shannon, 1948) was calculated as follows: $H = -\Sigma(p_i)(\ln p_i)$ where p_i is the proportion of an individual OTU relative to all sequences analyzed or the proportion of an individual T-RF peak area relative to the cumulative peak area. The evenness (e) of OTUs was calculated as follows: $e = H/\ln(S)$.

To calculate diversity indices from the T-RFLP profiles, data were first normalized by dividing peak areas of each sample by the sum of all peak area values from the corresponding sample. This step compensated for differences in PCR product quantity and T-FRLP fingerprint intensity among samples. Peaks differing by 0.7 bp or less were considered as a consensus peak (based on T-Align in Smith et al. (2005)). A derivative profile containing only the most conservative T-RF information was created for each sample, by identifying the subset of T-RFs that appeared in all three replicates of one sample, while irreproducible T-RFs (i.e. fragments that were not observed in all the replicate profiles) were discarded (Dunbar et al., 2001). Average relative peak areas for these peaks were calculated and peaks with less than 1% of the sum of all peak areas were excluded from further analysis. Diversity indices were determined as described above.

3. Results

3.1. Comparison of bacterial communities in the reactors by T-RFLP

The influence of pharmaceuticals on bacterial community structures in activated sludge was determined by T-RFLP analysis of 16S rRNA genes. At all three sampling times, T-RFLP profiles of the bacterial community from reactor R50 clustered separately from profiles of reactors R0 and R5. Profiles of reactors R50 and R0 were app. 82% similar, while the similarity between the replicate profiles was at least 92% based on Pearson's correlation coefficient (Fig. 1). Minor shifts were also detected in bacterial community profiles with time; however, irrespective of the sampling time, bacterial community profiles from reactors R0 and R50 clustered

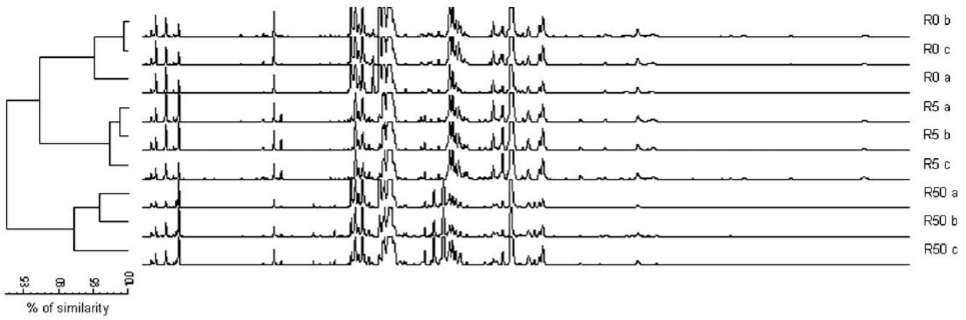

Fig. 1 – Comparison of bacterial community profiles from the three reactors. Profiles of only one sampling time (December samples) are shown as the other two were very similar. The dendrogram of T-RFLP profiles was generated based on Pearson's correlation using the UPGMA method. R0, reactor without pharmaceuticals; R5, reactor with 5 $\mu g\,L^{-1}$ of pharmaceutical mixture; R50, reactor with 50 $\mu g\,L^{-1}$ of pharmaceutical mixture; a, b, c represent DNA isolations from triplicate samples.

separately, while profiles from reactor R5 were more similar to those from reactor R0 (Fig. 2).

Three additional bioreactors were inoculated with biomass from reactor R50 to investigate whether higher concentrations of pharmaceuticals have a stronger influence on activated sludge bacterial communities, and to assess adaptation of these communities during the first few months after inoculation. After the first month, a reduction in biomass yield was observed in the two reactors with higher concentrations of pharmaceuticals (app. 0.5 $g\,L^{-1}$ in R200 and R500) as compared to the biomass in reactor R50P (app. 5 $g\,L^{-1}$). Sampling was performed two months after inoculation, when the quantity of activated sludge biomass in the R200 and R500 reactors was comparable to that in the reactor R50P (app. 5 $g\,L^{-1}$). T-RFLP profiles of bacterial communities in the new reactors containing very high concentrations of pharmaceuticals were clearly distinguished from those in the reactor R50P (less than 60% similarity with R50P and R50, based on Pearson's correlation), and also clearly different from well-adapted sludge in reactors R5 and R0 (Fig. 3).

3.2. *Composition of the activated sludge bacterial communities*

Detailed bacterial community composition of activated sludge in the reactors was determined by analysis of 16S rRNA gene clone libraries from reactors R0 and R50. Of the 177 partial sequences obtained from both libraries, each consisting of approximately 830 nucleotides, 6 potential chimeras were identified and excluded from further analyses. The remaining 171 sequences were initially analyzed using BLAST searches at NCBI. Only 11 out of 84 sequences from the R50 clone library and 20 out of 87 sequences from the R0 clone library were less than 95% similar to database sequences. Among these, only 1 and 6 sequences from the R0 and R50 clone libraries, respectively, were less than 90% similar to database sequences. Sequences were then affiliated with phylogenetic groups using BLAST searches, RDP classifier (http://rdp.cme.msu.edu/classifier) and reconstructed phylogenetic trees. All clones with 16S rRNA gene sequence similarity of at least 97%

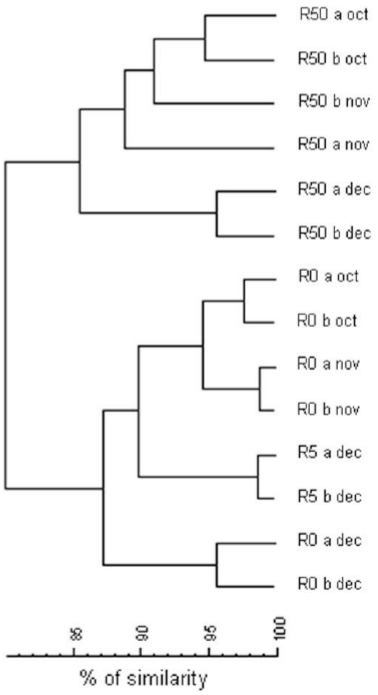

Fig. 2 – Comparison of the bacterial community profiles in the three reactors sampled at monthly intervals. The dendrogram of the T-RFLP profiles was generated based on Pearson's correlation using the UPGMA method. R0, reactor without pharmaceuticals; R5, reactor with 5 $\mu g\,L^{-1}$ of pharmaceutical mixture; R50, reactor with 50 $\mu g\,L^{-1}$ of pharmaceutical mixture; a, b, c represent DNA isolations from triplicate samples; dec, December samples; nov, November samples; oct, October samples.

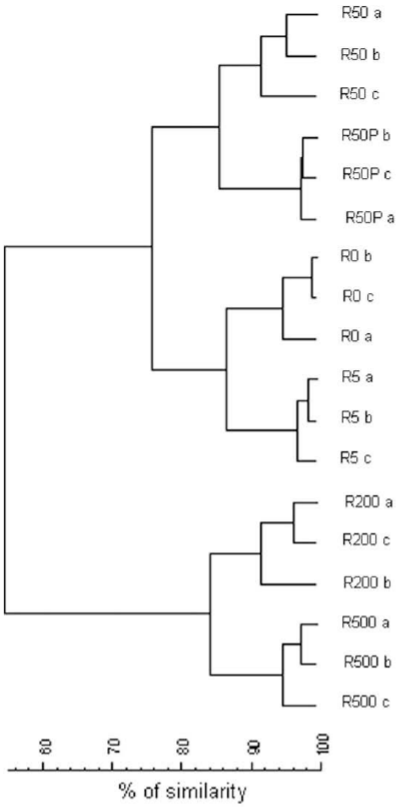

Fig. 3 – Comparison of activated sludge bacterial community structures in the reactors supplied with 0, 5, 50, 200 and 500 μg L^{-1} of pharmaceuticals. Activated sludge from reactor R50 was used as inoculum for the reactors R50P, R200 and R500. Sampling was performed in December, two months after the start-up of the reactors R50P, R200 and R500. The dendrogram of the T-RFLP profiles was generated based on Pearson's correlation using the UPGMA method. R50, original reactor with 50 μg L^{-1} of pharmaceutical mixture; R50P, reactor with 50 μg L^{-1} of pharmaceutical mixture; R200, reactor with 200 μg L^{-1} of pharmaceutical mixture; R500, reactor with 500 μg L^{-1} of pharmaceutical mixture; R0 and R5, reactors with 0 and 5 μg L^{-1}, respectively, of pharmaceutical mixture; a, b, c represent DNA isolations from triplicate samples.

were grouped into an operational taxonomic unit (OTU). The distribution of sequences (and OTUs) from the two clone libraries among phylogenetic groups is shown in Table 1. The majority of sequences from both clone libraries were related to the *Proteobacteria*, mainly to the *Betaproteobacteria*, among which *Acidovorax*, *Thauera*, *Ideonella* and *Sphaerotilus*

Table 1 – Distribution of sequences and operational taxonomic units (OTUs) from the two clone libraries (R0 and R50) and their relationships to various phylogenetic groups

Clone library	R0		R50		R0 + R50
Phylogenetic group	Clones	OTUs	Clones	OTUs	Shared OTUs
Proteobacteria	47	25	56	15	6
Alphaproteobacteria	7	4	4	2	0
Betaproteobacteria	36	17	46	9	5
Gammaproteobacteria	3	3	4	3	1
Deltaproteobacteria	1	1	2	1	0
Nitrospira	7	3	0	0	0
Gemmatimonadetes	4	2	2	1	1
Acidobacteria	9	5	10	4	3
Chloroflexi	17	7	11	5	4
Genera incertae sedis OP10	0	0	1	1	0
Unclassified	3	2	4	3	1
Total number	87	44	84	29	15

An OTU represents a group of sequences with at least 97% of similarity.

sequence types dominated. Phylogenetic tree reconstruction of the *Betaproteobacteria* from both clone libraries is shown in Fig. 4. The second most abundant phylogenetic group in both clone libraries was *Chloroflexi*. The most significant difference between the two libraries (as assessed also by RDP library compare program; http://rdp.cme.msu.edu/comparison) was in the phylum *Nitrospira*, which was found only in the R0 clone library, from the reactor without pharmaceuticals. A phylogenetic tree of *Nitrospira* sp. sequences from this library and from databases (Fig. 5) indicated that reactor sequences were mostly related to sublineage I of the genus *Nitrospira*.

To identify the most abundant phylotypes as indicated by whole community T-RFLP profiles, some of the most abundant clone sequences were amplified with the T-RFLP primers and digested with *Hae*III restriction enzyme and T-RF lengths were analyzed by capillary electrophoresis. Comparison of the T-RFLP profiles obtained with the whole community profiles, in combination with the *in silico* T-RFLP analyses of the sequences from the two clone libraries with *Hae*III restriction enzyme (http://www.biorcgld.org/tools/restriction/t_DistinctiEnz.pl), revealed that the majority of the highest peaks in the T-RFLP profiles could be assigned to the bacterial groups of the most abundant clone sequences in the libraries (Fig. 6), such as groups related to *Acidovorax* sp. (*in silico* T-RF length 165 bp and 163 bp), *Sphaerotilus* sp. (140 and 159 bp), *Ideonella* sp. (144 and 163 bp), *Nitrosomonadaceae* (146 and 139 bp), and *Chloroflexi* (250 bp). However, the T-RFLP data presented in Fig. 6 only partially confirm the trends detected in the clone libraries. The highest peaks were not always represented by the most abundant clone sequences; this is not surprising, as both methods are semi-quantitative, and therefore only serve to detect trends. Furthermore, *in silico* T-RFs of the *Nitrospira* sp. from the clone library were distributed among 4 distinct T-RFs (210, 213, 124 and 277) and *in silico* T-RFs for some

Fig. 4 – Phylogenetic reconstruction of partial 16S rRNA gene sequences affiliated with the *Betaproteobacteria*, the most abundant group in the two clone libraries. The phylogenetic tree was constructed using the neighbour-joining method. *Bacteroides fragilis* (AB050106) was used as the outgroup species. Sequences from the R50 clone library are designated with R and from the R0 clone library with 0. Lengths of the *in silico* restriction digests with *Hae*III restriction enzyme are given in parentheses.

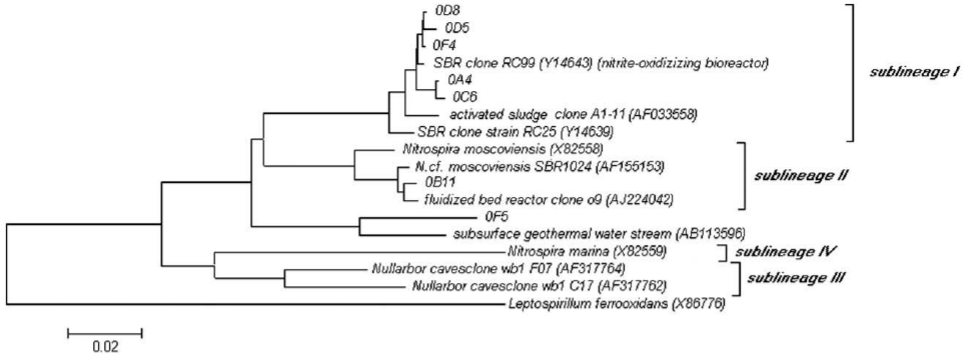

Fig. 5 – Phylogenetic tree showing the affiliation of the *Nitrospira*-related clones obtained in the R0 clone library (designated with 0) to known species in the genus *Nitrospira*. *Leptospirillum ferrooxidans* (X86776) was used as the outgroup species. Four sublineages of the genus *Nitrospira* are indicated.

Acidobacteria (213) were very similar or the same as for the majority of the *Nitrospira* sequences found in the R0 clone library, preventing identification of the T-RF representing the majority of the clones affiliated with *Nitrospira* sp.

3.3. *Bacterial diversity in activated sludge*

While the previous section focused only on abundant populations, the overall bacterial diversity was assessed by comparing three diversity indices: the number of OTUs or T-RFs present, richness, *S*; the evenness of OTUs abundance, *e*; and the Shannon diversity index, *H*. The numbers of distinct OTUs in the two clone libraries and distribution in different phylogenetic groups are shown in Table 1. Analysis of 87 sequences of the 16S rRNA genes from the R0 clone library revealed 44 distinct OTUs while only 29 distinct OTUs out of 84 analyzed sequences were found in the R50 clone library. Among the 58 distinct OTUs out of a total 171 sequences recovered from the two clone libraries, only 15 OTUs were found to be present in both populations. The number of distinct betaproteobacterial OTUs in the R50 clone library was significantly lower than those in the R0 clone library (Table 1). Collector's curves showing the number of different clones (with more than 3% different nucleotide sequences) versus the total number of clones analyzed revealed plateau-shaped plots, indicating lower diversity in the R50 clone library (Fig. 7). Additionally, library coverage *C* was calculated for both libraries according to the formula $C = (1 - (n_1/N)) \times 100\%$, where n_1 was the number of OTUs containing only one sequence, and *N* the total number of 16S rRNA gene sequences analyzed (Good, 1953). This indicated a high library coverage in R50 clone library (88%), and much lower coverage (40%) in the R0 library.

Diversity indices, calculated from the two clone libraries by applying either number of OTUs or number of *in silico* T-RFs to define richness (*S*), and from T-RFLP analyses of experimental whole community T-RFLP profiles, indicated a reduction in bacterial diversity (Table 2). A distinct T-RF length represents an OTU and a T-RF peak area represents its abundance (Osborne et al., 2006). However, a distinct T-RF length could represent more than one OTU, defined by 97% sequence similarity, which could be reflected from the sequence data analysis of the clone libraries (Table 2 and Fig. 4). *In silico* T-RFLP of the clone sequences with *Hae*III restriction enzyme revealed only 31 and 25 unique T-RFs in the R0 and R50 clone libraries, respectively, indicating a lower absolute diversity and difference between the two communities as compared to calculations based on the whole sequence similarities of the clone libraries. When diversity indices were calculated from experimental whole community T-RFLP profiles using 1% of the total fluorescence as a threshold for the peak, only 23–26 distinct T-RFs were obtained, and differences in diversity indices between the two communities were even lower.

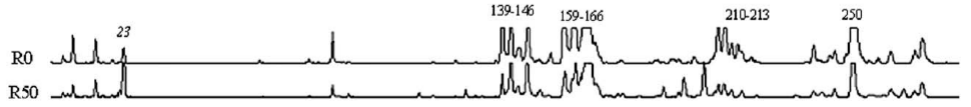

Fig. 6 – The most abundant clone sequences in two clone libraries indicated as the length of *in silico* T-RFs, assigned to the whole community T-RFLP profiles. 23: *Thauera* sp.; 139–146: *Nitrosomonadaceae*, *Alphaproteobacteria*, *Sphaerotilus* sp., *Ideonella* sp.; 159–166: *Sphaerotilus* sp., *Ideonella* sp., *Acidovorax* sp., *Chloroflexi*, *Acidobacteria*; 210–213: *Nitrospira*, *Acidobacteria*; 250: *Chloroflexi*.

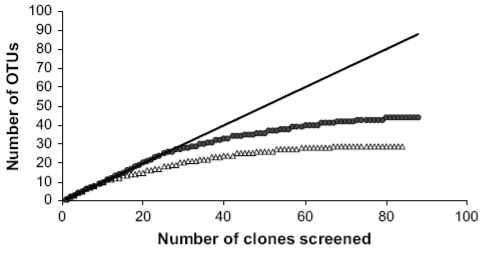

Fig. 7 – Collector's curves of the unique *E. coli* clones with 16S rRNA gene inserts versus total number of clones screened from clone libraries 0 and R. • R0 clone library; △ R50 clone library; — maximum theoretical diversity.

4. Discussion

4.1. *Influence of pharmaceuticals on bacterial community structure*

Wastewater treatment processes rely on the composition and activity of their microbial communities. To our knowledge, there has previously been no study addressing the influence of pharmaceutical residues in wastewater on the bacterial community structure of activated sludge. It is not known how different commonly used pharmaceuticals affect bacterial communities in activated sludge and if they influence the removal efficiency of other pollutants.

T-RFLP analysis of activated sludge in the three reactors operating under constant conditions for two years (pharmaceutical and NH_4^+ removal rate as well as some other reactors' parameters were relatively constant throughout the experiments) demonstrated minor but consistent differences in the structure of bacterial community in the presence of 50 $\mu g\,L^{-1}$ of selected pharmaceuticals. This was indicated by separate clustering of R50 T-RFLP profiles at all sampling times (Fig. 1), suggesting that the concentration of 50 $\mu g\,L^{-1}$ of pharmaceuticals had a stronger influence on community structure than time variability. However, the lower pharmaceutical concentration of 5 $\mu g\,L^{-1}$ resulted in only a minor structural shift, to a similar extent as that detected over time, and T-RFLP profiles of the reactor R5 at different sampling times could not be distinguished from those in reactor R0 (Fig. 2).

Table 2 – Diversity indices calculated from the sequence data (OTUs and *in silico* T-RFs from the clone libraries) and from whole community T-RFLP profiles of the two sampling times (nov: November and dec: December) for the two reactors R0 and R50

Index	Library (based on OTUs)		*In silico* T-RFLP (based on *Hae*III T-RFs)		Whole community T-RFLP (based on *Hae*III T-RFs)			
	R0	R50	R0	R50	R0 nov	R50 nov	R0 dec	R50 dec
S	44	29	31	25	26	23	23	25
H	3.53	2.88	3.16	2.71	2.90	2.66	2.74	2.71
e	0.93	0.86	0.92	0.84	0.89	0.85	0.87	0.84

S, richness (number of OTUs or T-RFs); *H*, Shannon index; *e*, evenness.

Pharmaceuticals are designed to interfere with biological systems and are, either alone or in combination with other compounds and/or metabolites, likely to affect microorganisms in the environment. However, the selected pharmaceuticals are aromatic carbohydrates, which are widely found in wastewaters, and their degradation pathways, at least in part, are probably similar to the pathways used for various aromatic compounds. Degradation of many pharmaceuticals is most likely the inherent ability of the sludge communities. Therefore, major changes in bacterial communities after exposure to the widely used pharmaceuticals were not expected. However, high concentrations that are possible for some periods and on special locations, especially in hospital sewage, could lead to greater structural shifts.

Concern over contamination of the environment with pharmaceuticals has increased in the recent years and is currently mainly restricted to surface waters and receiving waters where several pharmaceuticals have been detected and their concentrations determined to reach on average up to several $\mu g\,L^{-1}$. Concentrations in Slovenian WWTP used for seeding were measured only in the effluents and were usually less than 1 $\mu g\,L^{-1}$ (unpublished data). However, it should be noted that concentrations in the influent wastewaters could be much higher than those reported for WWTP effluents and for the surface waters. For example, Ashton et al. (2004) have detected 27 $\mu g\,L^{-1}$ and Farré et al. (2001) even 85 $\mu g\,L^{-1}$ of ibuprofen in the effluent samples. If we consider our and some other reported results for ibuprofen degradation that could be up to 99%, the concentrations in the wastewater treatment plants theoretically could reach over 200 $\mu g\,L^{-1}$ which is in the same range as in our experiments. In fact, Gómez et al. (2007) have detected 34–168 $\mu g\,L^{-1}$ of ibuprofen in the influents from a municipal sewage treatment plant located in the southeast of Spain (Alméria). Thus, although the concentrations of pharmaceuticals used in this study are higher than the typical environmental concentrations detected, the results of this study could be important for at least some of the wastewater treatment plants, especially with the increasing worldwide consumption of various pharmaceuticals.

Analyses of reactors R50P, R200 and R500, which were set up with the sludge containing communities already adapted to pharmaceuticals at 50 $\mu g\,L^{-1}$, indicated a strong influence of higher concentrations (200 and 500 $\mu g\,L^{-1}$) on bacterial community structure two months after exposure (Fig. 3). Although a community shift was also observed in the reactor R50P, operated under the same conditions as the reactor from which the inoculum originated, T-RFLP profiles of communities in reactors R200 and R500 indicated only approximately 60% similarity with R50, while the similarity between the reactors R50P and R50 was app. 86%. Some studies have shown divergence of communities with time and lack of reproducibility, even in replicate laboratory-scale reactors operated under identical conditions (Boon et al., 2000;

Kaewpipat and Grady, 2002). However, the activated sludge in the new reactors (R50P, R200 and R500) was at the time of sampling most likely still in the phase of development and adaptation, so the reactor R50P could not be directly considered as a replicate reactor of the reactor R50. In addition, our recent analyses after one year of acclimatization of the 'new' reactors indicated that reactors R50 and R50P converged to 89% similarity and the reactors R50 and R0 were still 81% similar (data not shown), which is supporting our conclusions. Furthermore, reduced growth and much greater structural divergence in reactors R200 and R500 from reactor R50 as compared to divergence between reactors R50 and R50P, strongly suggest that pharmaceutical concentrations determine the extent of structural shifts in activated sludge bacterial communities.

4.2. *Bacterial community composition*

Sequencing of the clone libraries constructed from the reactors R0 and R50 provided detailed information on the phylogenetic composition in the activated sludge of the two reactors. *Proteobacteria*-associated sequences dominated both libraries (54% from R0 and 67% from R50 clone library), particularly *Betaproteobacteria* (41% from R0 and 55% from R50). Only two other phylogenetic groups were present by more than 10% of the sequenced clones: *Chloroflexi* (19% from R0 and 13% from R50) and *Acidobacteria* (10% from R0 and 12% from R50). The phylum *Chloroflexi* constitutes a specialized group of aerobic, filamentous bacteria, consuming primarily carbohydrates (Kragelund et al., 2007) and contains diverse environmental clones retrieved from various wastewater treatment plants with only a few cultured representatives. Thus, their roles in WWTPs are not well understood, but Miura et al. (2007) have reported *Chloroflexi* to be one of the dominant members in membrane bioreactors.

At the division level, results of this study agree with reports of dominance of activated sludge bacterial communities by *Betaproteobacteria*, as determined by analysis of 16S rRNA gene libraries and/or by fluorescence *in situ* hybridization (FISH) (LaPara et al., 2000; Juretschko et al., 2002; Eschenhagen et al., 2003). Many of the betaproteobacterial clones belonged to the *Acidovorax* sp., which have been reported at high abundance in activated sludge from a municipal WWTP (Schulze et al., 1999). Wang et al. (2004) demonstrated that *Acidovorax* spp. and *Comamonas testosteroni* (represented by three clones in the R50 clone library) could efficiently remove acrylonitrile from wastewaters. Many of the clones were affiliated with the recently described genus *Thauera*, which was the second most abundant betaproteobacterial group in FISH analyses of the nitrifying–denitrifying activated sludge of an industrial wastewater treatment plant (Juretschko et al., 2002). *Thauera* spp. have been reported as representing denitrifying bacteria in wastewater treatment processes (Tarlera and Denner, 2003) and can be abundant phenol degrading members of reactor communities (Manefield et al., 2002). The most significant difference in bacterial community composition between the two libraries was in the genus *Nitrospira*, which was found only in the reactor without pharmaceuticals representing 8% of the total community. Juretschko et al. (2002) also detected *Nitrospira* in significant numbers (12% of the total bacterial counts as determined by quantitative FISH) in nitrifying activated sludge of an industrial WWTP. Chemolithoautotrophic nitrite oxidizers of the genus *Nitrospira* are a monophyletic but diverse group of organisms, which are widely distributed in many natural habitats, and play a key role in nitrite oxidation during biological wastewater treatment (Maixner et al., 2006). Our findings suggest that these key nitrite-oxidizing bacteria are affected by pharmaceuticals, with a potentially important influence on the treatment plant operation. The effect of pharmaceuticals (including diclofenac and clofibrate) on bacterial nitrite oxidation was investigated by Dokianakis et al. (2004), who found inhibition by some of the tested pharmaceuticals on the nitrite reduction rate performed by nitrite-oxidizing bacterial culture isolated from activated sludge. Their findings suggest that neither diclofenac nor clofibrate inhibit bacterial nitrite oxidation, but they did not study the influence of naproxen, ketoprofen and ibuprofen and, in particular, the influence of combination of these pharmaceuticals, which might exert a more pronounced effect. In addition, they investigated effects on nitrite-oxidizing enrichment cultures, which may react differently than complex bacterial communities.

4.3. *Diversity of bacterial communities*

Diversity indices were calculated for clone libraries from the reactors R0 and R50 to compare bacterial diversity and indicated reduced diversity following supply with pharmaceuticals at 50 $\mu g L^{-1}$ (Table 2). The number of OTUs was most significantly reduced within the *Betaproteobacteria* although the total number of clones affiliated with this group was higher. Different percentages of library coverage in two clone libraries also indicated a lower diversity in R50 clone library. Reduction in species diversity is often reported in response to stress factors, e.g. phenol shocks, elevated temperatures, addition of nonylphenol polyethoxylates and solids retention time (Eichner et al., 1999; LaPara et al., 2000; Lozada et al., 2004; Saikaly et al., 2005).

Diversity indices calculated from T-RFLP data indicated only a slight reduction in diversity in the R50 library. However, calculation of diversity indices from T-RFLP profiles is highly dependent on thresholds for the peaks used (threshold of 1% was used in this study). Blackwood et al. (2007) have shown that the relationships between T-RF diversity indices and true indices were sensitive to the relative abundance threshold, with greatly improved correlations observed using a 0.1% threshold, which is rarely possible with current technology. In addition, some organisms may produce more than one T-RF because of *rrn* operon copy number heterogeneity (Crosby and Criddle, 2003), and a distinct T-RF may represent more different species. This was indicated by diversity indices that were calculated based on the *in silico* T-RFLP of the sequences from the clone libraries. Indices were lower than those based on sequence data, suggesting that bacterial diversity determinations for complex bacterial communities based on T-RFLP profiles have a lower resolution than sequence data. This is in agreement with Blackwood et al. (2007) who suggested that for highly complex communities, calculation of diversity indices from T-RFLP data provides inaccurate estimates of true diversity in microbial communities. Therefore,

diversity indices calculated from the sequence data that have indicated a lower diversity in R50 as compared to R0, would be more reliable, which is supporting our conclusions. Cultivation-independent, molecular techniques currently provide the best approach for analysis of complex natural microbial communities. In interpreting results using this approach, however, potential biases associated with differences in DNA extraction efficiency, gene copy number or PCR amplification and primer biases, must be considered. Clone frequencies in clone libraries may not always reflect environmental relative abundances. However, in this study, sludge samples investigated were processed using identical techniques and data analyses, and biases are likely to have been similar, thus differences detected would be significant and would arise from structural differences in the examined samples.

Results of this study suggest that the presence of selected pharmaceuticals in wastewaters at concentrations of 50 $\mu g\,L^{-1}$ affects bacterial community structure. Since a decrease in species diversity may reduce the chance of obtaining species with different complementary physiological traits that are better adapted to specific environmental perturbations, a reduction in bacterial diversity may affect the essential functions of activated sludge wastewater treatment systems.

5. Conclusions

Analyses of T-RFLP profiles and two clone libraries indicated that a mixture of pharmaceuticals containing commonly used NSAIDs and clofibric acid at concentrations of 50 $\mu g\,L^{-1}$ caused shifts in the structure of activated sludge bacterial communities and reduced bacterial diversity in the reactors. The inability to detect *Nitrospira* in the reactor with addition of pharmaceuticals suggests an important effect on bacteria that play a key functional role in nitrogen removal from wastewater. However, to confirm some of the indications obtained in this study, further research of specific bacterial groups is required for a more detailed view of community structural and diversity shifts in activated sludge in response to pharmaceuticals. Since microorganisms represent the key components in wastewater treatment systems and the effects of changes in bacterial community structure may affect the wastewater treatment system as a whole, these investigations are important for future predictions of the effects of environmentally present pharmaceuticals on wastewater treatment plant operation and on their release to the environment.

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3.3 Transformation of pharmaceuticals during biological water treatment

While the transformation of pharmaceuticals in the human body and in other mammals has been extensively studied, the kinetics, breakdown pathways and mechanisms of their degradation during water treatment and in the environment remain largely unknown. Because of this poor knowledge of transformations of pharmaceuticals subjected to treatment processes, recognizing their identity was one of the core subjects in this thesis. Compared to other treatment processes the studies on biotransformation are especially demanding, since these studies by nature involve complex matrices containing significant quantities of organic matter, which may interfere with the detection and identification [78]. Another issue hindering the identification is that biodegradation experiments do not allow adapting the concentration of the analytes as this can potentially alter the structure of the microbial community [235]. Recent studies indicated that enzymatic transformations provoke only slight alterations in chemical structures of recalcitrant pharmaceuticals [206], which normally results in increased polarity of the TPs.

In parallel to structural elucidation studies, scientific interest focuses on technological advances and developments achieved in mass spectrometry, where increased sensitivity and selectivity play important roles. To gain sufficient data for secure structure elucidation of (bio)degradation products, a combinatory analysis should be performed combining MS^2 techniques (e.g., QqQ, IT or Qq-LIT), with HRMS (e.g., TOF or Fourier transform) for accurate-mass measurements. Alternatively, hybrid MS systems, such as QqTOF or LTQ-Orbitrap, can be applied for the same purpose. Thus, the scientific papers included in this chapter discuss the capabilities of different MS techniques for structural elucidation of pharmaceutical TPs and, by the use of QqTOF, describe two studies in the field of pharmaceutical biotransformation. Biotransformation experiments were performed in laboratory-scale bioreactors and involved two chlorine-bearing pharmaceuticals, DF and CLA. Even though the applied levels of pharmaceuticals exceeded typical environmental concentrations by factor $5 – 10^3$, this was considered an acceptable compromise between environmental concentrations and the detection limits of TPs, which are usually in percentage or promile amounts [236]. Because of the low levels of the TPs and high amount of data that the QqTOF instrument provides, detection of the TPs in complex WW matrices was a particularly challenging task. To facilitate the detection, this study exploited an in-source fragmentation, which was based on diagnostic fragment ions of DF (*m/z* 214) and CLA (*m/z* 127). An alternative detection principle was based on data processing by a spectral and chromatographic search algorithm MetaboLynx™ (Waters Corp.), which highlighted any suspect TPs in treated samples, irrespective of the structural relationship to the parent compound.

It was shown that the biotransformation pathways partially mimic abiotic processes or human metabolism. Thus, the biodegradation products identified in this study matched those reported to be abiotic TPs, i.e. 4-chlorophenol was formed from CLA [156,237] and 1-(2,6-dichlorophenyl)-1,3-dihydro-2H-indol-2-one from DF [238,239,240], or human metabolites (hydroxy-diclofenac [241]). Alternatively, biotransformation yields also unusual and unexpected TPs, such as a nitro-analogue of DF. Research further suggests that MS alone is sometimes insufficient for a complete structural elucidation of unknown compounds, and complementary investigations applying NMR are necessary. Finally, this research demonstrated that the toxicity of a WW may increase during biological wastewater treatment. This supports the need for the toxicity evaluation of transformation products, and the further development of new treatment techniques to achieve complete mineralization of emerging contaminants is justified.

The results of this research are in details presented in three papers and a book chapter:

- Mass spectrometry for identifying pharmaceutical biotransformation products in the environment (TrAC, Trends in Analytical Chemistry, 2007)
- Metabolism studies of diclofenac and clofibric acid in activated sludge bioreactors using liquid chromatography with quadrupole - time-of-flight mass spectrometry (Journal of Hydrology, 2009)
- The use of quadrupole time-of-flight mass spectrometer for the elucidation of diclofenac biotransformation products in wastewater (Journal of Chromatography A, 2008)
- The challenge of the identification and quantification of transformation products in the aquatic environment using high resolution mass spectrometry (Spinger Book Edited Series: Xenobiotics in the Urban Water Cycle, in press 2009).

3.3.1 Scientific paper: “Mass spectrometry for identifying pharmaceutical biotransformation products in the environment”

Trends Trends in Analytical Chemistry, Vol. 26, No. 11, 2007

Mass spectrometry for identifying pharmaceutical biotransformation products in the environment

T. Kosjek, E. Heath, M. Petrović, D. Barceló

Many classes of pharmaceuticals have been detected in wastewaters and surface waters around Europe, but little is known about their occurrence, fate and potential harmful effects on the environment, and that makes them an important group among those compounds considered to be new emerging contaminants. To understand the cycling of pharmaceuticals and their metabolites, it is essential to possess qualitative and quantitative information on their presence in the environment. This review covers the current status and future prospects of advanced hyphenated mass spectrometric (MS) techniques (gas chromatography-MS (GC-MS) and liquid chromatography-MS (LC-MS)) in elucidating the structures of trace contaminants, namely pharmaceutical biodegradation products in complex environmental matrices. The article is oriented towards technique and method and discusses capabilities, potential and limitations of different GC and LC mass analyzers (quadrupole, ion trap, time-of-flight and hybrid techniques) in dealing with analytical challenges of complex matrices and trace contaminants. We also give practical examples of their applications. The main scope of this article is to support and to facilitate the on-going research on pharmaceutical biodegradation products in environmental samples.

Keywords: Biodegradation; Environmental sample; Gas chromatography; Hyphenated technique; Identification; Liquid chromatography; Mass spectrometry; Metabolite; Pharmaceuticals

T. Kosjek, E. Heath*
Jožef Stefan Institute, Department of Environmental Sciences, Ljubljana, Slovenia

M. Petrović
ICREA, Catalan Institution for Research and Advance Studies, Barcelona, Spain

D. Barceló
IIQAB-CSIC, Department of Environmental Chemistry, Barcelona, Spain

*Corresponding author.
Tel.: +386 1 477 3584;
E-mail: ester.heath@ijs.si

1. Introduction

Emerging contaminants have long been present in the environment; however, until recently, they have not gained scientific or public attention. These pollutants are not yet included in routine monitoring programmes at the European level, but, depending on ecotoxicity results and data regarding their occurrence and fate in the environment, they may be candidates for future regulations [1]. They are believed to pose a burden on the environment and human health.

Among new emerging contaminants, pharmaceuticals belong to a group of increasing interest due to their pharmacological activity and rising consumption deriving from their use in human and veterinary medicine [2,3]. Moreover, due to their ubiquitous presence in the environment arising from continual input into the aquatic compartment, they are considered as ''pseudo'' persistent pollutants [4]. The discharge of therapeutic agents in effluents from production facilities, hospitals, and private households, improper disposal of unused drugs, and the direct discharge of veterinary medicines all lead to contamination of environmental waters, and wastewater-treatment plants (WWTPs) are considered to be a major source [2,3,5–7].

Once they reach a WWTP, these compounds are completely mineralized, partially degraded, or pass through unaltered. Since WWTPs provide the first, and possibly the only, opportunity for removal of pharmaceutical residues, it is important to characterize their fate during WWT [8]. Pharmaceuticals require a certain chemical stability to avoid degradation before they have their medicinal effect [9]. Rather than hydrolytic processes [10], enzymatic transformations are therefore of major importance for the degradation (transformation) of pharmaceuticals during the biological WWT process [11]. Enzymatic reactions are often complex, involving various competing or parallel pathways, which, due to enzyme

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induction or inhibition processes, lead to multiple reaction products that may:

(a) retain the properties of the parent compound [12];
(b) show greater toxicity than the parent compounds [13–15];
(c) lead to synergistic or additive pharmacological effects in combination with other compounds present; or,
(d) lose their pharmacological activity [10].

As pharmaceuticals are present in the environment in trace concentrations, their effects are chronic rather than acutely toxic. Their effects depend on bioavailability, susceptibility to the compound and duration of exposure to the non-target organism [16]. In contrast to human metabolism of pharmaceuticals, which has to be studied in detail before pharmaceuticals are approved, microbial degradation of such compounds, their transformation pathways and products have gained attention only recently [11]. Due to the different enzyme systems involved, the enzymatic transformations in waste and environmental waters are generally not comparable to those in mammals. This assumption was supported by Jjemba [16], who showed that drugs highly metabolized in target organisms (and therefore excreted in low proportions) may have an inherently low environmental (bio)degradability. Other studies [8,17] reported similar compounds originating from enzymatic biotransformation and human metabolism, but this might, however, have applied to a minor extent of biotransformation products, which were easily identified by targeted search of metabolites.

The main drawback of the conventional analytical approach is target-compound monitoring, which is often insufficient to assess the environmental relevance of emerging contaminants [4]. To better understand the risk that they pose to the environment, it is essential to consider the occurrence of both parent pharmaceuticals and their transformation products [3]. The occurrence of parent pharmaceuticals [18–22] and their human metabolites [23–26] in the aquatic environment has been the subject of numerous studies; however, only a few scientific publications concern their breakdown products [3,4,11]. There are several reasons for this but the most important is their identification, which requires application of advanced instrumental methods. Among these methods, liquid chromatography with mass spectrometry (LC-MS) has experienced impressive progress, in terms of both technology development and application [10,27]. Transformation products are numerous and vary greatly in chemical structure, and, in many cases, they are not commercially available. This means that in-house chemical synthesis of authentic transformation products is often the only option available to verify their structure, and that can be complex, time consuming and expensive.

In structure elucidation of pharmaceutical photodegradation products, there have been a few studies published recently [10,28–30]. Due to the greater complexity of the task (e.g., screening for trace levels of unknown chemical structures in complex media (wastewater) that involve biomass activity), there have been fewer studies involving identification of their biodegradation products [3,17,31,32]. However, a growing number of publications on this subject justifies a literature review on recent developments in hyphenated MS techniques regarding identification of biodegradation products in environmental samples.

This article sets out the current status and the future prospects of MS techniques, focusing on their capabilities and potential with respect to their application in elucidating the structure of trace contaminants in complex environmental matrices. As an example of trace contaminants with unknown chemical structure, we studied products of microbial degradation of pharmaceuticals. Only one review has been published recently on the use of MS techniques to determine pharmaceutical biodegradation products and human metabolites [32]. Even though human metabolites are important source of drug metabolites in the environment, this study does not specifically address them, as, by default, they have to be identified during clinical studies required for marketing authorization permit. The present work focuses on research on identifying drug metabolites in the environment. Biodegradation mechanisms and organisms involved in these processes are beyond the scope of this work.

2. Capability and potential of instrumentation

Depending on the objective of a particular analysis, analytical methods can generally be classified into categories of methods (i.e. screening, quantitative, confirmatory and elucidation) [33].

Elucidation methods are crucial in identifying pharmaceutical biodegradation products and should discover the identity of a suspected or unknown analyte that was previously detected by a screening method but not confirmed afterwards. Screening for unknown non-polar and thermally stable contaminants can be performed by GC–MS. Identification of the unknown compounds is then based on comparing their mass spectra with special mass spectral libraries (e.g., NIST), together with interpreting very abundant fragment-ion patterns obtained by electron ionization (EI)-MS [3,17,34,35]. However, single MS coupling will not give sufficient information to confirm the chemical structure of an unknown, even though the resolved chemical structure is in agreement with the library report. Also, comparison with authentic standard compounds is possible, but they are rarely commercially available. More recently, LC-MS (or

LC-tandem MS (LC-MS^2)) methods have been replacing GC-MS (or GC-MS^2) as they present obvious advantages (e.g., reduced sample pre-treatment and their capacity to determine polar or thermally stabile compounds (i.e. numerous pharmaceuticals and their transformation products)).

Table 1. Chemical structures of parent compounds, identified biotransformation products, MS identification methods and type of biodegradation experiments

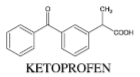
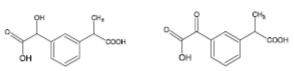
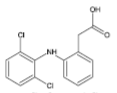
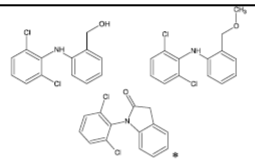
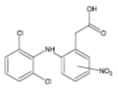
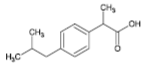
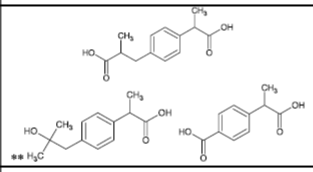
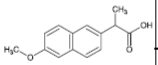
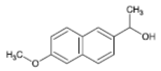
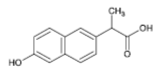
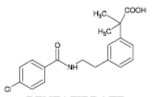
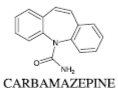
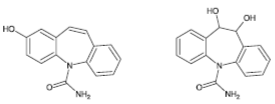
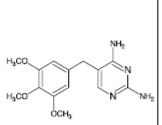
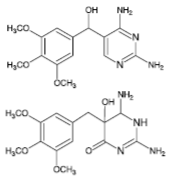
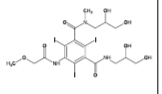
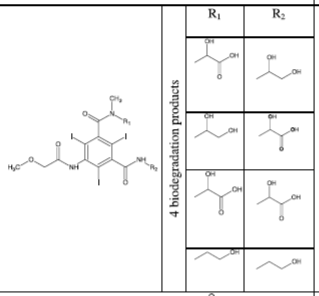
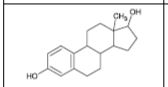
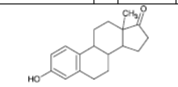
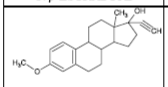
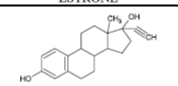
Parent compound	Biotransformation product(s)	chr. separation / MS detection	Biodeg. experiment	comments	Ref.
 KETOPROFEN		LC / ESI-MS^2 (QqQ)	*In-vitro* batch experiment with active sludge		[11]
 DICLOFENAC	 *	GC / EI-MS (Q)	Laboratory scale active sludge reactors	*: Confirmation with authentic standard	[3]
		LC / ESI-QqToF	Laboratory scale active sludge reactors		[46]
 IBUPROFEN	 **	GC / EI-IT-MS	Laboratory-scale biofilm reactors & *in-vitro* batch experiment with active sludge		[17]
	**2-hydroxy-ibuprofen (also reported by [17]) & hydroxy-ibuprofen (position of –OH moiety is unknown)	LC / ESI-MS^2 (QqQ)	*In-vitro* batch experiment with active sludge		[11]
 NAPROXEN		GC / EI-MS (Q)	Laboratory scale active sludge reactors		[3]
		LC / ESI-IT-MS	*in-vitro* batch experiment with three *Cunninghamella* sp.	Contirmation by ^{1}H-NMR	[42]
 BENZAFIBRATE		LC / ESI-MS^2 (QqQ)	*In-vitro* batch experiment with active sludge		[11]
 CARBAMAZEPINE		LC / ESI-MS^2 (QqQ)	WWTP (PE = 75 000)	Determination of human metabolites → biotransformation in WWTP	[8]

Table 1 (*continued*)

 TRIMETHOPRIM		LC / ESI-IT-MS, LC / ESI-QqToF-MS	*In-vitro* batch experiment with nitrifying activated sludge	Confirmation of chemical structures with H/D-exchange experiment	[31]
 IOPROMIDE	 4 biodegradation products	LC / ESI-IT-MS	*In-vitro* batch experiment with (nitrifying) activated sludge	H/D-exchange experiment essential for structure elucidation	[43]
 17β-ESTRADIOL	 ESTRONE	GC / EI-IT-MS	Aerobic *in-vitro* batch experiment with activated sludge		[37]
 MESTRANOL	 17α-ETHINYLESTRADIOL				

In MS, two common strategies are applied, depending on the instrumentation used:

- one relies on MS measurement of accurate molecular mass, and subsequently, the determination of empirical formula using orthogonal acceleration time-of-flight (oaToF) instruments; and,
- the other involves structural elucidation on the basis of structural information gained in MS (MS^2) experiments that can be accomplished either by coupling mass analyzers in series (triple quadrupole (QqQ)) or by using a single ion-trap (IT) analyzer [10].

Among recent advances in MS, a new powerful identification tool has become available, combining the advantages of the ion separation and detection principle of ToF with the fragmentation obtained with MS (MS^2). A hybrid quadrupole-ToF mass spectrometer (QqToF) permits the acquisition of full-scan product ion spectra, with the accurate mass of the product ions, thus yielding results of much higher degree of certainty and making it useful for structure elucidation of unknown compounds [27], as well as for identifying target compounds. The use of QqToF instruments in the environmental sciences is still rare, mainly because of their high cost; however, application of this technique is increasing simultaneously with new trends in environmental analysis (e.g., identification of biodegradation or photodegradation products of environmental contaminants).

3. GC-MS

Electron impact ionization (EI), like chemical ionization (CI), is commonly used in GC-MS instruments. EI is a ''hard'' ionization technique and yields mass spectra with abundant fragment ions and thus a lot of structural information. However, EI often leads to the loss of an intact molecular ion, so the origin of an unknown can be determined only using characteristic ion fragments. Also, GC is appropriate for the determination of relatively volatile compounds and it usually requires cumbersome

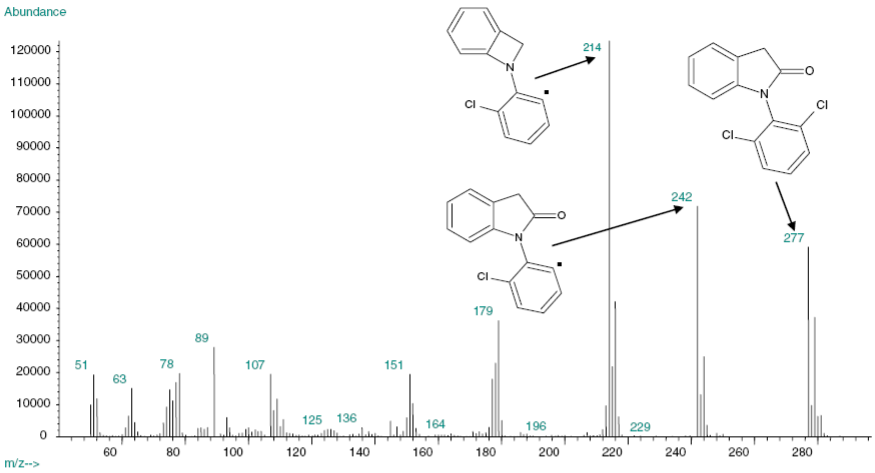


Figure 1. EI mass spectrum obtained from a degradation product [(2,6-dichlorophenyl)-1,3-dihydro-2H-indol-2-one] of diclofenac (adapted from [3]).

sample preparation, so it is important to consider that "degradation products" identified may not only originate from environmental processes, but also be generated due to relatively harsh conditions of sample preparation and analysis (e.g., thermal instability during GC analysis). Furthermore, GC often requires derivatization of polar compounds, and that reduces the chance of finding a suitable match in MS databases.

In practice, there have been few studies that reported the application of GC-MS to identifying pharmaceutical biodegradation products [3,17,37]. Kosjek et al. [3] identified three degradation products of diclofenac and one of naproxen (Table 1) in a pilot WWTP effluent using single-quadrupole-MS to induce in-source fragmentation. Identification was based on fragmentation of the parent compounds and comparison to spectra held in the NIST Library. One compound structure was confirmed by comparison with commercially available authentic standard compound (Fig. 1). To determine and to confirm the proposed chemical structures of degradation products, high-resolution and/or tandem MS should be used.

Zwiener et al. [17] reported biotransformation of ibuprofen in activated sludge and biofilm reactors. Ibuprofen is, together with naproxen and diclofenac [3], a non-steroidal anti-inflammatory drug (NSAID) commonly used for its analgesic and antipyretic activity. NSAIDs are also chronically applied in treatment of rheumatic disease. Three major metabolites of ibuprofen (Table 1) were identified using GC with an IT detector. Their identification was performed in MS full-scan mode and was based on the EI mass spectra of their methyl esters and comparison with data in the literature [36]. For selective determination and quantification of ibuprofen and its metabolites, MS^2 detection by selected reaction monitoring (SRM) of the mass transition precursor ion → product ion was achieved. One or two, where possible, additional product ions were recorded to confirm peak identity. Since authentic standards of the biotransformation products identified were not available, quantification of the compounds was based on the SRM response of ibuprofen for the hydroxylated metabolite and on the SRM response of the terephthalic acid for both carboxylated compounds [17].

In another study, Ternes et al. [37] used GC-EI-IT-MS to study the biodegradability of estrogens in an aerobic batch reactor with activated sludge from a municipal WWTP. The experiments revealed that 17β-estradiol was oxidized to estrone and mestranol was transformed in small portions into 17α-ethinylestradiol. Further, two glucuronides of 17β-estradiol (17β-estradiol-17-glucuronide and 17β-estradiol-3-glucuronide) were cleaved in contact with the diluted activated sludge solution, so 17β-estradiol was released.

4. LC-MS

Since the introduction of atmospheric pressure ionization techniques, such as electrospray ionization (ESI), LC-MS has played an increasingly important role in environmental analysis. ESI and atmospheric pressure chemical ionization (APCI) can analyze a broad range of compounds, including non-volatile, thermally-labile and

polar species. In addition, ESI and APCI provide high sensitivity, which is essential for environmental analysis where contaminants are found at trace (ng/L or μg/L) levels [38,39].

To cope with sample composition that is complex and chromatographic peaks that are not fully resolved, MS^2 methods are predominant in environmental analysis, applying QqQs or ITs. LC-MS with single quadrupole mass spectrometers can also be used to produce fragmented spectra. This is done using ''in-source collision-induced dissociation'', which can provide higher sensitivity in some cases, but much less selectivity than MS^2, because, in this process, co-eluting analytes and matrix components are also fragmented and can result in a mixed mass spectrum of the analyte and interfering compounds [38].

4.1. QqQ-MS

QqQs are very widely used for sensitive and selective quantification of target compounds that show specific mass transitions in the multiple reaction monitoring (MRM) mode. For structure elucidation, neutral loss scan, precursor-ion scan or product-ion scan modes of QqQ operation may be appropriate [38,40]. The product-ion scan mode was applied by Quintana et al. [11] for analyses of pharmaceutical biotransformation products by recording product-ion spectra at different collision energies. Of five acidic pharmaceuticals tested, four gave metabolites (ketoprofen, naproxen, benzafibrate, ibuprofen; Table 1), but no transformation was found for diclofenac. Ketoprofen was the only compound found to be metabolized as a single substrate and two of its metabolites were detected at a relatively high intensity. In parallel with LC-MS analyses, which were employed mainly to follow the removal of pharmaceuticals, dissolved organic carbon (DOC) was determined to distinguish between mere transformation of the parent compound and complete degradation (i.e. mineralization). The transformation pathway of ketoprofen was confirmed by visible changes from UV chromatograms, which were recorded simultaneously. Addition of an external carbon source allowed co-metabolic degradation of the other pharmaceuticals; however, the DOC data were less significant concerning mineralization, as the external carbon source was applied in much higher concentration than pharmaceuticals. Although DOC might have provided valuable information regarding the fate of the tested pharmaceuticals, it was not crucial for resolving the chemical structure of metabolites examined, so additional confirmation methods (e.g., high-resolution MS (HRMS) and authentic standards are required to confirm the proposed structures of biotransformation products. Quintana et al. [11] analyzed biodegradation products using the same chromatographic separation as in target analyses of acidic pharmaceuticals, as well as mass detection in negative-ionization mode. The latter is only appropriate for a small range of pharmaceuticals (e.g., those containing a carboxylic group). This can also be observed from the identified biodegradation products, which contain at least one carboxylic group each. The product-ion scan with QqQ, as used by Quintana [11], is not widely applied in identifying unknowns. One reason is the lower sensitivity of QqQs in the scan mode, which is a major disadvantage of these instruments compared to IT and ToF instruments [38]. QqQ represents primarily an instrument of choice in target quantitative analysis and possesses excellent sensitivity; however, preselection of fragments is required, which leads to a loss of qualitative information on the compound examined.

Another approach, adopted by Miao and Metcalfe [41], was applied when identifying carbamazepine biodegradation products (Table 1) with QqQ MS. The study primarily focused on the determination of carbamazepine and its human metabolites in aqueous samples collected from WWTPs. Carbamazepine is used widely as an antiepileptic, applied in high daily dosages (600–1200 mg). It has been reported as resistant to biological treatment [5,7]; and, because of the continual input into the environment, it causes, together with its active metabolites, significant environmental concern. Mass determination was performed in ESI positive-ion mode using direct infusion of individual standard solutions of the analytes. Product-ion mass spectra were recorded to resolve fragmentation of each analyte and according to the characteristic m/z values MRM channels were set. After LC-ESI-MS^2 conditions were optimized, target analysis for carbamazepine and its human metabolites was carried out. However, the concentrations of two metabolites, 2-hydroxycarbamazepine and 10,11-dihydro-10,11-dihydroxycarbamazepine (Table 1), increased significantly in the treated wastewater relative to the untreated wastewater, which suggests that the compounds were microbiologically transformed during WWT, most likely by deconjugation [8] of glucuronide conjugates from human biological fluids. This study shows the importance of understanding the processes occurring in the environment, in particular during WWT to foresee the metabolites that could be formed during WWT.

4.2. IT-MS

Three-dimensional quadrupole IT-MS instruments allow MS-MS in a time-sequenced series of ion isolation, fragmentation and trapping of the product ions formed. IT-MS uses three electrodes to trap ions, where a mass spectrum is generated by changing the electrode voltages to eject ions from the trap. They are unique in that they allow MS^n, which is particularly attractive in identifying new compounds, since it provides fragmentation pathways that, in many cases, are not as obvious in product-ion spectra obtained by QqQ and QqToF.

In general, quadrupole ITs have high sensitivity in the scan mode, but neutral loss scans are not possible with

this technique, and quantification is less reliable than MRM with a triple-quadrupole instrument [38]. Zhong et al. [42] used an IT mass spectrometer to identify microbial transformation products of naproxen. Metabolites of naproxen produced by *Chunninghammella* species were isolated and identified using a combination of MS and nuclear magnetic resonance (NMR). Compared to the controlled samples, three deprotonated molecular ions $[M\text{-}H]^-$ were observed, where two of them belonged to biotransformation products of naproxen, while the third belonged to the parent compound. The chemical structures of biodegradation products (desmethyl-naproxen and its sulphate conjugate; Table 1) were elucidated based on comparison with-typical fragmentation pattern of the parent compound in MS, MS^2 and MS^3 full-scan spectra. The compounds identified by IT were further confirmed by ^{1}H-NMR analysis. To isolate a sufficient quantity of biotransformation products to allow ^{1}H-NMR analysis, naproxen was applied in these tests in a high concentration (250 mg/L) [42]. Importantly, two objectives should be considered when assessing the reliability of this study; first, is naproxen biologically transformed under the same pathways in high concentrations comparing to trace concentrations in wastewater, and, second, is the *in vitro* batch biodegradation experiment (bottle test) sufficient to imitate real conditions in a WWTP?

In another study, MS^n data from IT-MS were complemented by accurate-mass measurements using ToF-MS to elucidate the structures of metabolites [31]. Unfortunately, not many environmental laboratories have access to HRMS. Another possible way to establish the identities of novel metabolites is to use complementary analysis involving hydrogen/deuterium (H/D) exchange experiments. Such an approach is useful in the quest for pharmaceutical metabolic products, because many biotransformations that render the compound more polar tend to form metabolites that are not significantly changed in terms of overall chemical structure [43].

Eichhorn et al. [31] identified major biodegradation products of antimicrobial trimethoprim, a first-choice drug for treatment of bacterial urinary infections, which is commonly used in combination with sulfamethoxazole. The biodegradation products were generated in a small-scale laboratory batch reactor. The chemical structures of two biotransformation products (Table 1) were elucidated by applying multi-stage fragmentation studies in combination with H/D-exchange experiments using an ESI-IT mass spectrometer. In addition, a quadrupole ToF mass spectrometer was used to provide accurate-mass measurements. Of the two metabolites produced in the nitrifying activated sludge, the first to elute was α-hydroxy-trimetoprim (t_R = 8.6 min, Fig. 2) with a molecular weight of 16 Da ($[M + H]^+$, m/z 307) higher than trimethoprim (t_R = 10.1 min, Fig. 2), suggesting that the parent compound had undergone an oxidative transformation. The second-eluting metabolite of trimethoprim (t_R = 8.9 min, Fig. 2) had a molecular weight of 34 Da, also higher relative to the parent

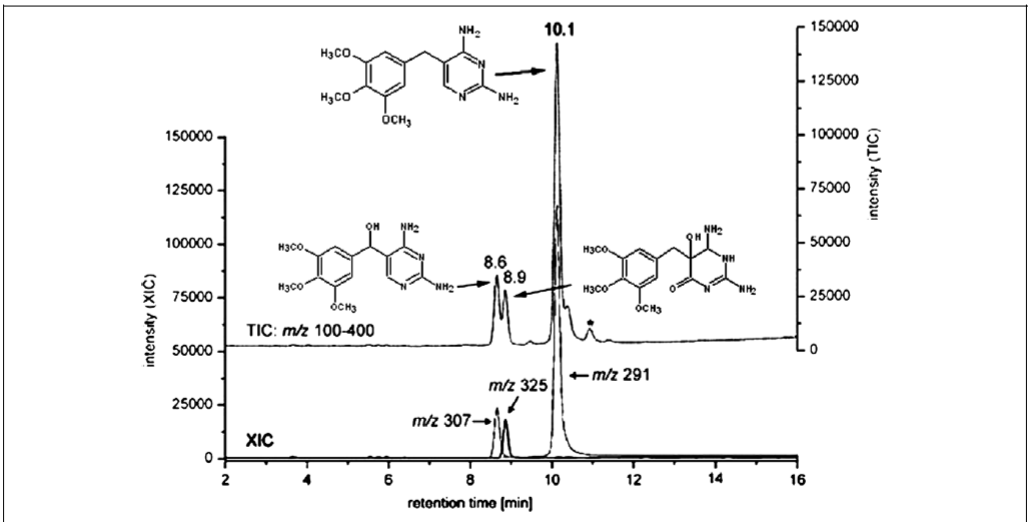


Figure 2. LC-ESI-MS chromatogram obtained from sample from batch reactor. Upper mass trace shows total ion chromatogram (TIC) acquired over mass range of m/z 100–400 in positive-ion mode. Extracted ion chromatograms (XIC) are shown in the lower part of the graph. Chemical structures of parent compound and both metabolites are shown, together with the corresponding peaks (adapted from [31]).

compound, indicating oxidation or the addition of a functional group.

(+)-ESI-MS^2 and (+)-ESI-MS^3 mass spectra of characteristic product ions were recorded for both biotransformation products, as well as for the parent compound. Further, H/D exchange was performed and the increments of ion masses were analyzed in the same manner to confirm the proposed fragmentation. Also, accurate-mass measurements of both metabolites were provided by QqToF-MS, achieving absolute mass errors <5 mDa for all fragment ions, and that confirmed the postulated elemental compositions. To ensure that the trimethoprim degradation pathway was independent of its initial concentration, the experiment was carried out at 20 mg/L and later repeated at a concentration three orders of magnitude lower [31]. Namely, depending on their concentration, pharmaceuticals can affect biomass structure [44], which may alter their biodegradation pathways and thus lead to the formation of different biotransformation products.

Pérez et al. [43] reported the biotransformation of iopromide in an *in vitro* biodegradation experiment with conventional and nitrifying activated sludge collected from a municipal WWTP. Iopromide is an iodinated X-ray contrast compound, which is not used for treatment or prevention of illnesses, such as other pharmaceuticals, but for diagnostic purposes (e.g., imaging human tissues and organs). The group identified four iopromide biotransformation products using LC and (+)ESI-IT-MS in combination with H/D-exchange experiments. Two compounds were generated upon oxidation of the primary alcohols on iopromide side chains to carboxylic acid, a third was the subject of double carboxylation (on two side chains), while the fourth biotransformation product underwent dehydroxylation on a side chain. Table 1 shows the chemical structures of the parent compound and the biotransformation products.

Of IT instruments, we also found that Qq-linear IT (Qq-LIT) is a promising configuration for identification of pharmaceutical metabolites. The linear two-dimensional IT mass spectrometer is based on an ion path of a QqQ mass spectrometer, using the collision cell of the final mass analyzer as the LIT. The LIT has two major advantages over conventional three-dimensional IT: larger ion-storage capacity; and, a higher trapping efficiency. With a QqQ instrument and a Qq-LIT, significantly enhanced product-ion scanning performance can be obtained while retaining all the QqQ capabilities, such as precursor-ion and neutral-ion scan [43]. As attention to the subject is so recent, to our knowledge, there is not yet any published data available on identifying pharmaceutical metabolites in environmental samples.

4.3. ToF and QqToF instruments

ToF instruments measure the mass-dependent time it takes ions of different mass-to-charge ratios to move from the entrance of the analyzer, where they are orthogonally accelerated in a pulsed fashion, to the detector. Full-scan sensitivity, high-mass resolution and mass accuracy provided by ToF-MS are especially suited for the identification of trace-level unknowns in complex environmental samples. ToF instruments provide mass determinations with an error typically lower than 2 mDa; however, structural elucidation is feasible primarily for compounds with easy in-source fragmentation or compounds having a characteristic isotopic pattern [10].

Although ToF instruments have been described as powerful tools in structure elucidation of unknown compounds [28,45], we did not find any publications regarding their use in determination of pharmaceutical biotransformation products. However, two studies [31,46] applying QqToF instruments have been published. The main advantage of QqToF is its ability to perform accurate product-ion mass scans. Thus, while the accurate mass obtained from ToF allows us to establish the elemental composition of a compound, QqToF reveals the elemental compositions of all product ions obtained; this is a crucial feature of this analyzer in the elucidation of chemical structure of an unknown compound [33]. In studies of microbial transformations of pharmaceuticals, QqToF was used solely as a tool for structure elucidation in one case [46], while Eichhorn et al. [31] combined it with IT. Kosjek et al. [46] used QqToF in a study of biodegradation of pharmaceuticals in a pilot WWTP. In this experiment, the biotransformation product of diclofenac was recognized in a pilot WWTP effluent on the basis of its typical isotopic pattern. The ESI negative-ionization MS method was optimized by applying various cone voltages to obtain the most favorable fragmentation for accurate-mass measurements of fragment ions and for further fragmentation by collision-induced dissociation (CID). The mass-accuracy measurements taken for the precursor ion plus the five most abundant product ions showed an error typically lower than 2 mDa, which is generally accepted as an accurate-mass measurement [35,47]. The mass-accuracy measurements obtained were assessed according to 2002/657/EC [48], the quality criteria for MS identification and confirmation of organic residues and contaminants, based on the use of identification points (IPs) and Hernández et al. [49], who modified the IP assessment for measurements with HRMS. According to these criteria, the total IPs earned was 16, showing that QqToF is an excellent instrument for confirmation and identification.

5. Conclusions and future trends

As a result of legislation that demands the study of major metabolic routes prior to obtaining an authorization

permit for marketing new pharmaceuticals, human-drug metabolites have been well studied. However, this is not the case with biological degradation metabolites formulated as a result of environmental microbiological action on pharmaceutical residues discharged into the environment. The latter have been recognized only for a small number of pharmaceuticals, possibly because of the burden of untargeted analysis to be carried out in complex environmental matrices at trace concentrations.

In parallel to studies of pharmaceutical fate and effects in the environment, scientific interest focuses on technological advances and developments achieved in hyphenated MS techniques, where increased sensitivity and selectivity play important roles. To gain sufficient data for secure structure elucidation of the proposed (bio)degradation products, a combinatory analysis should be performed combining MS^2 techniques (e.g., QqQ, IT or Qq-LIT), with HRMS (e.g., ToF or Fourier transform) for accurate-mass measurements.

Alternatively, QqToF alone can be applied for the same purpose. However, due to the price and the lower sensitivity of QqToF instruments, the number of environmental applications remains low. Yet, developments expected in QqToF instruments may bring improved sensitivity and linear dynamic range, which will contribute to wider acceptability of these instruments in environmental analysis.

Recently, another hybrid MS system has been launched, which may represent an alternative to QqToF. LTQ-MS^n-FT Orbitrap combines LIT with the Orbitrap analyzer, using Fourier-transform MS to attain a high-resolution mass performance. The instrument may become very important in the field of untargeted environmental analysis, due to not only its high resolution and high mass accuracy, but also especially its wider dynamic range, when compared to that of QqToF.

However, when considering pharmaceutical biodegradation products in environmental samples, one should bear in mind that the experimental set-up and sampling conditions will greatly influence the availability and the identification of stable biodegradation products. Only when studies of pharmaceutical biodegradation products are combined with detailed toxicity studies can we attempt to assess the threat that they pose to human health and to the environment.

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3.3.2 Scientific paper: “Metabolism studies of diclofenac and clofibric acid in activated sludge bioreactors using liquid chromatography with quadrupole - time-of-flight mass spectrometry”

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Metabolism studies of diclofenac and clofibric acid in activated sludge bioreactors using liquid chromatography with quadrupole – time-of-flight mass spectrometry

Tina Kosjek [a], Ester Heath [a,*], Sandra Pérez [b], Mira Petrović [b,c], Damia Barceló [b]

[a] Jožef Stefan Institute, Department of Environmental Sciences, Ljubljana, Slovenia
[b] IDAEA – CSIC, Department of Environmental Chemistry, Barcelona, Spain
[c] ICREA, Catalan Institution for Research and Advance Studies, Barcelona, Spain

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SUMMARY

Two environmentally relevant pharmaceuticals, the non-steroidal antiinflammatory drug, diclofenac and the pharmacologically active metabolite of several serum triglyceride-lowering pharmaceuticals, clofibric acid, were subjected to microbiological transformation in activated sludge bioreactors, and the production of breakdown products was studied. For separation, detection and identification of diclofenac's metabolites a UPLC-(+)ESI-QqToF-MS was employed, which enabled the detection of seven transformation products of diclofenac, all including the diagnostic fragment ion at *m/z* 214. The chemical structure of one metabolite was proposed, which was produced by dehydratation and lactame formation. Further investigations revealed additional two metabolites, which were isomeric structures with an elemental composition $C_{13}H_{10}NCl_2$; however, their chemical structures were not completely resolved. In addition, another biodegradation product showed an abundant fragment ion at *m/z* 295, the elemental composition of which was confirmed with a high degree of certainty as $C_{14}H_{11}NO_2Cl_2$. The biodegradation of clofibric acid revealed one metabolite in the (-)ESI-QqToF chromatogram, 4-chlorophenol, which is known to exhibit a higher toxicity than the parent compound. This study confirms that further research is needed on the formation of stable metabolites both during wastewater treatment and in the environment. It also highlights the need for parallel toxicity testing. In addition, this study suggests that more needs to be known about the environmental fate of pharmaceuticals so that we are able to provide a comprehensive risk assessment.

Introduction

Pharmaceutically active substances are emerging as a new group of environmental contaminants. Their effects on humans and animal species are, by default, thoroughly investigated during preclinical and clinical studies required for a marketing authorisation permit as a part of a drug development process; however, their behaviour, effects and fate in the environment have only in the last decade begun to gain scientific and public attention.

Pharmaceuticals are predominantly synthetic chemicals, designed to produce a pharmacological response at specific sites of action within the target organism, and require a certain chemical stability in order to reach these sites in an unaltered form. Their metabolic stability may be environmentally manifested in their insufficient removal during wastewater treatment and by their persistence to environmental degradation. However, due to the strong dissimilarities between human and environmental metabolic pathways, there is no direct correlation in the behaviour and fate of pharmaceuticals as subjected to human or environmental degradation. The persistence of the parent pharmaceuticals together with their continuous intake and release are leading to detectable concentrations in surface waters (Wiegel et al., 2004) that may represent a threat to the aquatic wildlife and a potential health risk to humans via the consumption of drinking water (Hao et al., 2006). So far the actual concentration levels of pharmaceuticals in the environment are three to four orders of magnitude lower than that needed to produce a human pharmacological effect; therefore the likelihood of any acute health risk to humans is low (Fent et al., 2006). Diclofenac has, however, been associated with hepatotoxicity in humans, which was caused independently of the administered dose (Boesterli et al., 1995) and was possibly related to the formation of pharmacologically active metabolites (Ngui et al., 2000). Diclofenac is reported to be ecotoxic in rainbow trout (Triebskorn et al., 2004; Schwaiger et al., 2004) and was found responsible for an unusually high death rate among Asian vultures, fed with diclofenac treated livestock (Oaks et al., 2004),

* Corresponding author. Tel.: +386 1477 35 84; fax: +386 1251 93 85.
E-mail address: ester.heath@ijs.si (E. Heath).

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which suggests that its pharmacological response may be species specific. In addition, it is important to consider bioaccumulation, possible additive or synergistic effects and especially, the toxicity of their microbial or physico-chemical transformation products compared to parent compound (Isidori et al., 2005).

Numerous investigations regarding the occurrence of pharmaceuticals in the aquatic environment have been carried out (Petrović et al., 2007; Kosjek et al., 2005; Zuccato et al., 2004), but there remains a major gap in our knowledge particularly concerning their fate and microbial transformations (Kosjek et al., 2007a). The identification of these compounds is essential, not only to provide a comprehensive risk assessment on drug residues in the environment, but also for designing improved treatment technologies.

The objective of this study is to examine the microbial fate of two chlorine-bearing pharmaceuticals: diclofenac (DF) and clofibric acid (CLA). Diclofenac (DF) is a *cyclooxygenase* inhibitor, a non-steroidal anti-inflammatory drug (NSAID) with anti-inflammatory, analgesic and antipyretic effects. It is widely used for treatment of rheumatic disease and for mild to moderate pain relief. DF is frequently detected in wastewater treatment plant (WWTP) influents and effluents in $\mu g\,L^{-1}$ range (Metcalfe et al., 2004; Zwiener and Frimmel, 2004), as well as in surface waters in $ng\,L^{-1}$ (Wiegel et al., 2004; Buser et al., 1998). While DF was shown to be persistent to biological treatment (Zwiener and Frimmel, 2004; Kosjek et al., 2007b; Quintana et al., 2005), it is more susceptible to undergo rapid decomposition when exposed to natural sunlight in surface waters (Buser et al., 1998; Tixier et al., 2003; Agüera et al., 2005; Bartels and von Tümpling Jr., 2007). Clofibric acid, is a bioactive metabolite of serum triglyceride-lowering pharmaceuticals (ethofyllinclofibrate, etofibrate and clofibrate), used as antilipaemic agents, and has also been detected in waste and surface waters (Metcalfe et al., 2004; Zwiener and Frimmel, 2004). The environmental concern related to CLA arises from its environmental persistence (Doll and Frimmel, 2003) and resistance to water treatment technologies, both biological treatment and advanced oxidation processes (Zwiener and Frimmel, 2004; Tixier et al., 2003; Ternes et al., 2002). Diclofenac and clofibric acid are carboxylic acids with pK_a values of 4.2 (Rafols et al., 1997) and 3.2 (Scheytt et al., 2005a), respectively, and are thus negatively charged at environmental pH. The result is a notably higher mobility than that indicated by their organic carbon normalized sorption coefficients K_{OC} (HSDB, 2009). For this reason the sorption to sludge, suspended matter and sediments is not considered to be an important contribution to their actual elimination from waste and surface waters (Fent et al., 2006; Bartels and von Tümpling, 2007; Ternes et al., 2002; Scheytt et al., 2005b). Instead, microbial degradation is most likely to be the dominant sink in wastewaters (Fent et al., 2006; Quintana et al., 2005; Gröning et al., 2007).

In contrast to the majority of previously published studies (Quintana et al., 2005; Zhong et al., 2003; Eichhorn et al., 2005) our experiments used activated sludge flow-through reactors (Kosjek et al., 2007b), that mimicked real wastewater treatment processes and worked with concentrations of pharmaceuticals close to those found in actual WWTP (0.1–30 $\mu g\,L^{-1}$; Metcalfe et al., 2004). Albeit the applied levels still exceeded typical environmental levels by factor 5 to 10^3, this was considered an acceptable compromise between environmental concentrations and the detection limits of stable pharmaceutical metabolites, which are usually in percentage or promile amounts (Heath and Leskovšek, 1999) relative to the parent compound. This approach, in tandem with quadrupole time-of-flight mass spectrometry (QqToF-MS), allows us to move towards the goal of identifying previously unknown and unpublished diclofenac and clofibric acid biotransformation products.

Experimental

Pharmaceutical standards, solvents and calibrants

Diclofenac sodium salt and clofibric acid (both 97% purity) were obtained from Sigma–Aldrich (St Louis, MO, USA). 1-(2,6-dichlorophenyl)indolin-2-one (CAS: 15,362-40-0; >98.5% purity) was purchased from Chemosyntha NV (Meulebeke, Belgium). All organic solvents were Chromasol LC grade. Water, acetic acid and ammonium hydroxide were purchased from Sigma–Aldrich (Munich, Germany), acetonitrile and methanol, were both obtained from Riedel de Haen (Steinheim, Germany). Formic acid Suprapur (>98%), hydrochloric acid (37%), sodium hydroxide and acetone were obtained from Merck (Darmstadt, Germany).

Biodegradation experiments

To study pharmaceutical biodegradation we employed flow-through bioreactors on a lab-scale with 4 L aerated volume, which contained activated sludge obtained from a municipal WWTP. A detailed description of the bioreactor's configuration and their operation can be found in Kosjek et al. (2007b). The bioreactors had been continuously operated for 3 years and as a result harboured adapted microorganisms capable of utilising the two test pharmaceuticals (Kosjek et al., 2007b; Kraigher et al., 2008). For the purpose of this study, five flow-through parallel bioreactors (R0, R-5, R-50, R-200 and R-500) were operated and were fed by mineral–nutrient medium, containing yeast and meat extract, casein peptone and minerals (Kosjek et al., 2007b). The studied pharmaceuticals were spiked into the mineral–nutrient medium of R-5, R-50, R-200 and R-500, while R0 served as the control study, without the addition of pharmaceuticals. Operating conditions in the bioreactors e.g. hydraulic retention time (HRT), and the substrate composition and concentration, were altered according to experimental progress and are described below. After the experimental conditions were changed the biomass was allowed to acclimatise for 2 weeks, before the sampling was performed.

Standard operating conditions

Pharmaceuticals in concentrations of 5, 50, 200 and 500 $\mu g\,L^{-1}$ were spiked into the inlet containers of the bioreactors R-5, R-50, R-200 and R-500, respectively. For the purpose of producing diverse transformation products in sufficient amounts to allow their identification, we tested the bioreactor's performance at different HRT: 48, 24 and 12 h. As a result, an HRT of 24 h was found optimal for the formation of DF degradation products, while at an HRT 12 h we were able to successfully identify the CLA biodegradation product.

'Sole nutrient source' ('SNS') operating conditions

To examine the effect of a carbon source on DF depletion activity we spiked DF at 200 $\mu g\,L^{-1}$ into the R-200 bioreactor inlet. On the 2nd day of sampling we cut-off the mineral–nutrient medium (Fig. 4: *x-axis*) and used tap-water spiked with DF (keeping the same concentration) in its place. Sampling was then performed every 24 h, where besides the parent compound, also the formation of the most abundant degradation product (DP1) was measured simultaneously.

Control studies

To eliminate the effects of temperature and light the bioreactors were operated in darkness at 19 ± 1 °C. Further, during sampling, storage and sample preparation the samples were light protected. Standard solutions for spiking CLA and DF into the bioreactor inlet containers were kept under identical conditions (temperature and

light) as the bioreactors. The absence of degradation products in standard solutions for spiking was confirmed, which additionally excluded the possibility of abiotic degradation. In addition, the possibility of pharmaceutical transformation due to a low pH was eliminated by comparing the standard solutions in acidified (pH 2; set with hydrochloric acid 37%) and neutral methanol/MilliQ water (25/75) media. For additional confirmation, parallel acidic and neutral extractions of spiked deionised water samples were carried out.

Moreover, the bioreactor R0 was used as a control, in order to distinguish between those chromatographic peaks representing products of matrix biodegradation or bacteria lyses and the actual pharmaceutical degradation products.

Sampling and sample preparation

Samples were collected according to experimental progress and taking into account changes in the operating conditions of the bioreactors. The samples were collected and kept in the dark at 4 °C and processed within 24 h. Sample preparation consisted of filtering 200 mL of sample through a 1.2 μm microfibre glass filter (GF/C Whatman, UK) and 0.45 μm membrane filter (Sartorius, Goettingen, Germany) to remove suspended matter. The filtrate was then acidified to pH 2–3 prior to solid phase extraction (SPE), while comparative tests to eliminate the influence of pH on the production of degradation products were performed in parallel at neutral pH. Solid phase extraction was then performed using Oasis® HLB reversed-phase sorbents (60 mg/3 mL, Waters, Corp., Milford, MA, USA) and aided by a 12-fold vacuum extraction box (Supelco, Bellefonte, USA). The Oasis® HLB reversed-phase sorbents, assured good recovery for compounds having a broad spectrum of polarities. The following SPE procedure was applied for sample pre-concentration and clean-up: conditioning with 3 mL ethylacetate and 3 mL methanol, respectively, followed by equilibration with 3 mL aqueous hydrochloric acid solution at pH 2–3 and an enrichment step at a flow-rate 4–5 mL min^{-1}. The cartridges were then dried for 30 min under vacuum and the extracts eluted with 2 mL of an acetone:ethylacetate (1:1, vol.%) mixture, followed by 2.5 mL of ethylacetate. The eluants were concentrated to approx. 0.75 mL, transferred to 1.5 mL glass vials and dried under nitrogen. The dry extracts were then reconstituted with 0.5 mL of a methanol:MillQ water mixture (0.25:0.75, vol.%).

LC-MS analysis

After the sample preparation the extracts were screened for transformation products. First, the LC separation was performed by ultra performance liquid chromatography (UPLC) using a Waters Acquity UPLC instrument, equipped with a binary solvent delivery system and an autosampler. Separation was achieved with a 10 cm × 2.1 mm internal diameter Waters Acquity C_{18} 1.7 μm column. The injection volume was 10 μL. DF and its transformation products were analysed under positive ion (PI) conditions and eluted from the column using (A) 5 mM aqueous ammonium acetate/acetic acid (pH 4.8) and (B) acetonitrile/methanol (2:1, vol.%) mobile phases. The elution gradient began after 1 min and was linearly increased from 5% B to 60% B in 7 min, and further increased to 90% B in 2 min, kept isocratic for 1.5 min and finally decreased back to 5% B in 0.5 min. The total runtime was 12.0 min and the flow rate was kept at 400 μL min^{-1}. CLA and its degradation product were analysed under negative ion (NI) conditions. They were eluted by (A) water and (B) methanol. The elution gradient was linearly increased from 10% B to 90% B in 4 min, then increased to 95% B in 1 min, kept isocratic for 1 min and was finally decreased back to initial conditions in 1.5 min. The total runtime, including conditioning of the column to the initial conditions, was 10 min.

The LC system was coupled to a quadrupole time-of-flight tandem mass spectrometer, QqToF-Micro (Waters Corp., Milford, MA, USA). The nebulizer gas was set to a flow rate of 600 L h^{-1} at a temperature of 350 °C working under PI conditions, and to 500 L h^{-1} at a temperature of 400 °C under NI conditions. The cone gas flow rate was set to 50 L h^{-1}, and the source temperature was 120 °C, in PI and NI modes. The capillary voltages were set to 3000 V and 2800 V in PI and NI modes, respectively, and the cone voltages were varied from 10 to 40 V. The instrument was operated in the wide pass quadrupole mode, for MS experiments, with ToF data collected between *m/z* 50 and 600 and, to check for possible transformation products with higher molecular masses, also between *m/z* 100 and 1000 in ToF-only mode with low collision energy (4 eV). Data were collected in the centroid mode, with a scan time of 0.48 s with an interscan delay of 0.1 s. The MS/MS experiments were performed using variable collision energy (10–40 eV) in order to acquire greatest extent of structural information on each transformation product. All analyses were acquired using an independent reference spray via the Lock spray interference to ensure accuracy and reproducibility; the $[M+H]^+$ and $[M-H]^-$ ions of Val-Tyr-Val were used as a lock mass (*m/z* 380.2185 and 378.2029, respectively) at a flow rate of 10 μL min^{-1}. The accurate masses and elemental composition for the precursor and product ions were calculated using the MassLynx v.4.0 software. External mass calibration for positive and negative ESI modes was conducted prior to analysis over the mass range *m/z* 80–500 by infusing a solution of acetonitrile/0.1 M sodium hydroxide/10% formic acid (98:1:1) at a flow rate of 10 μL min^{-1}.

Results and discussion

Products of diclofenac's (bio)transformation

The detection and identification of transformation products require the application of sophisticated instrumentation, of which mass spectrometry (MS) is considered to be leading the field, both in terms of technology development and application (Petrović and Barceló, 2006). In this study a hybrid mass detector QqToF was applied, which combines the advantages of the ion separation and detection principle of the ToF and the fragmentation obtained with MS (MS^2). QqToF permits the acquisition of full-scan product ion spectra, with the accurate mass of the product ions, thus yielding results of much higher degree of certainty making it useful for structure elucidation of unknown compounds, as well as for identifying target compounds.

The usual approach to identify transformation products using LC-QqToF-MS was by screening the total ion chromatogram, acquired in full-scan mode and then selecting a specific protonated molecule for further product ion scans. The MS^2 experiments improved the selectivity of the analyses, providing the structural information together with accurate mass measurements of product ions, and thus allowed the precision in the low ppm range (Reemtsma, 2003). Complementary to the described procedure, an additional, rather uncommon approach was used for screening and identification of diclofenac's (bio)transformation products, which involved in-source fragmentation (Marquet et al., 2000) and application 'pseudo-MS^3' to a preselected diagnostic fragment ion.

The fragmentation of the parent compound, diclofenac, under (+)ESI-ToF (CV = 20–30 V; Fig. 1a) and collision induced dissociation (CID; CE = 30–40 eV; Fig. 1b) showed *m/z* 278, which corresponded to the neutral loss of H_2O (18 Da) from the protonated molecule $[M+H]^+$ with *m/z* 296. By further loss of CO, a cation $[M+H-H_2O-CO]^+$ with *m/z* 250 was produced and was followed by cleavage of ·Cl radical to form $[M+H-H_2O-CO-Cl]^{\cdot+}$ with *m/z* 215, or loss of HCl to $[M+H-H_2O-CO-HCl]^+$ with *m/z* 214. Acquisition at higher CE typically increased the abundance of the latter,

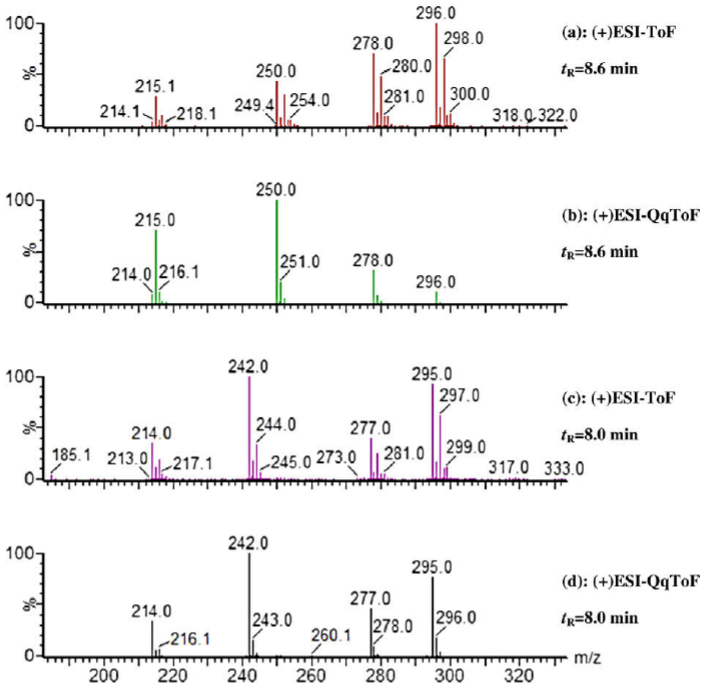


Fig. 1. (a) (+)ESI-ToF DF mass spectrum (CV = 20 V); (b) (+)ESI-QqToF DF mass spectrum of *m/z* 296 (CV = 15 V, CE = 10 eV); (c) (+)ESI-ToF mass spectrum of DP1 (CV = 30 V); (d) (+)ESI-QqToF spectrum of *m/z* 295 (CV = 20 V, CE = 10 eV).

which was by detailed inspection of the treated samples observed to be a diagnostic fragment ion of the residual parent compound and the breakdown products. Accordingly, selecting *m/z* 214 (7-[(2-chlorophenyl)imino]cyclohepta-1,3,5-trienylium fragment) in the Q1 of the hybrid mass analyzer for product ion generation allowed us to track down several breakdown products of diclofenac at the following retention times (t_R): 8.0, 8.8, 9.3, 9.6, 9.8, 10.2 and 10.7 min, indicated as DP1, DP2, DP3, DP4, DP5, DP6 and DP7, respectively in Fig. 2a (see also the Supplementary material for mass spectra). Further investigations, aiming to identify the detected compounds, were based on MS/MS fragmentation of the proposed precursor ions (protonated molecules), which were recognised at the set retention times (Table 1) in (+)ESI-ToF mode and were backed-up by accurate mass measurements. The identification of the DF degradation products was facilitated by recognising the isotopic peak pattern at A/A+2/A+4 with the nominal intensity ratio of 9:6:1, a characteristic of the presence of two chlorine atoms. The isotopic pattern was observed in the mass spectrum of protonated diclofenac (296/298/300, Fig. 1a), as well as in the (+)ESI-ToF mass spectra of the chlorine atoms-bearing degradation products of diclofenac (i.e. Fig. 1c). Accordingly, the chromatographic peak at 9.3 min (DP3) gave a typical di-Cl mass spectra with $[M+H]^+$ at *m/z* 278, 280 and 282 (mass spectrum not shown), which indicated a decrease in molecular mass for 18 Da, suggesting a dehydratation of the parent compound. The presence of the protonated molecule peak $[M+H]^+$, at *m/z* 278 was confirmed by its sodium adduct $[M+Na]^+$, at *m/z* 300. The MS/MS spectrum of *m/z* 278 showed a neutral loss of CO (28 Da), producing a cation $[M+H-CO]^+$ at *m/z* 250, and a loss of ·Cl at *m/z* 243 ($[M+H-Cl]^{\cdot+}$). The most abundant product ion, which also appeared in the (+)ESI-ToF mode, is $[M+H-CO-Cl]^{\cdot+}$ at *m/z* 215, while further cleavage of the second chlorine as a radical gave *m/z* 180, $[M+H-2Cl-CO]^+$. In addition, the product ion at *m/z* 208 was observed in the MS/MS spectrum, resulting from the loss of two chlorine atoms from the DP3 protonated molecule ($[M+H-2Cl]^+$). The analysis of *m/z* 278.0135 for the elemental composition yielded six hits within the set limits (settings: ±10.0 ppm mass error, C: 0–20, H: 0–20, N: 0–5, O: 0–5, Cl: 0–2; odd and even), among which only the formula $C_{14}H_{10}NOCl_2$ was plausible showing mass error of −1.4 ppm and best i-FIT at 0.6. DP3 was according to its fragmentation, accurate mass analysis and similarities to the published mass spectra, identified as 1-(2,6-dichlorophenyl)-1,3-dihydro-2H-indol-2-one (DP3, Table 1), and was formed by dehydratation with concurrent intramolecular lactame formation of diclofenac (Galmier et al., 2005). The identity of DP3 was ultimately confirmed by analyzing the commercially available authentic standard of the proposed compound, which matched the retention time and fragmentation pattern of the protonated molecule.

According to European Pharmacopoeia (2005) DP3 can be formed as an impurity during the synthesis of DF, but it also appears as a product of DF's thermal decomposition (Tudja et al., 2001; Roy et al., 2001), or may also be driven by the presence of the free ·OH radicals (Gaudiano et al., 2003). Additionally, in our preliminary experiments we compared control (untreated) samples, which were subjected to the described sample preparation procedure, followed by GC or LC analysis. The results confirmed the presence of DP3 as an artifact formed by thermal degradation

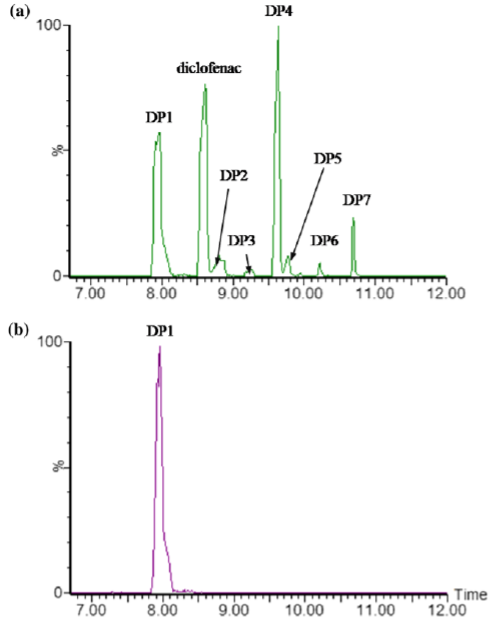


Fig. 2. (a) (+)ESI-QqToF chromatogram acquired by CID of *m/z* 214 with a chromatographic peak representing residual DF (t_R = 8.6 min) and as a minimum seven of its degradation products eluting at t_R: 8.0, 8.8, 9.3, 9.6, 9.8, 10.2 and 10.7 min, assigned by DP1, DP2, DP3, DP4, DP5, DP6 and DP7, respectively (acquired at CV = 35 V and CE = 40 eV); (b) (+)ESI-QqToF chromatogram acquired by CID of *m/z* 242 showing the DP1 peak, the only degradation product with a fragment ion at *m/z* 242, while the further fragmentation showed fragments 1 Da lower than the parent compounds' (CV = 30 V, CE = 20 eV).

during GC analysis, while the compound was absent in the samples analysed by LC. As in the present biodegradation study DP3 occurred only in the treated samples, it can be deduced that its formation was mediated microbiologically.

To elucidate the chemical structures of the remaining degradation products, initially low CV (10–15 V) was applied in (+)ESI-ToF scan mode in order to determine molecular weights, where their protonated molecules ($[M+H]^+$) were found at *m/z* 250.0202 and 250.0211, for DP4 and DP7, respectively. This finding suggested that DP4 and DP7 were isomeric structures holding the identical elemental composition ($C_{13}H_{10}NCl_2$). Their elemental composition was determined at ±10.0 ppm error (settings: C: 0–20, H: 0–20, N: 0–5, O: 0–5, Cl: 0–2; odd and even). Between the three resulting elemental formulae for DP4, only the elemental composition $C_{13}H_{10}NCl_2$ was found to be reasonable, in respect to the protonated parent compound's elemental formula ($C_{14}H_{12}NO_2Cl_2$) and a feasible C/H/O/N relationship in each of the given elemental compositions. Further, $C_{13}H_{10}NCl_2$ also showed the most favourable isotopic ratio (i.e. ion-FIT; i-FIT 1.0) and the mass error of 4.8 ppm. Correspondingly, among the four results yielded for DP7's elemental composition calculation, $C_{13}H_{10}NCl_2$ was again the only plausible and revealed the best i-FIT of 0.5, while the mass error was 8.4 ppm. The common fragmentation pattern and a molecular mass decrease of 28 Da (carbonyl group) in comparison to DP3, imply that the compounds DP4 and DP7 contained the 2,6-dichloro-N-(phenyl)aniline structural fragment. An additional -CH_2- group was bonded to the proposed fragment, which was by considering the parent compound's structure, most likely placed on the *ortho* position on the phenyl ring. However, due to the insufficient MS data, its position was not confirmed and its type (i.e. ring or double bond) remained unclear. The most plausible route of DP4 and DP7 biodegradation pathway seems to be the decarboxylation of diclofenac followed by oxidation, i.e. dehydrogenation. However, their insufficient production did not allow studies on time-concentration dependent profile. For this reason, no further conclusions on their precursors and further degradation could be made at this point.

The mass spectra of DP1 revealed most abundant fragment ions with *m/z* 295, 277, 242 and 214 (Fig. 1d). The fragmentation of DP1 (Fig. 1c and d) was strongly related to the fragmentation of the parent compound (Fig. 1a and b), apart from the fragment ion *m/z* 242, which was not detected for diclofenac. Fig. 2b shows a chromatogram acquired using MS/MS at *m/z* 242, where only the peak at t_R = 7.95 min (DP1) was evident (the mass spectrum is not shown). Further, comparison of Fig. 1a and b with Fig. 1c and d proved that DP1 lacked the fragment *m/z* 250, which was, on the other hand, characteristic for the parent compound as well as for the majority of the degradation products (DP4, DP5, DP6 and DP7). In order to study its chemical structure, the elemental compositions of the most abundant DP1 fragment ions were calculated, which are given, along with the mass errors, in Table 2. Thus, the proposed elemental composition of the positively charged species $C_{14}H_{11}NO_2Cl_2$ was determined with a mass error of 0.0 ppm within a ±10.0 ppm tolerance (settings: C: 0–20, H: 0–20, N: 0–5, O: 0–5; Cl: 0–2 odd and even). The protonated molecule was not found within the scan range of *m/z* 50–600; therefore, a supplementary (+)ESI-ToF screening in the extended mass range from *m/z* 100 to 1000 was performed under gentle ionisation conditions (CV 10–15 V). Based on the nitrogen rule, the integer DBE value and the absence of only one hydrogen atom when compared to DF's, we assume that the compound either passed the QqToF sample cone as a radical cation or it was subjected to in-source degradation thus showing a radical fragment instead of its protonated molecule. Possibly, the lacking hydrogen atom was substituted by another functional group in the DP1 molecule, which was degraded in-source to show only the *m/z* at 295. However, additional studies possibly involving NMR analysis are required to elucidate its structure.

As suggested above, the diagnostic fragment ion with *m/z* 214 was confirmed by MS/MS fragmentation, revealing the matching product ion spectra as shown on Fig. 3 (one of the identical product ion spectra is shown in the figure; for the remaining spectra see 'Supplementary material'). At CV 35 V and CE 40 eV two abundant fragment ions with *m/z* 178 and *m/z* 151 were formed from *m/z* 214, which corresponded to loss of HCl (36 mDa) and subsequent ring opening with, presumably, cleavage of ethenyl radical (27 mDa). Supported by the accurate mass determinations of the product ions the identical structure of *m/z* 214 in all components was confirmed, thus suggesting their formation from DF.

As a result of the structure elucidation study, chemical structure of DP3 was determined, another two (DP4 and DP7) were not completely identified, while for the DP1 the elemental composition of its highest detected fragment ion was resolved. The chemical structures of the remaining three degradation products (DP2, DP5 and DP6) are still to be elucidated, which was due to their poor fragmentation patterns and low concentrations.

Studying its behaviour, we attempted to obtain supporting information to facilitate the structural elucidation of DP1. Because of the non-detection of DP1 in the abiotic control, as well as in the inlet of the bioreactor, the compound was believed to be the product of biological transformation. Further, the area-under-curve (AUC) relationship between DP1 and DF in bioreactor effluents was found to be inversely proportional, suggesting that DP1 was an intermediate produced by a direct, single-step enzymatic

Table 1
Diclofenac and its degradation products defined by their retention time, abbreviation, protonated molecule (fragment ion), elemental composition and, where possible, proposed chemical name and structure.

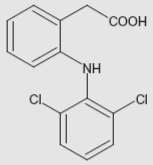
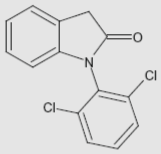
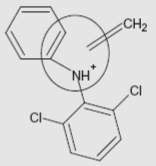
t_R (min)	Abbreviation	*m/z*	Elemental composition	Chemical name	Chemical structure	Comments
8.0	DP1	295 (highest fragment ion)	$C_{14}H_{11}NO_2Cl_2$	–	–	Degradation in-source
8.6	DF	296 $[M+H]^+$	$C_{14}H_{12}NO_2Cl_2$	{2-[(2,6-dichlorophenyl) amino]phenyl}acetic acid		Parent compound: diclofenac
9.3	DP3	278 $[M+H]^+$	$C_{14}H_{10}NOCl_2$	1-(2,6-dichlorophenyl)-1, 3-dihydro-2H-indol-2-one		Confirmed by authentic standard
9.6 10.7	DP4 DP7	250 $[M+H]^+$ 250 $[M+H]^+$	$C_{13}H_{10}NCl_2$ $C_{13}H_{10}NCl_2$	Contains structural fragment "2,6-dichloro-N-(phenyl)aniline"		Isomeric structures; the position and nature of $-CH_2$-group not defined

Table 2
Accurate mass measurements of the most abundant fragment ions in the mass spectrum of DP1.

Proposed elemental composition	Mass accuracy					Remarks
	Theoretical mass	Measured mass	Mass error (mDa)	Mass error (ppm)	DBE	
$C_{14}H_{11}NO_2Cl_2$	295.0167	295.0167	0.0	0.0	9.0	CV 20 V
$C_{14}H_9NOCl_2$	277.0061	277.0064	0.3	1.1	10.0	CV 30 V
$C_{14}H_9NOCl$	242.0373	242.0383	1.0	4.1	10.5	MS/MS 242 at CV 35 V, CE 20 eV
$C_{13}H_9NCl$	214.0424	214.0445	−0.7	3.3	9.5	MSMS 295 at CV 30 V, CE 20 eV

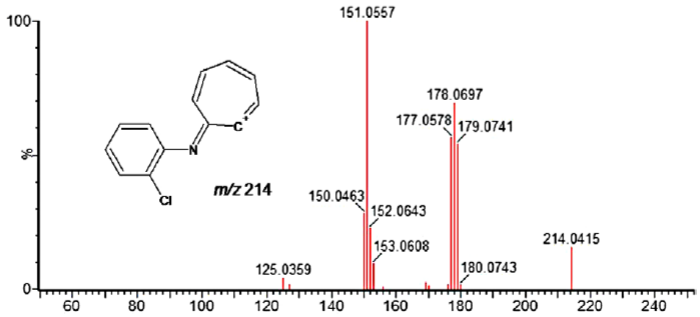


Fig. 3. Product ion mass spectrum of fragment ion with *m/z* 214 (postulated structure is shown: 7-[(2-chlorophenyl)imino]cyclohepta-1,3,5-trienylium) at CV 35 V and CE 40 eV (DP7; t_R = 10.7 min).

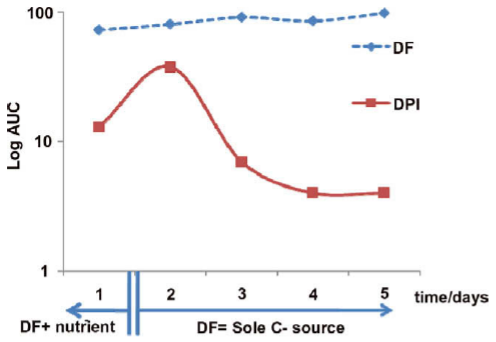


Fig. 4. Effect of nutrient–mineral medium on the DF depletion activity and behaviour of DP1. DF was applied as the sole C-source (200 μg L^{-1}) from the 2nd to 5th day of the experiment.

conversion of DF. In addition, by rapid increase of DF concentration at factor 100 in R-5 it was shown that the previous adaptation of microbial community was not essential to produce DP1 in amounts comparable to those in R-500. DP1 occurred at each of the tested inlet DF concentrations, including the lowest at 5 μg L^{-1}. This finding implied that DP1 may also be produced by biotransformation of DF at typical WWTP and environmental concentrations (ng–μg L^{-1} range; Gröning et al., 2007).

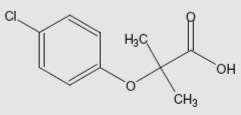
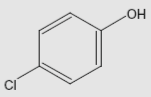
In another experiment, which principally focused on the DF biotransformation mechanism, we compared the DF-depleting activity for the biomass under the ordinary and 'SNS' operating conditions. As illustrated in Fig. 4 a slight decrease in the DF elimination was observed under 'SNS' conditions (days 2–5); yet, the nutrient source affected the DP1 response in particular. Thus, an apparent lack of degradative potential was noticed for DP1, which was reflected in a sharp raise in the DP1 concentration on the day when the 'SNS' conditions were established (day 2). However, due to a dilution effect in the flow-through bioreactors and a simultaneous decline in the DF degradation potential, the concentration of DP1 decreased during the following 3 days. This dependence of the biodegradation process on the presence of the nutrient–mineral medium as a carbon source revealed the cometabolic nature of the diclofenac transformation. In addition, Gröning et al. (2007) observed a poor potential of microbial community to degrade diclofenac and could not achieve the enrichment of diclofenac-depleting microbial activity, which was, agreeing with our results, reasoned by the cometabolic conversion of this compound.

Product of microbial clofibric acid transformation

During the LC-(-)ESI-ToF-MS analysis of the bioreactor outlet samples in scan mode an additional peak emerged at a retention time of 2.8 min. The absence of this peak from abiotic control and inlet samples, spiked with CLA, make it likely to be a product of CLA biotransformation. The parent compound was eluted at t_R 1.7 min and revealed a deprotonated parent molecule at $[M-H]^-$ 213 and a fragment ion at *m/z* 127, which was believed to be a 4-chlorophenolate fragment ion. Both of them showed a typical monochlorine 3/1 (213/215 and 127/129) isotopic pattern in (-)ESI-ToF mode. Similarly, the mass spectra of the suspected biotransformation product peak showed *m/z* 127, a deprotonated parent molecule. The elemental composition measurements implied only one result within a ±10.0 ppm mass error window (settings: odd and even ions, C: 0–20, H: 0–20, O: 0–5, N: 0–5; Cl: 0–1) for a deprotonated molecule having an elemental composition was C_6H_4OCl. The elemental formula was calculated with a minimal mass error of 0.8 ppm, a considerably low error for a small organic molecule with a molecular weight of only 128. Further, the product ion spectrum of the metabolite matched with the product ion profile of the CLA fragment ion with *m/z* 127 (CV 15 V, CE 10 eV), showing a single fragment ion at *m/z* 91, which corresponded to a neutral loss of HCl (36 Da), thus suggesting a cyclohexa-1,3-dien-5-yl-1-oate fragment ion. The biodegradation product was identified as 4-chlorophenol (4-CP, CAS 106-48-9), generated by the microbial cleavage of the ether bond of CLA (Table 3). The compound had previously been recognised as a breakdown product of CLA; however, only as a product of abiotic transformation mechanisms, i.e. photolytic (Doll and Frimmel, 2003) and photocatalytic (Doll and Frimmel, 2004) degradation.

In our experiment, 4-CP was found only in the effluent samples obtained from reactors operating at higher influent concentrations of CLA (200 and 500 μg L^{-1}) and its amount increased proportionally to the increasing concentration of the parent compound in the influent (data not shown). This finding suggested that 4-CP was also produced at lower concentrations of CLA in the influent; however, we were unable to detect it due to its insufficient amount in the sample. Further, the biodegradability of CLA was in our preliminary experiments estimated to as low as 30% even at highest tested HRT (48 h), while on the other hand, a strong relationship between degradation of 4-CP and hydraulic retention time (HRT) was observed. Comparison of 4-CP AUCs in the effluent samples at a HRT of 12 and 24 h showed a 15-times higher AUC at the shorter HRT. Similar findings were obtained by Kargi and Konya (2007), reporting that a HRT of 15 h led to 90% removal of 4-CP in their pilot flow-through bioreactors, while HRTs of 5 and 10 h resulted in

Table 3
Clofibric acid and its degradation product defined by their retention time, abbreviation, deprotonated molecule, elemental composition, chemical name and structure.

t_R (min)	Abbreviation	Deprotonated molecule $[M-H]^-$	Elemental composition	Chemical name	Chemical structure	Comments
1.9	CLA	213	$C_{10}H_{11}O_3Cl$	2-(4-Chlorophenoxy)-2-methylpropanoic acid		Parent compound: clofibric acid
2.8	4-CP	127	C_6H_5OCl	p-Chlorophenol		

15% and 34% removal, respectively. The improved removal efficiency at high HRTs can be justified by the good settling properties and consequently high biomass concentration (Kargi and Konya, 2007). Unfortunately the concentration of 4-CP was too low to attempt to characterise its metabolic transformation. It had been shown, however, that 4-CP can only be utilized as a cometabolite (Hill et al., 1996; Saez and Rittmann, 1991).

4-CP shows local antibacterial activity (Lemke, 1995) and is a suspected gastrointestinal or liver toxicant and neurotoxicant (Scorecard, 2008). 4-CP belongs to a class of chlorophenols, which can cause severe toxic effects on microorganisms and on the environment (Kargi and Konya, 2006; Cruz da et al., 2007; WHO working group, 1989), making this a good case in point where a product of microbial transformation may exhibit increased toxicity in comparison to its parent compounds (Kosjek et al., 2007a; Catalkaya et al., 2003).

Conclusions

For polar acidic pharmaceuticals, such as DF and CLA, microbial degradation is the principal elimination mechanism in classical wastewater treatment, whereas in surface water their breakdown is primarily influenced by daylight. The degradation processes often do not lead to their complete mineralization; instead breakdown products emerge, which as well as the parent compounds represent environmentally relevant xenobiotics.

This study investigated the breakdown of DF and CLA in activated sludge flow-through bioreactors, aiming to identify their (bio)transformation products. LC-QqToF was used for detection and identification purposes. The characterisation method was based on comparison of the fragmentation pathways of the parent molecule and the degradation products, where the elemental compositions of the proposed precursor and product ions were confirmed by their accurate masses. Seven DF's transformation products were detected, involving a common diagnostic fragment ion at m/z 214 and the chemical structure of one degradation product (1-(2,6-dichlorophenyl)-1,3-dihydro-2H-indol-2-one) was successfully resolved and confirmed by the authentic standard. In addition, elemental formulae ($C_{13}H_{10}NCl_2$) of two isomeric diclofenac's degradation products were proposed and confirmed by MS/MS fragmentation; yet their identity could not be completely resolved. Further identification studies proposed the elemental composition of a fragment ion with m/z 295 ($C_{14}H_{11}NO_2Cl_2$), which stands for a radical fragment ion of another DF's biodegradation product and was produced by the in-source degradation in the QqToF sample cone. The remaining three DF's transformation products, despite shown to involve the common 7-[(2-chlorophenyl)imino]cyclohepta-1,3,5-trienylium ion fragment, occurred in a concentration insufficient for their structures to be determined.

In researching the breakdown pathway of CLA, 4-chlorophenol was identified as a product of microbial transformation, while to the authors' knowledge the literature has only reported its production by abiotic mechanisms. In agreement with what had been published, we found the 4-chlorophenol degradation largely depended on hydraulic retention time in the bioreactor. Finally, the occurrence of 4-chlorophenol demonstrated that the toxicity of a WWTP influent may increase during biological wastewater treatment. This supports the need for the toxicity evaluation of transformation products, and the further development of new treatment techniques to achieve complete mineralization of emerging contaminants is justified.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhydrol.2009.04.006.

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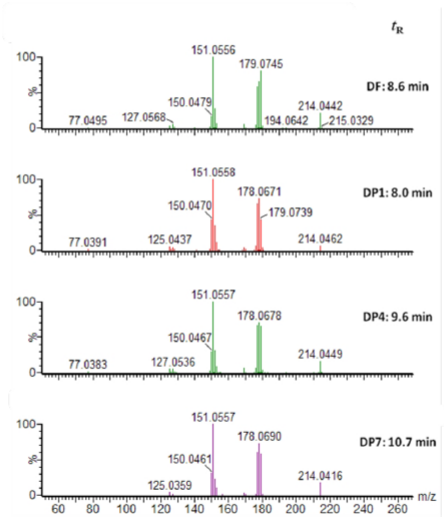
Metabolism studies of diclofenac and clofibric acid in activated sludge bioreactors using liquid chromatography with quadrupole – time-of-flight mass spectrometry

Tina Kosjek, Ester Heath, Sandra Pérez, Mira Petrović, Damia Barceló

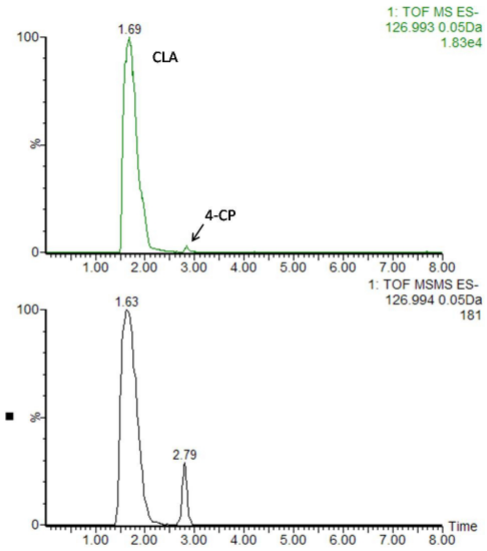
SUPPLEMENTARY MATERIAL

Caption		Page
Supplementary 1	MSMS spectra of diagnostic ion with m/z 214: DF (top: tR = 8.6 min) and three degradation products (abbreviations and retention times indicated on the right).	2
Supplementary 2	Top: extracted mass chromatogram at m/z 126.99 (± 50 mDa), showing the parent compound (CLA, tR = 1.7 min) and 4-CP at tR = 2.8 min. Bottom: (-)ESI-QqToF chromatogram acquired by CID of m/z 127.	2
Sepplementary 3	Top: (-)ESI-ToF mass spectrum of 4-CP (tR = 2.8 min). Bottom: (-)ESI-ToF mass spectrum of CLA (tR = 1.7 min).	3
Supplementary 4	Elemental composition report for 4-CP.	3

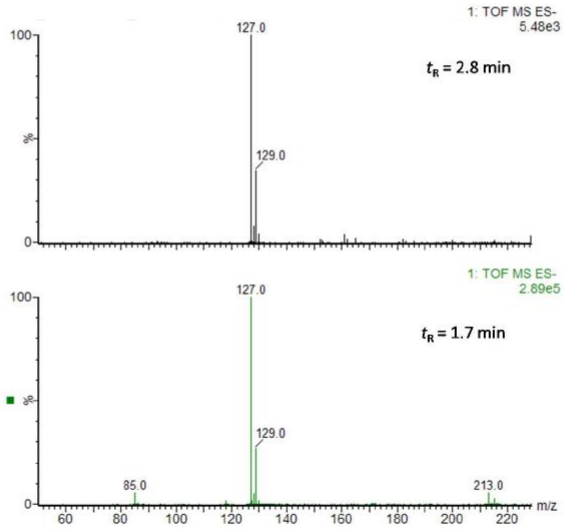
Supplementary 1: MSMS spectra of diagnostic ion with *m/z* 214: DF (top: t_R = 8.6 min) and three degradation products (abbreviations and retention times indicated on the right).



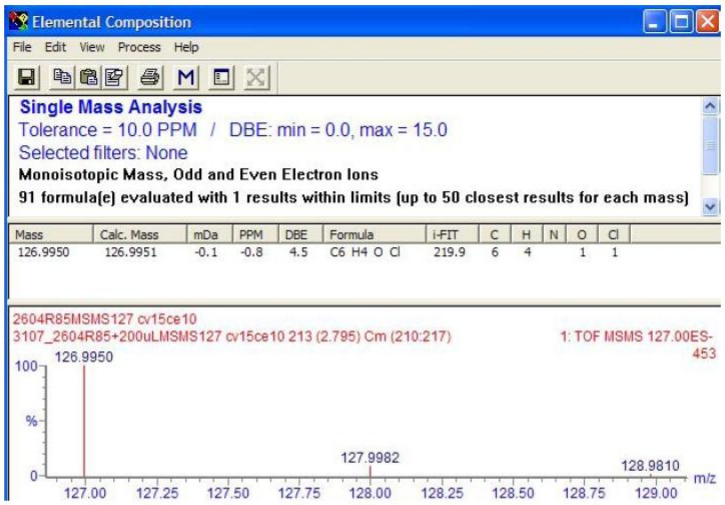
Supplementary 2: Top: extracted mass chromatogram at *m/z* 126.99 (± 50 mDa), showing the parent compound (CLA, t_R = 1.7 min) and 4-CP at t_R = 2.8 min. Bottom: (-)ESI-QqToF chromatogram acquired by CID of m/z 127.



Supplementary 3: Top: (-)ESI-ToF mass spectrum of 4-CP (t_R = 2.8 min). Bottom: (-)ESI-ToF mass spectrum of CLA (t_R = 1.7 min).



Supplementary 4: Elemental composition report for 4-CP.



3.3.3 Scientific paper: “The use of quadrupole time-of-flight mass spectrometer for the elucidation of diclofenac biotransformation products in wastewater”

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The use of quadrupole-time-of-flight mass spectrometer for the elucidation of diclofenac biotransformation products in wastewater

Tina Kosjek, Dušan Žigon, Bogdan Kralj, Ester Heath*

Jožef Stefan Institute, Department of Environmental Sciences, Jamova 39, Ljubljana, Slovenia

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ABSTRACT

The work presented herein discusses the potential of liquid chromatography–quadrupole-time-of-flight mass spectrometry (QqToF–MS) for the chemical structure elucidation of pharmaceutical degradation products (DPs). The model compound, the nonsteroidal anti-inflammatory drug diclofenac was subjected to microbiological transformation in a laboratory scale pilot wastewater treatment plant (WWTP) and its transformation products were detected in the outlet samples. Their chemical structures were elucidated using the principal features of the instrument, i.e. high resolution, accurate mass and MS/MS capability. A hydroxy-diclofenac, a benzoquinone imine derivative and a nitro analogue of diclofenac were successfully identified. The final structural elucidation of the fourth transformation product was not successful. Overall, the study emphasises the capabilities and potential of quadrupole-time-of-flight mass spectrometer showing it to be a powerful tool in both structure elucidation and confirming the identity of environmental contaminants.

1. Introduction

New emerging contaminants have long been present in the environment but only recently have scientists begun to study them in detail and as yet they are not covered by existing regulations governing water quality. Nevertheless, they are believed to pose a significant burden on the environment and are a potential threat to human health. Among the new emerging contaminants, pharmaceuticals represent an important group; the main reasons are their pharmacological activity and the fact that they are being used in ever increasing amounts in both human and veterinary medicine. The discharge of these therapeutic agents from the hospital, municipal or industrial wastewater treatment plants (WWTPs), or the improper disposal of unused drugs and the direct discharge of veterinary medicines, results in the contamination of environmental waters, where the WWTPs are considered to be a major source [1,2]. Once these pharmaceuticals enter the wastewater treatment process they are either completely mineralized, transformed into metabolites, or pass through the WWTP unaltered. Given that WWTPs provide the first and possibly the only opportunity for the removal of pharmaceutical residues, characterizing their fate during treatment is justified. Also, since the identity, toxicity and fate of the pharmaceutical metabolites as a result of microbiological transformations is yet to be fully investigated, the identification of pharmaceutical degradation products (DPs) is essential, not only to provide a comprehensive risk assessment on drug residues in the environment, but also for designing improved treatment technologies for persistent trace contaminants. We can attribute the degradation of pharmaceutical residues in natural environments or in WWTP to biotic (biodegradation) and abiotic (advanced oxidation, photolysis and hydrolysis) processes [3]. To date, a number of abiotic degradation products have been identified [4–9], but published peer reviewed studies dealing with the structural elucidation of the degradation products resulting from microbial transformation are scarce [10–12]. One obvious reason is that the screening and identification of the biotransformation products in complex environmental matrices, such as wastewater, are much more demanding in comparison to abiotic degradation studies in, for example, surface or drinking water.

The detection and identification of transformation products require the application of sophisticated instrumentation, of which mass spectrometry (MS) is considered to be leading the field, both in terms of technology development and application [4,13]. At the same time, the improvements in separation techniques, such as ultra performance liquid chromatography (UPLC) or rapid resolution liquid chromatography (RRLC), make this technology more attractive and powerful when combined with MS [14–16]. Because of the data intensive nature of LC–MS, considerable time and effort are required to interrogate the data in order to extract the results needed to identify unknowns. However, holding a certain structural relationship with their parent compound, the transformation products are not complete unknowns. There-

* Corresponding author. Tel.: +386 1477 35 84.
E-mail address: ester.heath@ijs.si (E. Heath).

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fore, the speed of their detection can be improved by applying spectral and chromatographic search algorithms, such as MetaboLynx from Waters, Analyst/MataboliteID from Applied Biosystems, Xcalibur/MetWorks from Thermo Fisher or MassHunter from Agilent. Such algorithm searches extracted mass chromatograms for expected metabolites based on predicted or unpredicted molecular changes relative to the parent compound and thus aids in the detection and identification of unknowns, particularly those buried in spectral noise. The software compares mass spectral chromatograms of a control versus analyte, i.e. metabolised, stressed or treated, sample, and automates the detection, identification and reporting of metabolites [17]. Such approach has been used before in a number of studies dealing with identification of drug metabolites in '*in vivo*' metabolism studies [18] and degradation products in food and environmental analytical chemistry [19–22].

The overall trends in analytical methods for structural elucidation and confirmation purposes include an increasing use of time-of-flight mass spectrometry (ToF–MS), quadrupole-time-of-flight (QqToF–MS) and Orbitrap mass spectrometry. ToF–MS and QqToF–MS provide an increased resolution capability (typically 10,000–12,000 resolution), while the Orbitrap by employing the Fourier transform algorithm allows empirical formula assignments with even higher precision [23]. This gives the analyst a much higher degree of certainty when identifying compounds in nontarget analysis, while positively and unequivocally confirming target compounds [13,24]. With these obvious advantages, particularly in pharmaceutical and bioanalytical field, QqToF has become an established technique [25–28]. On the other hand, in environmental analysis the benefit of ToF–MS and QqToF–MS can be seen merely in studies dealing with the structure elucidation of pesticide degradation products [29–32].

Often however, MS alone is insufficient to identify the exact position of transformation, to differentiate isomers, or to provide the precise structure of unusual and/or unstable transformation products. In addition, other substances present in samples can suppress ionization complicating metabolite identification. In such cases, multiple analytical and wet chemistry techniques, such as LC-nuclear magnetic resonance, chemical derivatisation, and hydrogen/deuterium-exchange (H/D-exchange) combined with MS are used to characterise the novel and isomeric transformation products of drug candidates [18].

The aim of this paper is to objectively assess performance of the UPLC-QqToF using the fate of diclofenac in waste water treatment as a case study. Diclofenac is a widely applied nonsteroidal anti-inflammatory drug, the human metabolism of which has been studied in detail [33]. Furthermore, there is evidence that it is susceptible to abiotic degradation and several studies have identified a number of transformation products [5,6,34], despite it being persistent to the biological treatment [2,11]. Most of the biodegradability studies involving WWTP discuss only the disappearance of the parent compound [11,35–37], and to the authors' knowledge there are only a few published reports on microbial transformation products [38,39]. It is therefore reasonable to focus more attention on this subject, in order to obtain a comprehensive assessment of diclofenac's environmental impact and the suitability of UPLC-QqToF for screening biodegradation products in complex matrices.

2. Experimental

2.1. Pharmaceutical standards, solvents and calibrants

A diclofenac sodium salt (97% purity) was obtained from Sigma–Aldrich (St. Louis, MO, USA). The calibration compounds used for tuning the mass spectrometer were sodium formate, prepared from 10% formic acid (Sigma–Aldrich Corp., Seelze, Germany) and 0.1 M sodium hydroxide (Merck, Darmstadt, Germany) in a 2-propanol (Sigma–Aldrich, Chemie GmbH, Germany): water solution (90:10), and leucine enkephaline (Sigma–Aldrich, St. Louis, MO, USA), 50 pg/μL prepared in 50:50 acetonitrile:water with 0.1% formic acid. Methanol (MeOH), ethylacetate (ETA), acetone, hydrochloric acid (HCl, 37%) and formaldehyde (37%) were obtained from Merck (Darmstadt, Germany), while MeOH and water used as the mobile phases and acetonitrile were of HPLC grade and were supplied by J.T. Baker (Phillipsburg, NJ, USA). Distilled water was used for sample preparation.

The preparation of the artificial wastewater used as the pilot wastewater treatment plant (PWWTP) influent by dissolving nutrients and minerals in tap water is described in Kosjek et al. [2].

2.2. Biodegradation experiments

To study pharmaceutical biodegradation we employed a laboratory scale PWWTP with 4 L aerated volume, which contained activated sludge from a municipal wastewater treatment plant. A detailed description of the PWWTP configuration and its operation is given by Kosjek et al. [2]. The PWWTP consisted of bioreactors, which had been continuously operated for 3 years and as a result harboured adapted microorganisms capable of utilising the test pharmaceutical [40]. For the purpose of this study three flow-through bioreactors (R0, R-200 and R-500) were in use and were fed by mineral–nutrient medium, containing yeast and meat extract, casein peptone and minerals (COD 600 mg/L) [2]. The biodegradation experiments were performed at different hydraulic retention times (HRT: 48, 24 and 12 h) and parent compound concentrations (200 and 500 μg/L of diclofenac in R-200 and R-500 influents, respectively) in order to produce highest amounts of biodegradation products for identification studies. In this view, the R-200 effluent samples at HRT of 24 h were selected for further identification studies. R0 served as the control and was operated under the same conditions as the other two bioreactors, but without the addition of diclofenac. Since the number of candidate peaks, which were only present in effluent samples was large, it was impossible to analyse each one of them. Accordingly, the R0 effluents were used to distinguish between those peaks representing products of matrix biodegradation or bacteria lyses and the actual pharmaceutical degradation products.

In addition, batch *in vitro* biodegradation test was carried out based on ISO 7827 (1995). A set of biodegradation tests was performed in six 0.5 L glass bottles (A, B, C, D, E, F) with a total wetted volume of 0.4 L, which was aerated with an aquarium pump. The bottles were kept at 23° and protected from light. Diclofenac was added into each parallel in concentration of 10 mg/L. The batches A, B, C and D contained 50 mL of active sludge taken from the bioreactors. Further, 350 mL of nutrient–mineral medium was added into A and B, while C and D were kept under the same volume of deionised water. The batches E and F contained 0.4 L of deionised water without addition of active sludge. B, D and F were biologically inhibited by adding 2% formaldehyde in order to account for possible degradation via abiotic mechanisms.

2.3. Sampling and sample preparation

To reach the aim of this study we did not need to sample consistently. Instead, samples were collected according to experimental progress and taking into account changes in the operating conditions of the bioreactors. However, our own studies performed on the PWWTP simultaneously, employing various sampling protocols are published separately [40]. Sample preparation consisted of filtering 200 mL of sample through 1.2 μm microfibre glass filter (GF/C Whatman, UK) and 0.45 μm filter (Sartorius, Goettingen, Germany)

to remove any suspended matter. The filtrate was then acidified to pH 2–3 with 37% HCl and stored at 4 °C. Solid phase extraction (SPE) was then performed with the aid of a 12-fold vacuum extraction box (Supelco, Bellefonte, USA) and Oasis® HLB reversed-phase sorbents (Waters, Corp., Milford, MA, USA), which are commonly used to achieve good recovery for compounds having a broad spectrum of polarities. The following SPE procedure was applied for pre-concentration and clean-up of 200 mL samples: conditioning with 3 mL ethyl acetate and 3 mL methanol, respectively, followed by equilibration with 3 mL aqueous HCl solution at pH 2–3 and an enrichment step at a flow-rate 4–5 mL min^{-1}. To further reduce background interferences, a mixture of ETA, acetone and MeOH in aqueous pH 2–3 HCl solution (1:1:1:8) was used as a wash solvent. The cartridges were then dried for 30 min under vacuum and eluted using 3 mL of ETA (3 × 1 mL ETA). The eluants were concentrated to approx. 0.5 mL, transferred to 1.5 mL glass vials and dried under a stream of nitrogen. The dry extracts were then dissolved in 0.5 mL of deionised water/MeOH (9:1) medium.

For the batch biodegradation study, 1.5 mL samples were taken every 20–40 h during a 18-day period. The samples were immediately frozen and kept until the sample preparation. After defrosting, the samples were centrifuged for 5 min at 3000 rot/min and 10-fold diluted in 0.5 mL of deionised water/MeOH (9:1) medium.

2.4. Liquid chromatography

The chromatographic separation was performed on a Waters Acquity UPLC system (Waters Corp., Milford, MA, USA), equipped with a binary solvent delivery system and autosampler. The injection volume was 5 μL (partial loop with needle overfill). Separation was achieved using a 5 cm long Waters Acquity C_{18} 1.7 μm column with a 2.1 mm internal diameter. Compounds were analysed under negative ion conditions and were eluted from the column using water (A) and methanol (B) as the mobile phases. Additionally, an attempt to resolve the structure of a biotransformation product has been made in positive ion mode, where water was replaced by 0.1% formic acid as a mobile phase A, while other LC conditions were kept unchanged. The elution gradient was linearly increased from 10% to 90% B in 6 min, and kept isocratic for 2 min, decreased back to 10% in 0.10 min and then finally kept isocratic for 1.90 min. The total runtime was 10.00 min. Flow rate was 0.3 mL min^{-1} and the column temperature was maintained at 35 °C.

2.5. Mass spectrometry

The UPLC system was interfaced to a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (QqToF Premier, Waters, Milford, Massachusetts, USA). The instrument was equipped with an electrospray ionisation interface operating in the negative ion mode (ESI(−)), and additionally, an attempt was made to resolve a structure of one biotransformation product in the positive ion mode ((ESI(+)). The capillary voltage was set to 2.8 kV (3.0 kV in ESI(+)), while the sampling cone voltage was varied between 15 and 40 V in order to optimise the m/z abundance for mass measurements on deprotonated molecule or fragment ions. Source and desolvation temperatures were set to 120 and 350 °C, respectively. The nitrogen desolvation gas flow rate was 600 L/h. For MS experiments the first quadrupole was operated in rf-only mode, while detection was performed in the ToF mass analyser. MS data were acquired over an m/z range 100–1000 at collision energy of 4 eV. For MS/MS operation the acquisition range was between m/z 50 and 1000, and argon was used as the collision gas at a pressure of 4.5×10^{-3} mbar in the T-wave collision cell. The MS/MS experiments were performed with collision energy (CE), varied between 10 and 40 eV, in order to generate product ion spectra providing the most structural information. Data were collected in centroid mode, with a scan accumulation time set to 0.25 s and an interscan delay of 0.02 s. The data station operating software was MassLynx v4.1.

Prior to analysis, the instrument was calibrated over a mass range 50–1000, using a sodium formate calibration solution. Reproducible and accurate mass measurements, at a mass resolution of 10,000, were obtained using an electrospray dual sprayer with leucine enkephalin ($[M-H]^-$ = 554.2615, $[M+H]^+$ = 556.2271) as the reference compound. The latter was introduced into the mass spectrometer alternating with the sample *via* Waters LockSpray device.

2.6. Identification approaches and data processing

Data were acquired in centroid mode and afterwards processed by the MetaboLynx™ application manager embedded into MassLynx v4.1. software (Waters). The algorithm was programmed to detect products of common metabolic pathways (i.e. hydroxylation, loss of CO_2, dechlorination), as well as to detect unknown/unexpected components, i.e. those not included in the software library. The latter were examined in m/z 100–400 scanning range with 10 Da size of a step scan. Metabolite traces were searched in the time window 1.00–9.00 min at 0.05 min peak separation. The presence of biotransformation products was investigated in R-200 and R-500 effluent samples, while R0 effluent, R-200 and R-500 influents were used as control samples.

Based on accurate mass possible elemental compositions were calculated using the Elemental Composition Calculator embedded into the MassLynx v4.1. software. A maximum deviation of ±10 ppm from a measured accurate mass was accepted, and parameters were set in relation to diclofenac structure to C: 0–15, H: 0–20, N: 0–3, O: 0–10. In addition, from the characteristic isotopic pattern of ^{37}Cl (31.98% relative abundance) the corresponding number of Cl atoms was determined. The double bond equivalent (DBE) was set from 4.5 to 15 and from 0 to 15 for the mass measurements of deprotonated molecules and product ions, respectively. Such DBE was chosen as the retention of the aromatic ring during degradation was assumed. Additionally, the option "even electron only" was selected for precursor ions and "odd- and even- electrons" for product ions.

3. Results and discussion

3.1. Methodology of detection

To recognise the biodegradation products we performed the initial experiments in ToF–MS mode, with the first quadrupole operated in rf-only mode. Due to the remarkably high amount of information that the ToF–MS spectra provide, especially in complex matrices such as wastewater, screening for possible transformation products is a time-consuming and demanding task. Clearly, in any direct comparison of the PWWTP inlet and the outlet total ion current (TIC) it is not possible to observe any trace amounts of degradation products. Here, an additional data interrogation becomes a critical issue and, to aid for the detection of biotransformation products, the post-acquisition data processing using the MetaboLynx software package was employed in the present study. By comparing the mass chromatograms of control versus analyte samples the algorithm-highlighted differences in the presence of compounds, which could be attributed to transformation processes [29]. However, a number of chromatographic peaks evident in the MetaboLynx report file, might not only originate from diclofenac biotransformation, but could also be attributed to the nutrient metabolism or bacteria lyses. What greatly facilitated the detection of biotransformation products was the characteristic isotopic profile of chlorine-bearing molecules, since the parent compound

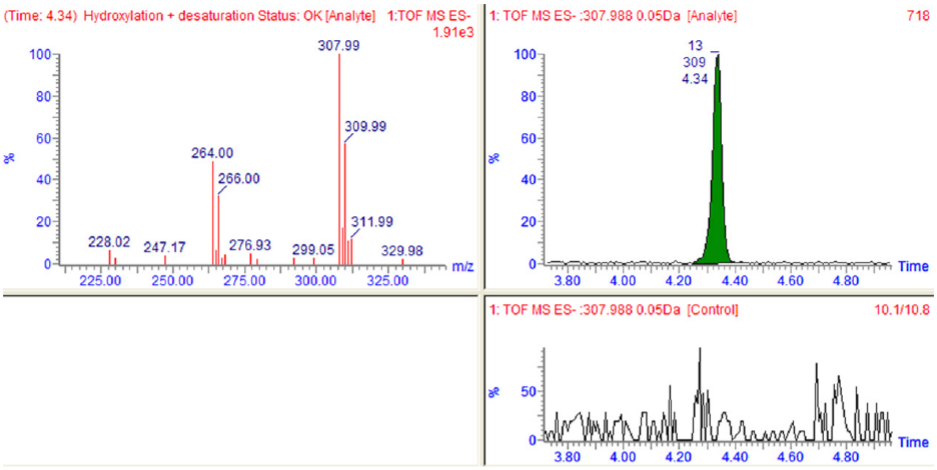

Fig. 1. Extracted from the MetaboLynx report file: analyte (top) and control (below) sample mass chromatograms and ToF-ESI(−) spectrum (left) of peak eluting at t_R 4.34 min (DP2). Due to the absence in the control sample and also for its typical dichlorine isotopic pattern this compound is considered as a biotransformation product of diclofenac.

contained two chlorine atoms in its chemical structure. As the influent samples were free of any other chlorine-bearing component (see Section 2), the detection of the chlorine isotopic profile in the effluent samples (R-200, R-500) implied that the attributed peaks were diclofenac degradation products. Accordingly, MetaboLynx pre-processing based on isotopic cluster analysis was performed, which reduced drastically the number of resulting peaks to those attributed to chlorinated diclofenac biotransformation products. The major peaks eluted at 4.1 (DP1), 4.3 (DP2), 5.0 (DP3) and 5.4 min (DP4), while the parent compound eluted at 4.7 min. Fig. 1 illustrates a segment from the MetaboLynx report file. The top chromatogram shows diclofenac biotransformation product, eluting at t_R 4.34 min, while this compound was absent in the control sample (Fig. 1, bottom).

3.2. Identification

The high resolution and mass accuracy measurements provided by ToF analyser allowed the assignment of highly probable empirical formulae for each DP. With the parameter settings defined under Experimental, the processing by the Elemental Composition Tool resulted in a single empirical formula proposed per each DP in most cases, as shown in Table 1.

Considering the mass differences between the parent molecule and DPs (Table 1), the m/z increases of 16, 14, 45 and 17 Da were shown for DP1, DP2, DP3 and DP4, respectively. In this view, DP1 is indicative of mono-hydroxylated diclofenac species, while, considering the mass to charge increase of 14 and the empirical formulae proposed in Table 1, DP2 corresponds to an addition of oxygen with a concurrent loss of two hydrogens. Further, the m/z increase of 45 in DP3 shows substitution of hydrogen atom by NO_2 group, while the most reasonable explanation for formation of DP4 is a decarboxylation of an intermediate, which was beforehand subjected to both transformations described above, i.e. hydroxylation and incorporation of a nitro-group.

In order to elucidate the chemical structures of DPs, first the mass behaviour of the parent compound was studied and afterwards compared with the mass fragmentation of detected DPs. Despite the soft ionisation normally achieved by ESI, the parent compound showed a considerable in-source fragmentation producing an abundant fragment ion with m/z 250 in addition to the deprotonated molecule ($[M-H]^-$) with m/z 294; both revealing the typical dichlorine isotopic pattern. Likewise (collision-induced dissociation) CID of m/z 294 showed the loss of CO_2 to produce the fragment ion with m/z 250, which was followed by two subsequent losses of HCl forming m/z 214 and 178 (Fig. 2, top).

DP1 in ToF–MS-ESI(−) mode resulted in mass spectra with $[M-H]^-$ ions with m/z 310, 312 and 314. Due to the in-source fragmentation (CV 20 V), similar behaviour was observed in DP1 as with the parent compound, producing $[M-H-CO_2]^-$ ions with m/z 266, 268 and 270. Product ions generated upon CID of the $[M-H]^-$ 310, again revealed the loss of CO_2 with m/z 266, and correspondingly to diclofenac, two subsequent losses of HCl resulting in m/z 230 and 194. Finally, the m/z 166 provided strong evidence that the hydroxylation occurred on the nonchlorinated ring (Fig. 2, middle). In comparison to DP1, DP2 reveals two hydrogens less and holds a DBE value of 1.0 U higher than DP1, thus supporting the formation of an additional double bond. Its ToF–MS-ESI(−) fragmentation

Table 1
The data regarding diclofenac and DPs attained by accurate mass measurements and post-acquisition processing: chromatographic elution time, theoretical and experimental accurate mass of a deprotonated molecule, mass error in mDa, empirical formula and mass difference in relation to the parent compound.

Compound	t_R (min)	$[M-H]^-$ (m/z theoretical)	$[M-H]^-$ (m/z found)	Mass error (mDa)	Empirical formula	Mass difference
Diclofenac	4.7	294.0089	294.0089	0.0	$C_{14}H_{11}NO_2Cl_2$	0.0000
DP1	4.1	310.0038	310.0040	0.2	$C_{14}H_{11}NO_3Cl_2$	+15.9951
DP2	4.3	307.9881	307.9886	0.5	$C_{14}H_9NO_3Cl_2$	+13.9798
DP3	5.0	338.9939	338.9945	0.5	$C_{14}H_{10}N_2O_4Cl_2$	+44.9856
DP4	5.4	310.9990	310.9990	0.0	$C_{13}H_{10}N_2O_3Cl_2$	+16.9901

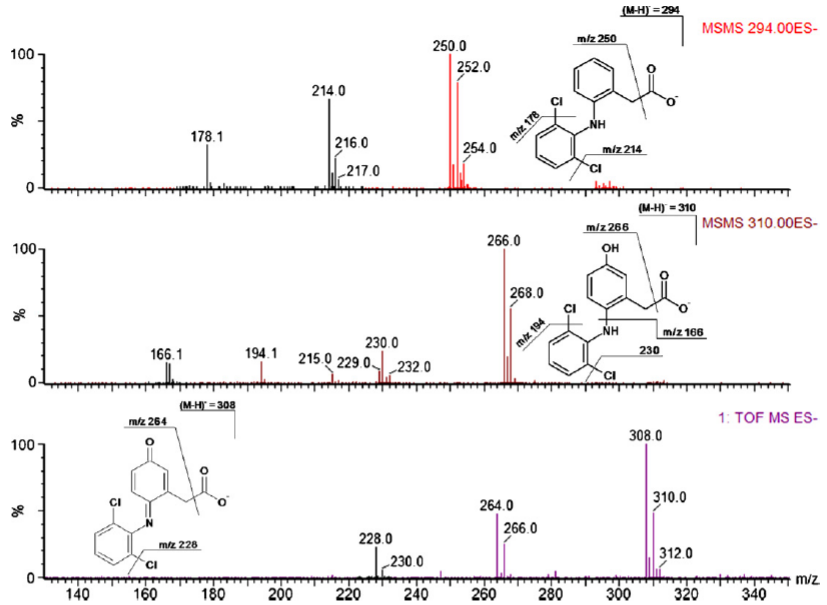

Fig. 2. From top to bottom: ToF–MS/MS-ESI(−) spectrum of diclofenac deprotonated molecule ([M−H]⁻ 294); ToF–MS/MS-ESI(−) spectrum of 5-hydroxy-diclofenac (DP1) deprotonated molecule ([M−H]⁻ 310); ToF–MS-ESI(−) spectrum of most plausible quinone imine species, i.e. diclofenac-2,5-quinone imine (DP2 ([M−H]⁻ 308)). The proposed origins of mass fragments of diclofenac and its transformation products are attached to the corresponding mass spectra.

showed [M−H]⁻ ions with *m*/*z* 308, 310 and 312 in ration 9/6/1, and again the loss of CO_2 to form *m*/*z* 264, 266 and 268 (Fig. 2, bottom), which confirms that the carboxylic group stayed intact during the biotransformation. In addition, *m*/*z* 228 was observed at ToF-ESI(−) conditions (Fig. 2, bottom), again correlating with the fragment ion *m*/*z* 230 in DP1. Thus, despite its unsuccessful further tandem mass fragmentation, but based on its strong structural relationship with hydroxy-diclofenac (DP1), the most probable structure of DP2 is benzoquinone imine species. In addition, the postulated chemical structure is supported by the study of Gröning et al. [39],

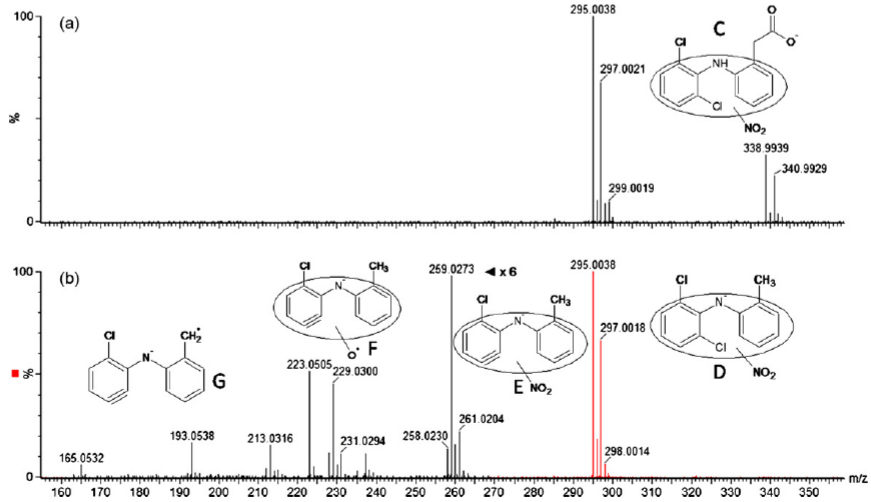

Fig. 3. Top: ToF-ESI(−) mass spectrum of DP3 (a); bottom: MS/MS spectrum of *m*/*z* 339 (b); proposed chemical structures of deprotonated DP3 molecule (*m*/*z* 339, C) and principal product ions 295 (D), 259 (E), 229 (F) and 213 (G).

Table 2
Elemental compositions of the DP4 deprotonated molecule and product ions, established in ToF-ESI(−) mass spectrum and ToF–MS/MS-ESI(−) spectrum from accurate mass measurements on QqToF.

Ion	MS measurement	Mass accuracy		
		Theoretical	Measured	Mass error (mDa)
$C_{14}H_9N_2O_4Cl_2$	ToF–MS-ESI(−)	338,9939	338,9941	0.2
$C_{13}H_9N_2O_2Cl_2$	ToF–MS-ESI(−)	295,0041	295,0037	−0.4
$C_{13}H_9N_2O_2Cl_2$	ToF–MS/MS-339.00 ESI(−)	295,0041	295,0040	−0.1
$C_{13}H_8N_2O_2Cl$	ToF–MS/MS-339.00 ESI(−)	259,0274	259,0269	−0.5
$C_{13}H_8NOCl$	ToF–MS/MS-339.00 ESI(−)	229,0294	229,0286	−0.8
$C_{13}H_7N_2O_2$	ToF–MS/MS-339.00 ESI(−)	223,0508	223,0501	−0.7
$C_{13}H_8NCl$	ToF–MS/MS-339.00 ESI(−)	213,0345	213,0342	−0.3
$C_{13}H_7NO$	ToF–MS/MS-339.00 ESI(−)	193,0528	193,0530	0.2
$C_{12}H_7N$	ToF–MS/MS-339.00 ESI(−)	165,0578	165,0574	−0.4

who found this compound as a product of diclofenac transformation in river sediments. Diclofenac-2,5-quinone imine is a stable oxidative product of 5-hydroxy-diclofenac, a minor diclofenac metabolite produced by microsomal enzymes from a group P450 [41,42]. Additionally, Poon et al. [43] proposed the formation of diclofenac-1′,4′-quinone imine from 4-hydroxy-diclofenac, again during the P450-mediated transformation. Thus, even though the most plausible structure of the quinone imine species is diclofenac-2,5-quinone imine, it cannot be ruled out that DP2 is its positional isomer that is formed from a short-living 4′-hydroxy metabolite occurring in the reactor effluent in an undetectable levels.

Analogous to the parent compound, DP3 (Fig. 3(a)) showed the deprotonated molecule $[M-H]^-$ with m/z 339 (assigned by chemical structure C, Fig. 3) and the elimination of CO_2 to form m/z 295, already under MS conditions. ToF-ESI(−) mass spectrum in Fig. 3(a) illustrates a dichloro isotopic pattern in both, m/z 339 and 295. In addition, the product ion m/z 295 corresponding to structure D, is evident in Fig. 3(b), illustrating the ToF–MS/MS-ESI(−) fragmentation of the m/z 339. CID of m/z 339 (Fig. 3(a)) resulted in further losses of HCl with m/z 259 (Fig. 3, structure E), and homolytic cleavages of NO· with m/z 229 (Fig. 3, structure F) or NO_2· with m/z 213 (Fig. 3, structure G). The product ion with m/z 223 is indicative of the loss of HCl from m/z 259, while m/z 193 corresponds to the loss of HCl from the product ion with m/z 229. The accurate mass measurements of the deprotonated DP3 molecule ($[M-H]^-$ = 339) and product ions (m/z 295, 259, 229, 223, 213, 193 and 165) were performed in order to confirm the fragmentation pattern proposed in Fig. 3. The accurate masses were determined based on average spectra and are given in Table 2. The measured mass error of the deprotonated molecule ($C_{14}H_9N_2O_4Cl_2$) and all seven product ions was below 2 mDa, which is generally accepted as an accurate mass measurement [44,45]. In conclusion, our results indicate that the biotransformation yields actually a nitro-analogue of diclofenac. However, the exact position of the nitro group in the molecule could not be derived from the MS/MS data, and further investigations applying nuclear magnetic resonance (NMR) are necessary. The incorporation of the NO_2 group into aromatic ring as an environmental transformation process had previously been reported by Hogenboom et al. [46], as well as recently by Hernández et al. [21]. In both cases the incorporation of the NO_2 group was a result of photodegradation experiments on pesticides as model compounds. The incorporation of NO_2 was according to Hernández et al. [21] reasoned by the influence of nitrate-containing surface water. Having a considerable amount of nitrate (10 mg/L) in the synthetic wastewater, and by discarding the possibility of abiotic DP3 formation with the control study, we assume that similar transformation occurred microbiologically in our bioreactors.

In contrast with the parent molecule and other biotransformation products, DP4 did not show the neutral loss of CO_2 generated by 'in-source' neither by the MS/MS fragmentation, what indicated that the transformation could have occurred on the carboxylic group of diclofenac. The mass fragmentation generated upon CID of m/z 311 revealed abundant fragment ions with m/z 161, 163 and 165, which corresponded to dichlorophenolate anion ($C_6H_3OCl_2$) implying that the hydroxylation must have taken place on the chlorine-bearing ring. With the exception of m/z 161 with its two isotopic ions no other abundant fragment ions were generated to enable the structural elucidation of DP4. In order to attain additional information on DP4 chemical structure, the compound was examined also under positive ion conditions, but it did not protonate readily and its final structural elucidation was hence not successful.

To investigate the stability of the diclofenac degradation products, the batch biodegradation tests were also performed in addition to the established PWWTP biodegradation experiments. However, among the biodegradation products identified in the bioreactor effluents, only 5-hydroxy-diclofenac was detected in trace amounts. Due to the lack of DP1 reference, only the peak areas could be compared, rating from 0.1% up to 0.5% of diclofenac peak area during 420 h of experiment. With exception of DP1 no other DPs were detected, which might be reasoned by the high concentration of the parent compound (10 mg/L) inhibiting the biodegradation pathways. This suggests that the standardised biodegradation tests do not necessarily yield same metabolic pathways as those occurring during the biological degradation in wastewater treatment. Moreover, under the batch conditions and high initial concentrations of parent compound, the environmental relevance of such tests is also questionable.

4. Conclusion

Microbiological transformations in WWTPs or in the environment do not necessarily lead to complete mineralization; instead, biotransformation products of unknown identity are often produced. In view of such transformations, it is not sufficient merely to measure the persistence/disappearance of the parent compound, but also to consider the breakdown pathways and the potential impacts of the transformation products, which may pose a threat to the environment. However, the latter is only truly becoming possible with advancements in identification techniques, such as quadrupole-time-of-flight mass spectrometry. QqToF is suited for the detection and nontarget analysis in complex mixtures; consequently, the main field of its application is qualitative analysis, a fact confirmed by this paper. The present study deals with the identification of diclofenac biotransformation products in a pilot wastewater treatment plant effluent. The transformation products were detected using the post-acquisitional data processing software, i.e. MetaboLynx, a data mining tool particularly essential in screening for nontarget metabolites, hidden in the

background noise in mass chromatograms of complex wastewater samples. Using QqToF we were able to positively identify hydroxy-diclofenac, a quinone imine derivative of diclofenac and a previously unrecognised nitro analogue of diclofenac. The final structural elucidation of the fourth transformation product was not successful. This study therefore confirms the key attributes of the instrument: MS/MS fragmentation, high resolution, good mass assignment accuracy, high sensitivity, and the ability to record a complete mass spectrum for each pulse of ions injected into the device. Finally, as virtually no information on metabolites of diclofenac transformation by microbial communities is currently available, the proposed biotransformation products bring an important contribution to recognising the fate of this pharmaceutical during biological wastewater treatment. Hopefully, because of the increasing attention given to the qualitative determination of pharmaceutical degradation products in the literature and the development of identification tools in general, knowledge about the fate of pharmaceuticals in the environment will become more comprehensive.

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3.3.4 Book chapter: “The challenge of the identification and quantification of transformation products in the aquatic environment using high resolution mass spectrometry”

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The challenge of the identification and quantification of transformation products in the aquatic environment using high resolution mass spectrometry

Juliane Hollender[1], Heinz Singer[1], Dolores Hernando[2,3], Tina Kosjek[4], Ester Heath[4]

[1]Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland, juliane.hollender@eawag.ch, heinz.singer@eawag.ch
[2]National Reference Centre for Persistent Organic Pollutants and Spanish REACH Reference Centre-University of Alcalá, 28871-Alcalá de Henares, Madrid, Spain
[3]University of Almeria, Spain, dhernan@ual.es
[4]Jožef Stefan Institute, Ljubljana, Slovenia, ester.heath@ijs.si, tina.kosjek@ijs.si

Abstract

The environment is contaminated by a number of micropollutants and their degradation products, many of which still remain undetected. Nowadays, several European regulations require the inclusion of transformation products in environmental risk assessment and monitoring. In the last decade, intense efforts have been taken to recognize the identity, quantity, and toxicity of unknown transformation products. Liquid chromatography combined with mass spectrometry has become a key technique for environmental analysis, now allowing the development of screening, identification, confirmatory and quantitative methods for the trace analysis of polar compounds in complex environmental matrices. The combination of modern technologies comprising high resolution, high mass accuracy and mass fragmentation enables the identification of compounds without having the authentic standards or even the detection of unknown analytes. However, a reliable confirmation of proposed structures using NMR spectroscopy or available standards is still desirable. This chapter presents new analytical strategies to identify and quantify transformation products generated by human metabolism, microbial degradation, or other environmental breakdown processes. Various hyphenated mass spectrometric techniques used for structure elucidation, such as liquid chromatography coupled to time-of-flight mass spectrometry, quadrupole-time-of-flight and linear ion trap-Orbitrap hybrid mass spectrometry are presented on three case studies of pharmaceutical and pesticide transformation products in environmental matrices, such as wastewater and groundwater.

Keywords

transformation product, groundwater, high resolution mass spectrometry, pesticide, pharmaceutical, wastewater treatment

1. Introduction

The environment is contaminated by a number of organic micropollutants released from urban, industrial, and agricultural activities, many of which still remain undetected. Although environmental monitoring includes more and more organic compounds, such as biocides, pesticides and pharmaceuticals, the analyses still mainly focus on parent compounds. However, the environmental exposure to their transformation products can be relevant as shown for pesticides in groundwater in the USA (Kolpin et al., 1997; 2004; Boxall et al., 2004) as well as in Switzerland (Hanke et al., 2007). In both studies, several pesticide transformation products (such as metolachlor-ESA or -OXA from the parent pesticide metolachlor) were found in higher concentrations in groundwater than the parent compounds. In the case of pharmaceuticals, human metabolites are excreted from the human body instead of or along with the parent compounds, often in considerable amounts. There is very limited knowledge on the environmental behaviour of those human metabolites. Some metabolites, such as conjugates of sulfamethoxazole and ethinylestradiol are cleaved back to the parent compound already in the sewer or in wastewater treatment plants (WWTP) (D'Ascenzo et al., 2003; Göbel et al., 2005). Few recent studies include the fate of persistent human metabolites of pharmaceuticals in the aquatic environment. Bendz et al. (2005) detected human ibuprofen metabolites not only in the WWTP as Buser et al. (1999), but also in the receiving river, while carbamazepine metabolites were found in WWTP effluent and even in drinking water (Miao et al., 2005; Hummel et al., 2006). In contrast to human metabolism of pharmaceuticals, which is studied in detail before pharmaceuticals are approved, their fate in the environment, including transformation pathways and formation of stable transformation products, has gained attention only recently. Only sparse information is currently available on transformation products of pharmaceuticals and their human metabolites formed in the environment or wastewater treatment plants (Kosjek et al., 2007).

As a consequence to findings of transformation products in the environment, the current European directive on drinking water as well as the guideline for groundwater quality with respect to pesticide contamination includes transformation products (Drinking Water Directive, 1998; European Guidance Document, 2003). Regarding chemical risk assessment, the need to identify and characterize relevant metabolites or transformation products is mentioned in several European directives and guidelines, for instance, in the EMEA guideline on the environmental risk assessment of medical products for human use (European Medicines Agency, 2006) and the Council directive concerning the placing of plant protection products on the market (European Directive, 1991). However, little concrete guidance on how to identify relevant transformation products is given.

Apart from the difficult selection of relevant transformation products for monitoring purposes, there are several challenges in analyzing transformation products in environmental samples such as surface and ground water. The first is, that the generally low but nevertheless potentially toxicologically relevant concentrations in the ng L^{-1} range require enrichment, separation from the matrix, and sensitive detection. The second challenge is the clear identification of transformation products without reference standards, which are often not available. An additional challenge is the identification of previously unidentified transformation products, which have never been described in the literature.

If the elemental composition is known to unequivocally identify the molecular structure of a transformation product without a reference standard, nuclear magnetic resonance (NMR) analysis coupled with liquid chromatography (LC) would be the method of choice. Although LC-NMR was successfully applied to environmental samples in a few cases (Levsen et al., 2000; Reineke et al., 2008), it requires costly equipment and is not yet sensitive enough for the low concentrations typically found in environmental samples. In contrast, GC-MS-(MS) and LC-MS-(MS) allow quantification in the concentration level down to a few ng L^{-1}. Without reference standards, a complicated interpretation of the fragmentation pattern in MS/MS or MS^n spectra is indispensable, which may give decisive hints for the identification of unknown transformation products. In modern GC-MS instruments, an electron impact (EI) ionization source is normally employed to provide a wealth of structural information in the mass spectra. EI is performed at 70 eV, thus yielding mass spectra which are identical over time

and between instruments for a given compound. The resulting spectra can then be matched against spectra of authentic compounds which may be found in extensive GC-MS libraries. This ability to match analytical data to known spectra can significantly facilitate the structural elucidation of unknowns (Chiron et al., 1997). On the other hand, many transformation products are polar compounds containing hydroxy-, carboxy-, or amino-functional groups which enable GC-MS analysis only after derivatization. Derivatization can be avoided by employing LC separation, followed by electrospray or atmospheric pressure chemical ionization and tandem mass spectrometry, which is therefore the preferred identification technique for polar transformation products (Eichhorn et al., 2005). Ionization under different conditions results in a number of possible fragmentation patterns for a given compound, and consequently no large LC-MS libraries are commercially available which complicates the identification procedure.

A new approach to overcome the limitations discussed for GC-MS and LC-MS is to employ high-resolution mass spectrometry detection technology. Table 1 provides an overview of existing commercially available mass spectrometric techniques with respect to resolution, mass accuracy and sensitivity. The most common mass spectrometer in organic trace analytics is the triple quadrupole mass spectrometer, which selectively filters ions based on their mass-to-charge ratio (*m/z*) in two consecutive quadrupoles combined by a collision cell. It uses oscillating electrical fields to selectively stabilize or destabilize the paths of ions passing through a radio frequency (RF) quadrupole field. The quadrupole ion trap and linear quadrupole ion trap work on the same physical principles as the quadrupole mass analyzer, but the ions are trapped and sequentially ejected. In contrast, in time-of-flight mass spectrometry, ions are accelerated by an electrical field to the same kinetic energy with the velocity of the ion depending on the *m/z* Thus, the time ions need to reach the detector can be used to determine the *m/z*. Sector field mass analyzers, which are nowadays rarely utilized in organic trace analytics, use an electric and/or magnetic field to affect the path and/or velocity of the ions. According to their *m/z* the ions are differently deflected. In the relatively new Orbitrap mass spectrometer ions are electrostatically trapped in an orbit and the mass is measured by detecting the image current produced by the ions oscillating in the presence of an electric field. The frequencies of these image currents depend on the *m/z* of the ions. Mass spectra are obtained by Fourier transformation of the recorded image currents. In very costly Fourier transform ion cyclotron resonance mass spectrometer the image current is produced in a magnetic field which enables superior resolution and mass accuracy.

Combination of two or more *m/z* separation devices of different types, the so-called hybrid mass spectrometer, can combine the advantages of two techniques. A triple quadrupole mass spectrometer with the final quadrupole replaced by a time-of-flight tandem mass spectrometry (QTOF) or linear ion trap combined with an orbitrap mass spectrometry (LTQ-Orbitrap) have especially been shown to enable fast, sensitive and reliable detection and identification of low molecular weight substances thanks to their high mass accuracy and mass resolution (Van Bocxlaer et al., 2005; Lacorte et al., 2006; Bueno et al, 2007; Krauss and Hollender, 2008). Full-scan mass spectra acquired with high mass accuracy and resolution allow selective searching for the molecular ions of transformation products based on their exact mass, while MS/MS technology provides structural information based on compound fragmentation.

Several studies report the use of high-resolution mass spectrometry to screen for transformation products in biodegradation experiments or photolysis studies carried out in the laboratory (Ibanez et al., 2004; Durand et al., 2006; Gomez et al., 2008; Ruan et al. 2008). In these studies, transformation of the parent compounds is studied at high initial concentrations in controlled matrix. In that case, classical techniques such as UV-VIS spectrometry can help characterizing the products as shown in Längin et al. (2009). Screening and identification of pesticides and their transformation products in environmental samples by the combination of LC-ion trap with LC-TOF instruments have also been described (Hernandez et al., 2004; 2005; Thurman et al., 2005). A systematic procedure to screen for large numbers of transformation products in environmental samples containing a variety of organic compounds at low concentrations in the ng L^{-1} range using an LTQ-Orbitrap has only recently been reported (Kern et al., 2009).

The scope of this chapter is to present the potential of new hybrid tandem mass spectrometers to identify and quantify transformation products generated by human metabolism, microbial degradation, or other environmental breakdown processes. The strategies to identify transformation products by different hyphenated mass spectrometric techniques are presented in case studies where the advantages and the limitations of the structure elucidation procedure are also discussed. The first case study deals with the identification of a transformation product which is produced from a pharmaceutical during microbial degradation in the wastewater treatment process. The QTOF technology may enable identification of degradation products that are not yet described in the literature. In contrast, human metabolites are studied in detail in drug development procedure and are stated in pharmaceutical dossiers. Reference standards are sometimes not available and therefore clear identification must be carried out using, for instance, LC-TOF as presented in the second case study for a human metabolite in wastewater. The compound structure was confirmed by a QTRAP. Finally, we present the identification of a pesticide transformation product from groundwater samples. In this case study, Orbitrap technology enabled the identification of the transformation product in low environmental concentrations concurrently with a targeted screening. The case studies presented herein not only include the application of three different hybrid LC-MS techniques, but at the same time we show the identification of transformation products in very different matrices from relatively pure groundwater to highly contaminated wastewater.

2.1 Case study 1: Identification of a biotransformation product of the pharmaceutical diclofenac in wastewater by ultra performance liquid chromatography hyphenated with quadrupole-time-of-flight mass spectrometry

Among the most powerful instruments for the identification of unknown analytes is the quadrupole – time-of-flight mass spectrometer (QTOF), a hybrid mass spectrometric system that combines the advantages of ion separation and the detection principle of time-of-flight (TOF) systems and the fragmentation obtained with MS^2 experiments. TOF instruments provide full-scan sensitivity, high mass resolution (10,000-20,000, full width at half maximum), good mass - accuracy (< 3 ppm), and theoretically limitless scan range (Campbell et al., 1998). However, structural elucidation with the stand-alone TOF is primarily feasible for compounds with easy in-source fragmentation or those having a characteristic isotopic pattern (Petrović and Barceló, 2007). As an alternative, the hybrid QTOF, in which the final resolving mass filter of a triple quadrupole is replaced by a TOF analyzer, also enables the acquisition of high resolution mass spectra with accurate masses for the product ions. This gives the analyst a much higher degree of certainty when identifying compounds in non-target analyses, by positively and unequivocally confirming target compounds (VanBocxlaer et al., 2005; Petrović and Barceló, 2006). While this instrument is already a well established tool for the confirmation of target micropollutants in environmental matrices, its use for the identification of complete unknowns or transformation products is still growing. So far only a few studies have reported the application of QTOF in this field (Eichhorn et al., 2005; Pérez et al., 2007; Kosjek et al., 2008; Kosjek et al, 2009).

As an example of use of QTOF-MS, in this paper we describe the separation, detection, and successful identification of a nitro-analogon of diclofenac, a biotransformation product produced in a pilot wastewater treatment plant. At the outset of the study, emphasis was placed on quality chromatographic separation, which is of great importance in a complex environmental matrix, such as wastewater. Thus, ultra performance liquid chromatography (UPLC) was employed and enabled elution of the analytes in narrow, concentrated bands resulting in improved resolution, increased peak capacity, and increased speed of chromatographic separation (Petrović et al., 2006). This was performed with a Waters Acquity UPLC system (Waters Corp., Milford, MA, USA), equipped with a C-18 column with a 1.7 µm particle size (Waters Acquity 50 × 2.1 mm) using water / methanol

gradient elution at a flow rate of 0.3 mL min^{-1}. The UPLC system was hyphenated to a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (QTOF Premier, Waters, Milford, Massachusetts, USA). To aid in the detection of biotransformation products, post-acquisition data processing was employed using the MetaboLynx™ software package. The algorithm, a part of MassLynx v4.1 software (Waters), searches extracted mass chromatograms for expected transformation products based on predicted or unpredicted molecular changes relative to the parent compound and thus aids in the detection and identification of unknowns, particularly those buried in spectral noise. The software compares mass spectral chromatograms between a control and an analyte (Freed et al., 2004). Thus, the use of MetaboLynx™ software for comparison of treated and untreated wastewater samples spiked with diclofenac resulted in detection of diclofenac biotransformation product, eluted at 5.0 min. Accordingly, the isotopic cluster analysis was performed in order to determine the isotope ratio between cluster ion fragments and yielded the same results. Figure 1 illustrates a segment from the MetaboLynx report file with the extracted mass chromatogram of the biotransformation product (top right), its mass spectrum (top left), and the absence in the control sample chromatogram (bottom).

The high resolution and accurate mass measurements provided by the TOF mass analyser identified the deprotonated molecular mass [M-H]$^-$ 338.9945. Using the "Elemental composition calculator" tool (± 10 ppm mass error, C:0-15, H:0-20, N:0-3, O:0-10, Cl:0-2) we were able to assign a highly probable elemental formula of the diclofenac biotransformation product ($C_{14}H_{10}N_2O_4Cl_2$). Comparing to the elemental formula of diclofenac ($C_{14}H_{11}NO_2Cl_2$), the biotransformation product shows a substitution of a hydrogen atom for a NO_2 group. The structure of the biotransformation product was studied based on its TOF-MS-(ESI-) and TOF-MSMS-(ESI-) fragmentation (Figure 2). Besides the deprotonated molecule $[M-H]^-$ (*m/z* 339), the elimination of CO_2 to form *m/z* 295 also occurred under MS conditions due to in source fragmentation. The TOF-ESI(−) mass spectrum in Fig. 2(a) illustrates a dichloro isotopic pattern in both, *m/z* 339 and 295. The collision induced dissociation of *m/z* 339 (Fig. 2(b)) resulted in further losses of HCl with *m/z* 259 and homolytic cleavages of $NO^{\cdot}$ with m/z 229 or $NO_2^{\cdot}$ with *m/z* 213. The product ion with *m/z* 223 is indicative of the loss of HCl from *m/z* 259, while *m/z* 193 corresponds to the loss of HCl from the product ion with *m/z* 229. The ion fragments proposed in Figure 2 were compared with fragmentation pattern of parent compound and confirmed by accurate mass measurements in which the mass error did not exceed 0.7 mmu. The identification procedure is described in detail in Kosjek et al. (2008). In conclusion, the results indicate that the biotransformation yields a nitro-analogue of diclofenac. However, the exact position of the nitro group within the molecule could not be derived from the MS/MS data, and further investigations applying nuclear magnetic resonance (NMR) are necessary for complete structure elucidation. The incorporation of the NO_2 group into the aromatic ring is a rather unusual transformation process, which does not occur during human metabolism. However, this transformation has previously been reported to occur on pesticides in the environment (Hernández et al., 2008) and is reasoned by the presence of nitrate in aquatic media (Hogenboom et al., 1999; Kosjek et al., 2008).

With this study the key attributes of the QTOF instrument were confirmed: MS/MS fragmentation, high resolution, good mass accuracy, high sensitivity, and the ability to record a complete mass spectrum for each pulse of ions injected into the device. Further, this study implies that environmental and wastewater treatment processes yield different transformation products than does human metabolism.

2.2 Case study 2: Identification of the human pharmaceutical metabolite N-acetyl-4-aminoantipyrine by liquid chromatography combined with time-of-flight mass spectrometry and quantification by quadrupole/linear iontrap mass spectrometry

Structural elucidation with a self-standing TOF is only feasible for compounds with easy in-source fragmentation or a characteristic isotopic pattern. If a hybrid system such as a QTOF is not available, additional measurements on a low resolution tandem mass spectrometer can be acquired to confirm the structure suggested based on the molecular ion obtained by TOF. As an additional benefit, target analysis based on MS/MS fragmentation by an triple quadrupole or ion trap provides excellent performance for quantitative analysis because of its inherent selectivity and sensitivity (Barceló et al. 2007; Hernando et al. 2007a,b). Ion traps (IT) are particularly powerful for unequivocal confirmation or elucidation of molecular structures, since very fast and sensitive full scan modes (including MS2 and MS^n) can be applied. The latest generation of linear ion trap (LIT) mass spectrometers enables the use of selected reaction monitoring (SRM) dwell times as low as 2 ms without loss of sensitivity enabling multi-target methods. QqLIT systems offer hybrid triple quadrupole/linear ion trap capabilities. Working in LIT mode, the QTRAP systems provide improved performance and enhanced sensitivity in full scan MS (EMS) and product ion scan (enhanced product ion (EPI)) modes. An extra operational mode of this hybrid system is the possibility of combining in the same run, SRM and EPI scans, by the built-in information-dependent acquisition (IDA) software, thus obtaining at the same time quantification and additional structural information.

This case study describes an analytical protocol that combines the use of QTRAP and TOF instruments to achieve both accurate and reliable target compound monitoring and identification of one of the major known metabolites of the antipyretic drug dipyrone (Bueno et al, 2007). The analytical strategy proposed in this work provides a comprehensive approach to increase the scope of a monitoring program for the identification of emerging contaminants (including transformation products and metabolites) in wastewater.

The chromatographic separations in both QTRAP and TOF systems were performed using an HPLC (series 1100, Agilent Technologies, Palo Alto, CA) equipped with a reversed-phase C-18 analytical column (Zorbax SB, Agilent Technologies) of 5-µm particle size, 250-mm length, and 3.0-mm i.d. Gradient LC elution was performed with 0.1% formic acid and 5% MilliQ water in acetonitrile as mobile phase A, and 0.1% formic acid in water (pH 3.5) as mobile phase B (for details see Bueno et al., 2007)

As an example, the identification of N-acetyl-4-aminoantipyrine (4-AAA) by a TOF system is shown in Figure 3. This major human metabolite of the antipyretic drug dypirone was identified for the first time in wastewater and surface water by Zuehlke et al. (2004). The strategy for identifying non-target analytes in the samples was based on three steps: i) selection of the extracted ion chromatogram (XIC) for the target m/z, (20 mmu); (ii) background-subtracted mass spectrum; (iii) verification of accurate mass and elemental composition of the molecule and fragment ions. The agreement between the measured and calculated masses within a <5 ppm error level, along with matching retention times and mass spectra if reference standards are available, provided an unequivocal confirmation of the compounds in the samples. Analysis of spiked wastewater extracts resulted in errors lower than 2 ppm for the target compounds. Applying this strategy, other metabolites were also identified (4-dimethylaminoantipyrine; N-formyl-4-aminoantipyrine; 4-amino-antipyrine; antipyrine), confirmed with the acquisition of the appropriate standards, and finally included in the monitoring program.

Identification of transformation products by QTRAP systems in wastewater samples was reinforced by the acquisition of three transitions. Additionally, confirmation by ratio of SRM transitions was also used as an identification criterion and as a way to detect possible contributions of matrix interferences to the transition intensities, thus avoiding overestimations or false positive findings in quantitative analysis. For instance, the metabolite of carbamazepine, carbamazepine 10,11-epoxide was confirmed by the acquisition of three SRM transitions (253.2→180.2; 253.2→236.2 and

253.2→210.2). By IDA software, QTRAP systems enable the application of survey scans in SRM mode and EPI mode in a single run. This alternative is useful for compounds for which the second transition is not detected or is present at low intensity and additional structural information is required for a suitable confirmation.

In summary, target analysis of contaminants by QTRAP provided quantitative results for a large group of selected compounds. The analyses by TOF-MS enabled the identification of non-target compounds in wastewater samples.

2.3 Case study 3: Identification of a transformation product of the pesticide chloridazon in groundwater by liquid chromatography combined with linear iontrap-Orbitrap mass spectrometry

Orbitrap technology was introduced to the market in 2005. The hybrid system of linear ion trap combined with the new orbitrap technology (LTQ-Orbitrap) combines high sensitivity with high mass resolution (R >100,000) and high mass accuracy (< 2 ppm) (Hu et al., 2005; Makarov et al., 2006). In recent years several studies reported the use of this new technology to identify unknown micropollutants, metabolites, and transformation products in laboratory studies or environmental samples including surface and groundwater (Petermann et al., 2006; Ruan et al., 2008; Reineke et al., 2008; Kern et al., 2009).

As an example of the use of LTQ-Orbitrap, we describe the separation, detection, and successful identification of a transformation product of chloridazon in groundwater parallel to a multi-targeted screening. As part of a Swiss national survey in 2008, we screened approximately 20 groundwater samples from various catchments within both agricultural and urban areas for the occurrence of more than 200 pharmaceuticals, pesticides, biocides, and their transformation products. Additionally, the samples were analysed for non-target compounds using accurate mass screening. For this purpose, all samples were enriched using solid phase extraction (SPE). Subsequently, 20 µl of the SPE extract were injected into the LC system. Chromatographic separation of the extracts was achieved on a C-18 column (XBridge, Waters, 50 x 2.1 mm, particle size of 3.5 µm) using gradient elution with methanol and water (0.1 % formic acid) at a flow rate of 200 µL min^{-1}. After electrospray ionisation in the positive and negative mode, ions were detected by a LTQ- Orbitrap XL mass spectrometer (Thermo Fisher Scientific Corporation). High-resolution mass spectra (HR-MS) with a resolution of 60,000 were recorded to extract the chromatograms of target and non-target analytes. To confirm peak findings, data-dependent high-resolution product ion spectra (HR-MSMS) at a resolution of 7,500 were also produced. In order to receive more than ten HR-MS scans for each peak and simultaneously enough HR-MSMS within one chromatographic measurement, the resolution had to be set to this low value. Mass calibration was carried out with external standard calibration compounds and a typical mass accuracy of < 3 ppm was achieved.

For the identification of more than 200 compounds, the accurate masses were extracted from the HR-MS-TIC with a mass filter of 5 ppm and confirmed by matching the HR-MSMS and the retention time with the related reference standards. To find unknowns, non-target compound detection was performed by filtering the total acquired mass range (115-1000 m/z) of the HR-MS-TIC (Figure 4 A) with a 5 ppm mass extraction window using the Formulator software (Thermo Fisher, USA). As a result, up to 5,000 extracted ion chromatograms containing peaks with a signal-to-noise ratio greater than 5 were collected per sample. After sorting the data set by retention time and peak intensity, compound peaks with high signal intensity and distinct isotopic patterns were processed further.

As an example, the protonated molecule $[M+H]^+$ with an accurate mass of 160.0272 occurred in nearly all groundwater samples with an intense peak at 2.2 min (see Fig. 4 B). By taking the accurate mass and the isotope pattern into account, the elemental composition $C_5H_6Cl_1N_3O_1$ could be

unequivocally assigned to this peak by constraining the atoms to C, H, N, O, S, Cl, and Br for the elemental formula fit. The excellent match of the measured and theoretical isotope pattern is depicted in Fig. 4 D and E. Searches in the Scifinder and Pubchem data base for $C_5H_6Cl_1N_3O_1$ resulted in approximately 100 possible chemical structures. By comparing the measured HR-MSMS with predicted mass spectra proposed by the software Massfrontier (Thermo Fisher, USA) along with estimated retention times for all possible structures from the data base search, the best match for the identified elemental composition was determined to be chloridazon-methyl-desphenyl. Because a reference standard was available for this compound, the retention time and the HR-MSMS were matched between the sample (Fig. 4 F) and the reference standard (Fig. 4 G). Due to this comparison it could be unequivocally confirmed that the unknown compound is indeed chloridazon-methyl-desphenyl..

Chloridazon is a systemic herbicide which is widely used for sugarbeet and beet crops. The biological formation of chloridazon-methyl-desphenyl from chloridazon takes place in soil (Roberts and Hutson, 2002; EU DG, 2006). Chloridazon (5-amino-4-chloro-2-phenylpyridazin-3(2H)-one) is first degraded to chloridazon-desphenyl which is further transformed to chloridazon-methyl-desphenyl (Fig. 5). The transformation product chloridazon-methyl-desphenyl was detected in many of the investigated groundwater samples in concentrations up to several 100 ng L^{-1}. This is in agreement with findings of Weber et al. (2007), who described the occurrence of this compound in surface, ground, and drinking water in Germany. The transformation product was most often found in higher concentrations than the parent compound which reinforces the need to include transformation products in environmental quality monitoring.

In summary, the LTQ-Orbitrap instrument concurrently enables a multi-targeted screening (with sensitivity comparable to a tandem mass spectrometer) and an identification of unknowns based on high mass resolution and mass accuracy for molecular ions and fragments.

3. Conclusions

The three case studies demonstrate that hybrid tandem mass spectrometry, which combines two mass spectrometric technologies including high resolution technique, opens possibilities for identification of polar transformation products without reference standards and even gives decisive hints for the identification of previously unknown transformation products. The hybrid mass spectrometry technology can be applied to different environmental matrices from relatively pure groundwater to highly contaminated wastewater. The new generation of instruments allows the detection of concentrations down to the low ng per liter range. Since the software tools for an automatic non-targeted screening mostly do not provide sufficient support and are demanding to work with, the detection of unknown transformation products is still time consuming and requires analysis by those with a high level of chemical expertise. The examples presented herein and described in the literature on the elucidation of transformation products are still scarce and more studies are needed to improve the knowledge about the occurrence of transformation products in the environment. Along with the identification and quantification of these compounds, the toxicity assessment is another important task, which may help to clarify the burden that the transformation products pose to human health and the environment.

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Table 1 Comparison of current mass spectrometers concerning resolving power, mass accuracy, and sensitivity.

mass spectrometer	**resolving power*** (FWHM)	**mass accuracy*** (ppm)	**sensitivity*** (absolute)
quadrupole (Q)	unit resolution**	100	fg-pg
quadrupole ion trap (QIT; linear, 3D)	20,000	50	fg-pg
time-of-flight (TOF)	20,000	3	pg
sector field (magnetic/electric)	80,000	2	fg-pg
orbitrap	100,000	2	fg-pg
fourier transform ion cyclotron resonance (FT-ICR)	1,000,000	1	fg-pg

* Common values for low mass range (about *m/z of* 400); mass resolution is dependent on different parameters like scan speed, mass, instrument design, etc.; special instruments can reach better values

** Unit mass resolution is the resolution for standard quadrupole instruments; with special hyperbolic quadrupole instruments a resolving power of 5000 and a mass accuracy of 5 ppm can be achieved

Figure captions

Fig. 1 Extracted from the MetaboLynx report file: analyte (top) and control (bottom) sample mass chromatograms and TOF-ESI(−) spectrum (left) of a peak eluting at t_R 4.95 min

Fig. 2 Top: TOF-ESI(−) mass spectrum of biotransformation product (a); bottom: MS/MS spectrum of m/z 339 (b); proposed chemical structures of deprotonated molecule (m/z 339, C) and principal product ions 295 (D), 259 (E), 229 (F) and 213 (G). (Reproduced from Kosjek et al., 2008 with permission of Elsevier).

Fig 3 Identification of *N*-acetyl-4-aminoantipyrine (4-AAA), a major metabolite of the antipyretic drug dipyrone, by TOF system

Fig. 4 Chromatograms and spectra of an enriched ground water sample for the identification of chloridazon-methyl-desphenyl as a mobile and persistent transformation product of the herbicide chloridazon
A: Total ion chromatogram of HR-MS scans (R 60'000, 115-2000m/z)
B: Extracted ion chromatogram (5 ppm) of $[M+H]^+$ 160.0272 m/z
C: Data dependent HR-MSMS scan of 160.0272 (R 7'500, 50-175m/z)
D: Measured HR-MS at 2.2 min (R 60'000, 115-2000m/z)
E: Theoretical HR-MS of the elemental composition $C_5H_6ClN_3O$ ($[M+H]^+$=160.0272)
F: Measured HR-MSMS of 160.0272 at 2.2min, marked fragments (*) were predicted by Massfrontier
G: Measured HR-MSMS of a standard solution of chloridazon-methyl-desphenyl

Fig. 5 Proposed formation of the transformation product methyl-desphenyl-chloridazon from chloridazon in soil (Weber et al., 2007)

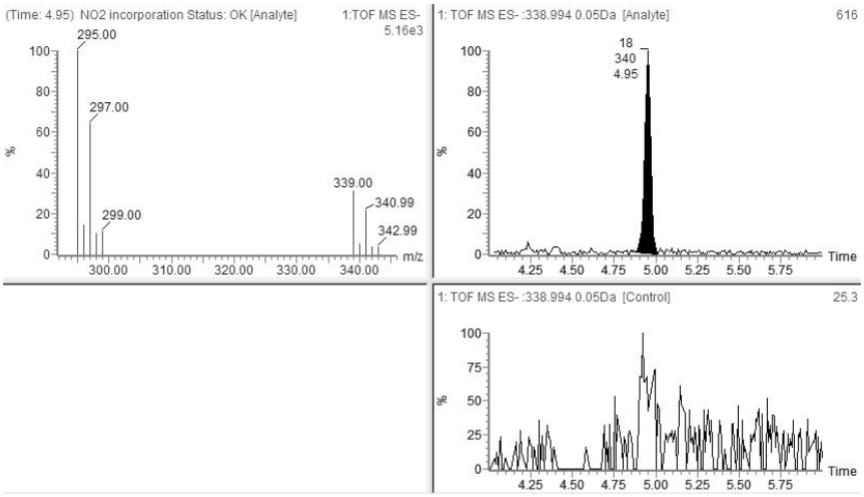

Figure 1

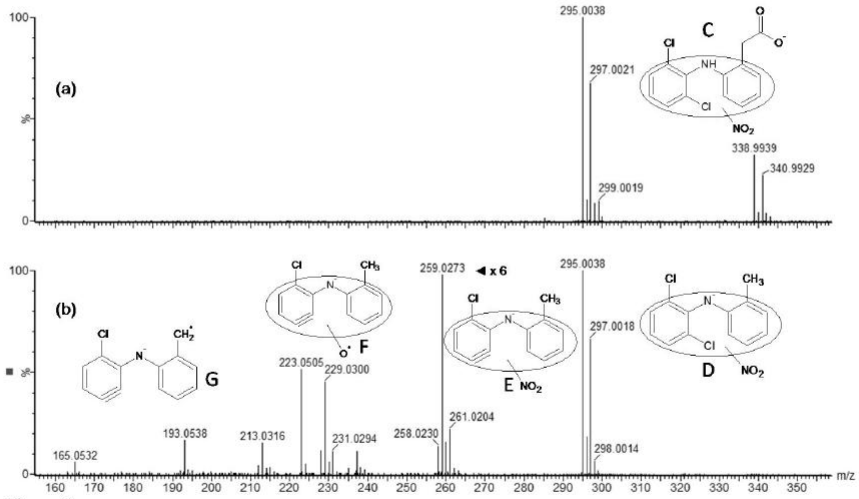

Figure 2

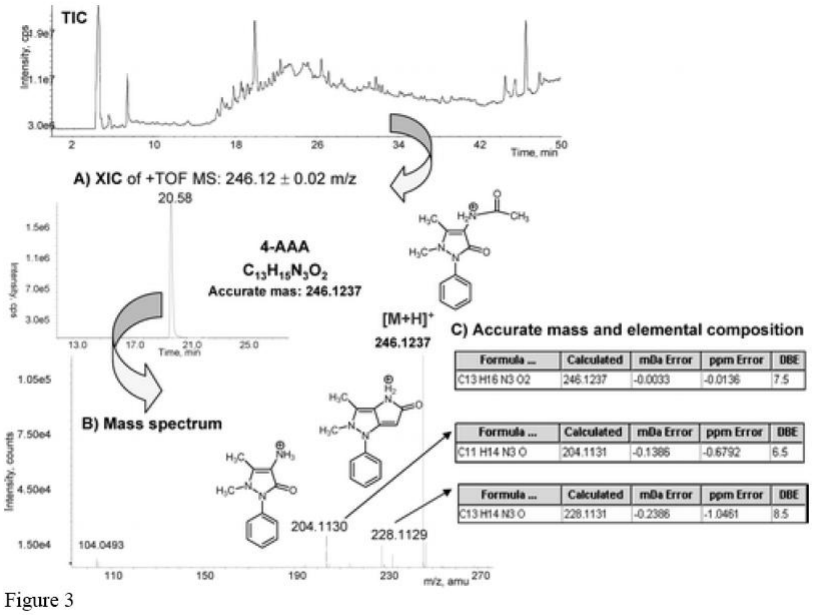

Figure 3

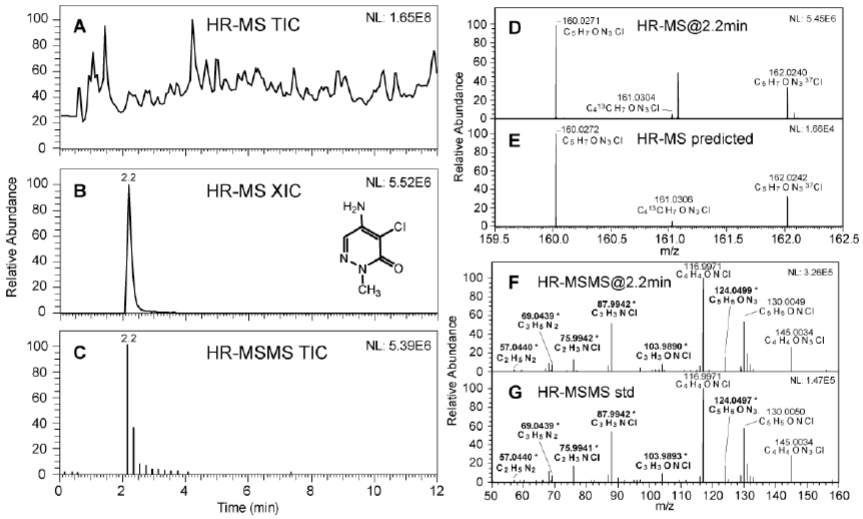

Figure 4

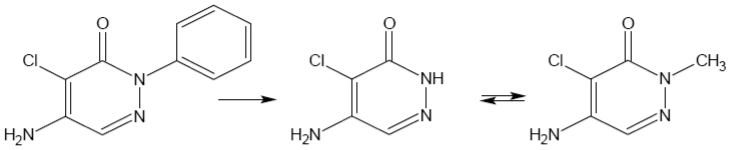

Figure 5

3.4 Transformation of pharmaceuticals during advanced oxidation and disinfection processes

Identification of TPs is justified for clarifying the reactions and the processes in water treatment, for improving it, and consequently for minimizing pharmaceutical residues in waste, environmental and potable water. In parallel, recognizing TPs and their individual and mixture toxicity, are grounds for a comprehensive environmental risk assessment on selected pharmaceuticals.

The breakdown pathways and the production of TPs depend on water treatment technologies, so different intermediates and reaction products may be formed. Breakdown during AOPs depends on the physicochemical characteristics of compounds, but typically involves hydroxylation of the aromatic ring by an electrophilic attack from ˙OH radicals, cleavage of C-O, C-N or S-N bond, cleavage on α-position from the aromatic moiety and ring opening, while chlorine disinfection merely yields chlorinated products. It was also observed that LC-MS and GC-MS yield distinctly different TPs, and that implies a need to combine both separation methods to obtain a comprehensive view of pharmaceutical transformations.

This study suggests that attention should be on the origin of TPs, i.e. whether they were formed as a result of actual water treatment or during sample preparation or analysis. Also, it highlights the hurdle related to the commercial unavailability of authentic TP standards, making in-house chemical synthesis the only option available, which indeed is a complex, time consuming and expensive task. Nevertheless, the authentic standards are of crucial importance, not only for confirming identity, but also for the continuation of the research in view of studying breakdown pathways and fate of the identified TPs. This study is also an example of the application of alternative identification methods to support structural elucidation. These methods can either involve other instrumental techniques (NMR, UV, complementary MS techniques), or use existing data on the investigated compounds found in either the NIST library or published mass spectra.

Amongst most important outcomes of this research was the finding that the UV irradiation and, to some extent, also the ClO_2 disinfection enhanced the biodegradability of CBZ residues. This suggests the use of sequential application of UV treatment with a second biological treatment step for advanced water treatment. The proposed coupled treatment technology for removal of CBZ residues may be relevant in a field of raw water for potable water production, employing UV treatment with a subsequent biological sandfilter. However, a sandfilter prior to UV is the preferred arrangement, since it improves the quality of water for UV to consume less energy. In this sense, the cost-effective solution would be to set up a system involving two sandfilters with a UV unit in between. Alternatively, the system involving ClO_2 oxidation with a subsequent biological treatment is then potentially useful for wastewater treatment, since together with an improved elimination of CBZ residues, there will be a decrease in the BOD created from COD during the ClO_2 oxidation. Naturally, the two proposed systems will require scale-up and further evaluation, both from a scientific and economic perspective.

Finally, this study sets an illustrative example, where the intermediate species are more toxic and hazardous than the parent compound. The major intermediates arising from ClO_2, UV and biological treatment of CBZ belong to the azaarenes, an established class of air and water pollutants, known for their photo-enhanced toxicity, mutagenic and carcinogenic activity [242,243,244]. This raises an important issue concerning the possible environmental impact of pharmaceutical residues in either domestic wastewaters or potable waters.

The results of this research are in details described in two scientific papers:

- Applications of mass spectrometry to identifying pharmaceutical transformation products in water treatment (TrAC, Trends in Analytical Chemistry, 2008)
- Fate of carbamazepine during water treatment (Environmental Science & Technology, 2009).

3.4.1 Scientific paper: “Applications of mass spectrometry to identifying pharmaceutical transformation products in water treatment”

Trends in Analytical Chemistry, Vol. 27, No. 10, 2008 Trends

Applications of mass spectrometry to identifying pharmaceutical transformation products in water treatment

Tina Kosjek, Ester Heath

Measuring the elimination of an organic pollutant is not sufficient to assess the efficiency of water-treatment techniques, as transformation products, more persistent and/or more hazardous than the parent compounds, may be generated. The identity, the fate and the effects of these compounds are the subjects of an increasing number of studies, in which the development of mass spectrometry (MS)-based methods has resulted in increased sensitivity and selectivity and has been widely applied in the investigation of breakdown products and pathways.

This review covers recent applications of MS to identifying pharmaceutical-degradation products formed *via* the oxidation and chlorination reactions utilized in water treatment. We give an overview of the current status and future prospects of advanced hyphenated MS techniques. We discuss the capabilities, the potential and the limitations of different mass analyzers for some of the most commonly studied pharmaceuticals.

Keywords: Advanced oxidation process; Disinfection; Gas chromatography; Identification; Liquid chromatography; Mass spectrometry; Pharmaceutical; Photolysis; Transformation product; Water treatment

Tina Kosjek,
Ester Heath*
"Jožef Stefan" Institute, Department of Environmental Sciences, Jamova 39, Ljubljana, Slovenia

*Corresponding author.
Tel.: +386 1 477 5384;
E-mail: ester.heath@ijs.si

1. Introduction

The presence of pharmaceutical residues in surface waters is an emerging environmental issue that provides a new challenge to treatment systems for drinking water, wastewater and water reuse. Pharmaceuticals after being administered to patients are normally excreted as a fraction of various metabolites together with the unchanged parent compound. Another common practice is the disposal of outdated or unused medicines down the drain or the toilet. In either case, significant quantities of pharmaceutical residues find their way to wastewater-treatment plants.

The occurrence of such compounds in surface waters and groundwaters can be explained in part by the inefficient removal processes of conventional water-treatment technologies [1], so the need for enhanced technologies that can reduce the discharge of pharmaceutical residues into the environment is clear. In this sense, ozonation and advanced oxidation processes (AOPs) are promising solutions to upgrade existing treatment [2]. Ozone (O_3), which is an effective disinfectant and powerful oxidizer, reacts directly with molecular O_3 or *via* the formation of free radicals (e.g., the hydroxyl radical). Molecular O_3 is a selective electrophile that reacts easily with double bonds, activated aromatic structures, or heteroatoms (e.g., nitrogen and sulfur), which are common substituent groups of many pharmaceuticals (e.g., diclofenac, carbamazepine and sulfamethoxazole). Alternatively, pharmaceuticals without reactive sites (e.g., bezafibrate, clofibric acid and iopromide) are more amenable to hydroxyl radicals ($^{\bullet}OH$), which react less selectively and at increased molecular rates [3,4].

In most water-treatment applications, the amount of $^{\bullet}OH$ produced from O_3 is generally low, so AOPs use a combination

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of O_3 with hydrogen peroxide (O_3/H_2O_2) to increase the concentration of $^{\bullet}OH$ in order to remove more recalcitrant compounds [5]. Furthermore, AOPs comprise numerous techniques (e.g., H_2O_2/UV, O_3/UV, γ-radiolysis, TiO_2 photocatalysis and photo-assisted Fenton) for producing highly reactive oxygen species for directly degrading organic pollutants present in drinking water and wastewater [1,6,7]. In addition, natural abiotic degradation processes (e.g., photolysis under sunlight) are believed to enhance the removal of pharmaceutical residues in surface waters significantly. Here again, the hydroxyl radicals, especially in photosensitized oxidation, are expected to play a key role [1].

Alternatively, disinfection processes for drinking water comprise non-radical oxidation reactions. Direct UV irradiation and O_3 are considered the disinfection agents, but the best known and historically most important are chlorinating agents (e.g., molecular chlorine, sodium hypochlorite, chloroamine and chlorine dioxide). Molecular chlorine is more reactive in oxidation reactions and in reactions with double bonds, while sodium hypochlorite has a higher activity in electrophilic aromatic substitutions [8]. However, trihalomethanes and halogenated acetic acids are generated during Cl_2 and NaClO treatment, and carcinogenic dimethylnitrosamine is also formed from chloroamine. Chlorine dioxide does not yield halogenated disinfection by-products, nor does nitrosamine under the correct operating conditions, and, in this sense, appears to be the chlorinating agent of choice [8,9].

The applications of AOPs and disinfection methods to recalcitrant pollutants have expanded in recent years, spurring extensive kinetic studies aimed at optimizing operational parameters [10]. However, the abatement of pharmaceuticals and other organic micro pollutants only provides a partial indication of the efficiency of the various treatment methods and the possible generation of toxic intermediates more resistant to degradation must not be overlooked [11]. Many gaps in our knowledge remain as to the degradation mechanisms, the identity of the transformation products (TPs), and their origin, fate and impact on the environment. Insights into this relatively unexplored region of environmental analytical chemistry are hindered by the need to contend with the quantity and the variety of products present at various degrees of oxygenation and degradation. Their identification also requires the application of sophisticated analytical tools capable of providing complementary information to complete the assignment of structure. Advanced MS techniques combined with either gas chromatography (GC-MS) or liquid chromatography (LC-MS) have, due to their high selectivity and sensitivity, gained in popularity and are now the preferred techniques for the structural elucidation of degradation products in the aquatic environment [12–14].

There are other reviews devoted to the subject of pharmaceutical environmental fate and behaviour during water treatment. For example, Zwiener [4] gave an insight into the pharmaceutical fate and removal by different drinking-water-treatment methods. Furthermore, Radjenović et al. [15] reviewed sample-preparation procedures based on analysis using GC or LC coupled to MS detection. In addition, they comprehensively discussed the removal of parent pharmaceuticals during biological wastewater-treatment technologies. Although these papers made important contributions to the field, they did not deal specifically with pharmaceutical TPs and with the potential of MS for their identification. Albeit, the applications of MS for qualitative determination of human metabolites [16], biodegradation [14,16] and photodegradation products [13] have recently been reviewed, and increasing concern about pharmaceutical residues in the environment is reflected in the increasing number of research papers and reviews.

In contrast to what has been published, this review principally covers pharmaceutical TPs (PTPs) generated by man-made AOPs and disinfection processes for water treatment. We do not specifically address natural photolytic processes (i.e. solar irradiation); yet, due to the strong similarities in the transformation mechanisms and in order to maintain the comprehensiveness of the review, we include environmentally-generated PTPs. In brief, in this article, we give an overview on the increasing role of MS methods for identifying PTPs and elucidating reaction pathways. The article is oriented towards techniques and methods, and discusses the capabilities and the limitations of established MS techniques by looking at examples. However, we do not address the breakdown processes in terms of reaction mechanisms, influencing factors and efficiencies, but focus strictly on the analytical challenges in the field of qualitative analysis with MS.

2. Capabilities of mass spectrometry for structural characterization

MS has revolutionized environmental analytical chemistry by allowing the analysis of complex organic mixtures for trace amounts of analytes.

MS analysis can not only generate informative fragmentation patterns that give an organic compound a unique molecular signature, which can be resolved using the principles of physical organic chemistry to reveal the chemical structure of an unknown compound, but also give an accurate mass to confirm the presence of the compound and reveal its elemental composition.

Complex environmental or biological samples require separation of components prior to the mass analysis, which justifies the use of combined systems (i.e. GC-MS

or LC-MS). In modern GC-MS instruments, an electron-impact (EI) ionization source is normally employed as an interface, providing a wealth of structural information in the mass spectra. EI is performed at 70 eV, thus yielding mass spectra, which are for the same molecule identical over time and between instruments. The resultant spectra can then be matched against spectra of authentic compounds, held in what have now become extensive GC-MS libraries. This ability to match to known spectra can significantly facilitate the structural elucidation of unknowns [17]. Examples taken from the recent literature, where this approach has been successfully applied to PTPs include 17β-estradiol [18], bezafibrate [19] and diclofenac [20].

Whereas EI can result in a loss of a compound's molecular ion in a mass spectrum, chemical ionization (CI) is a much ''softer'', lower energy alternative to EI that uses a reagent gas (usually ammonia or methane) in the ion chamber. The result is a reduction in the residual energy of the protonated molecules, so that fragmentation is greatly reduced and the molecular ion in the mass spectra is more prominent [12,20].

The coupling of high-performance LC with MS (HPLC-MS) has proved more challenging than that of GC with MS, since the aqueous HPLC effluent containing polar analytes must be converted to gas-phase molecules. Fortunately, this physical hurdle was overcome by the development of atmospheric pressure ionization (API) techniques [21]. API gives ''soft'' ionization with high efficiency, thus providing molecular mass information and excellent sensitivity. Despite this, the poor fragmentation makes identification much more challenging [22].

The most common API interfaces are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), while the more recently developed atmospheric pressure photoionization (APPI) is yet to be applied to the determination of PTPs. From the literature, it is mainly ESI that is applied for determining TPs [1,3,12,19,20,23–34]. Generally, ESI enhances the analysis of more polar compounds, while less polar and thermally-inert compounds are more amenable to APCI. APCI was used for the analysis of carbamazepine [3,32], sulfonamide [27,35] and clinafloxacin TPs [30]. In addition, matrix-assisted laser desorption/ionization (MALDI), despite normally being used in proteomics, was used to introduce photo-TPs of naproxen into a time-of-flight (ToF) mass spectrometer [36].

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 (MS^2) experiments; and,

2) highly accurate molecular mass measurements [13].

The simplest mass spectrometer is a single-stage quadrupole (Q), which, for the structural elucidation of PTPs, was employed in a GC-MS [12,18–20,27,36–38], or an LC-MS system [24,26,27,30,31]. In the Q mass analyzer, both analyte and matrix ions generated in the source undergo fragmentation, which results in complex, ambiguous spectral data and hence in non-selectivity that is its main disadvantage [21]. This non-selectivity of Q is overcome by tandem mass analyzers, which, due to their high specificity, can reduce the ''chemical noise''.

A triple-quadrupole (QqQ) mass detector is a ''tandem in-space'' instrument, comprising two mass analyzers with a collision chamber in between where ''collision-induced dissociation'' (CID) occurs. QqQ, by allowing multiple-reaction monitoring (MRM), precursor-ion scans (PISs) and constant neutral-loss scans (NLSs), affords much greater experimental flexibility and precision, when compared to the single Q, and provides a more comprehensive dataset for structure elucidation [3,23,25,29,39]. However, MS^2 fragmentation in QqQ can be limited or insufficient for full structure elucidation [40].

By contrast, ion trap (IT) mass detectors have high sensitivity, when in full-scan mode, and the unique ability to isolate and to accumulate ions, and that – by iterating ion trapping and scanning – allows the generation of CID spectra of the parent and fragment ions (and their fragment ions), resulting in a hypothetically infinite number of fragmentation patterns (i.e. MS^n) [21]. Combined with knowledge of the functional group fragmentation behavior, the MS^n spectra greatly facilitate the elucidation of the fragmentation mechanism of unknown species, and increase the level of confidence in assigning a particular structure [13]. Such multi-stage fragmentation has proved to be one of the most powerful techniques available for investigating suspected or unknown TPs [3,10,11,30–32,35,41–43].

The linear two-dimensional ion trap (LIT) has the advantage over the three-dimensional ion trap (3D-IT) as it omits the quadrupolar electric field in the axial direction. This means that fewer ions are lost during the process of filling and emptying the trap, and that, in turn, increases sensitivity [44]. So far, the LIT has found little application in studies of PTPs, when compared with 3D-IT [30,31,35].

Pérez et al. [28] have reported the use of a hybrid quadrupole LIT (QqLIT) as a confirmatory technique in the structural characterization of enalapril and enalaprilat TPs. The confirmation of the TPs was based on the instrument's MS^3 capabilities, while the identification relied principally on the use of another hybrid instrument (QqToF), exploiting its capabilities to determine the accurate mass of (de)protonated molecule and product ions [28]. The QqLIT operates the third quadrupole either as a mass-resolving quadrupole or as a LIT, and offers an flexible platform for a range of bioanalytical applications, enabling MRM, PISs and NLSs, together

with enhanced full-scan sensitivity and MS^3 or higher stage data acquisition for PTP-identification studies [21]. In spite of this, the mass resolution of the Q (qQ) or the IT mass analyzers is insufficient to determine the molecular formula of an unknown compound [40].

By contrast, orthogonal accelerating ToF (oaToF) mass spectrometers have a high resolution (10,000–20,000 FWHM), good mass-assignment accuracy (<3 ppm), sensitivity, and allow rapid mass scanning in a theoretically limitless scan range [44]. Accurate-mass determination provided by ToF instruments allows specific information to be obtained for a given molecule and enables almost unequivocal confirmation of the identity of the compound. However, structural elucidation is feasible only for compounds that easily fragment 'in-source' and/or have a characteristic isotopic pattern [13,45]. The advantages of the ToF instruments have been utilized in several studies, either solely [1] or complementary with other mass analyzers possessing fragmentation capabilities [12,19,20,31,33,36].

As an alternative, the hybrid QqToF, in which the final resolving mass filter of a QqQ is replaced by a ToF analyzer, not only allows MS^2 operation but also has the high accuracy and the resolution necessary to give exact-mass measurements in a single instrument. Despite these excellent characteristics for qualitative non-target analysis, there have been few applications of QqToF to studying PTPs [28,46].

Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometers provide even more resolving power than ToF instruments, but FT-ICR instruments are usually out of reach for most environmental applications [40]. However, Sakkas et al. [34] reported the application of a hybrid LIT with an Orbitrap mass analyzer (LTQ-Orbitrap), employing the FT algorithm for high-resolution mass analysis of salbutamol TPs.

Another promising technique for identifying unknowns involves coupling the ToF mass spectrometer to GC × GC. The structural assignment is possible using mass-spectral-database searches, where the added selectivity of full mass-spectral detection lends more credence to analyte identification [47]. However, no applications of GC × GC/ToF-MS have yet been reported in identifying PTPs.

3. Applications

Separation techniques, such as GC and LC, hyphenated to MS detectors are powerful tools for mixture analysis and have been widely used to study the breakdown of pharmaceuticals subjected to either AOPs or disinfection processes. Often however, MS alone is insufficient to identify the exact position of oxidation, to differentiate isomers, or to provide the precise structure of unusual and/or unstable TPs. In addition, other substances present in environmental samples can suppress ionization, complicating metabolite identification. In such cases, multiple analytical and wet-chemistry techniques, such as LC with nuclear magnetic resonance (NMR), chemical derivatization, and hydrogen/deuterium-exchange (H/D-exchange) combined with MS are used to characterize the novel and isomeric TPs of drug candidates [21]. Also, having authentic standards available, ultraviolet-visible (UV-Vis) spectroscopy is often applied, as it allows rapid and simple analyses of TPs. Table 1 shows the MS methods and complementary and/or confirmatory techniques for identification of PTPs.

In the following sections, we present the limitations and the potential of different MS techniques for identifying TPs, using examples of environmentally relevant pharmaceuticals [i.e. commonly used throughout the European Union (EU) (carbamazepine, diclofenac), resistant to conventional wastewater treatment (carbamazepine, bezafibrate, diclofenac) and/or potentially provoking antibacterial resistance (sulfonamides)].

3.1. Advanced oxidation processes

3.1.1. Carbamazepine. Carbamazepine is a dibenzazepine derivative with anti-epileptic and psychotropic activity; also, it is well established in the treatment of severe pain associated with neurological disorders (e.g., trigeminal neuralgia). It is administered chronically in doses ranging of 400–1600 mg daily, and is therefore continually being introduced into the environment, where it can be found in concentrations of a few hundred ng/L [48]. In addition, the drug has low sorption properties and resists biodegradation [49,50]. These factors make carbamazepine a pharmaceutical of considerable environmental relevance. By contrast with classical wastewater treatment, ozonation [3], H_2O_2/UV-induced photolytic degradation [11], photocatalytic degradation with TiO_2 [23] and direct photolysis [51] can remove carbamazepine.

McDowell et al. [3] used LC-MS^2, GC-MS^n and ^{1}H-NMR to characterize the TPs of carbamazepine formed during O_3 treatment. To obtain the molecular weights of three carbamazepine TPs, a QqQ mass analyzer coupled to an LC instrument was operated in both negative and positive modes of ESI. Increases in the mass-to-charge of 14, 30 and 46 were observed, and indicated the addition of one, two and three oxygen atoms along with removal of two protons in an elimination step (e.g., ring closure or double-bond formation) [3]. Complementarily, one of the TPs was introduced into a GC coupled to an IT mass detector. Using EI, a fragmentation pattern was obtained exhibiting m/z at 139, 167, 195 (base peak), 238 and 266 ($M^{+\cdot}$) (Fig. 1). The IT mass analyzer allowed subsequent GC-MS^2 experiments by trapping and fragmentation of the daughter ions at m/z 238, 195, and 167. The chemical structure of the TP being investigated was resolved by comparing the mass fragmentation with

Table 1. Overview of the pharmaceuticals degraded by advanced oxidation, disinfection or natural photolytic processes and the analytical methods employed for the identification of their transformation products (TPs)

Pharmaceutical	Treatment process	MS method	Complementary analytical methods	Ref.
β-blockers, other antihypertensives				
atenolol metoprolol propranolol	γ-radiolysis	LC/ESI-MS (ToF)	-	[1]
enalapril enalaprilat	Simulated sunlight	LC/ESI-MS^3(QqLIT), LC/ESI-MS^2(QqToF)	-	[28]
NSAIDs and analgesics				
paracetamol	O_3, H_2O_2 / UV	GC/MS^n (IT)	Authentic standards ^{1}H-NMR, HPLC-DAD*	[41]
	H_2O_2 / UV	GC/EI-MS^n (IT)	GC-MS / ^{15}N-labelling, HPLC-DAD, authentic standards, ^{15}N-, ^{1}H-, ^{13}C-NMR	[10]
dipyrone → 3 metabolites	Simulated sunlight	LC/ESI-MS (ToF), GC/EI-MS (Q)	-	[33]
naproxen	Simulated sunlight	GC/EI-MS (Q), MALDI-ToF	^{1}H-, ^{13}C-NMR	[36]
naproxen ketoprofen	UV, TiO_2/UV	LC-ESI-MS^2 (QqToF)	-	[46]
diclofenac	Photo-Fenton	GC/EI-MS (Q), GC/CI-MS (Q), LC/ESI-MS (ToF)	HPLC/UV-vis, ion chromatography, spectral libraries, "Frontier e^- density" theory	[20]
	H_2O_2 / UV, O_3	GC/EI-MS^n (IT)	HPLC-DAD, authentic standards, ^{1}H-, ^{13}C-NMR	[42]
	Heterogeneous catalytic oxidation with H_2O_2	GC/EI-MS^n (IT)	HPLC-DAD, ion chromatography	[43]
	Solar irradiation	GC/EI-QqQ	HPLC-PDA**	[39]
		GC/EI-MS (Q), GC/CI-MS (Q), LC/ESI-MS (ToF)	HPLC/UV	[12]
Psychoactive drugs				
carbamazepine	O_3	LC/ESI-MS^2 (QqQ), LC/APCI-MS^2 (QqQ) GC/EI-MS^n (IT)	^{1}H-NMR, IR	[3]
	H_2O_2 / UV	GC/EI-MS^n (IT)	HPLC-DAD, ^{1}H-NMR, authentic standards, spectral library	[11]
	Direct photolysis, UV/ $OH^•$ and $Cl_2^{•-}$	LC/APCI-MS^2/MS^3 (IT), LC/ESI-MS^n (IT)	HPLC/UV-vis	[32]
	TiO_2/UV	LC/ESI-MS^2 (QqQ)	HPLC-DAD/FLD***, authentic standards	[23]

(*continued on next page*)

Table 1 (*continued*)

Pharmaceutical	Treatment process	MS method	Complementary analytical methods	Ref.
fluoxetine flumeturon flutanil	Simulated sunlight, photosensitization	LC/ESI-MS^2 (QqQ)	HPLC-DAD, ion chromatography (F^-)	[29]
Estrogens				
17β-estradiol	NaHOCl	LC/ESI-MS (Q)	Structure deduction based on Cl-subst. mechanism / atom partial charge	[24]
	TiO_2/UV	GC/EI-MS (Q)	Spectral library, HPLC/UV, ''Frontier density'' theory	[18]
Synthetic antibacterials				
sulfamerazine sulfadiazine sulfadimethoxine sulfathiazole	TiO_2/UV	LC/APCI-MS^n (3D-IT)	Ion chromatography to observe a release of NO_3^-,NO_2^-,SO_4^{2-},NH_4^+	[35]
sulfamethoxazole	Cl_2	LC/ESI-MS (Q), GC/EI-MS (Q)	^{1}H-NMR, HPLC-DAD	[27]
clinafloxacin	Simulated sunlight	LC/APCI-MS (Q), LC/ESI-MS^n (3D-IT)	^{1}H-NMR	[30]
Antibiotics				
chlortetracycline	Simulated sunlight	LC/ESI-MS^n ((3D-IT), LC/ESI-MS (ToF), LC/DAD-ESI-MS (Q)	-	[31]
penicillin amoxicillin cefadroxil	ClO_2	GC/EI-MS (Q)	authentic standards, ESI-MS, ^{1}H-NMR, ^{13}C-NMR	[37]
Bronchodilators				
salbutamol	TiO_2/ simulated sunlight	LC/ESI-MS^2/MS^3/MS^4/ MS^5(LTQ-Orbitrap)	Ion chromatography	[34]
Anticancer drugs				
tamoxifen	Simulated sunlight, photosensitization	GC/EI-MS (Q)	Preparative TLC, ^{1}H-NMR	[38]
Lipid-regulating agents				
bezafibrate	TiO_2/UV	LC/ESI-MS (ToF) GC/EI-MS (Q)	HPLC-DAD, spectral library, authentic standards	[19]
	O_3	LC/ESI-MS (Q)	HPLC/UV-vis	[26]
clofibric acid (metabolite)	TiO_2/UV	LC /ESI-MS^2 (QqQ)	HPLC-DAD/FLD, authentic standards	[25]

Under ''MS method'', the separation method, the mass analyzer and, where stated, the ionization method are also given.
*DAD, Diode-array detector; **PDA, Photodiode-array detector; ***FLD, Fluorescence detector.

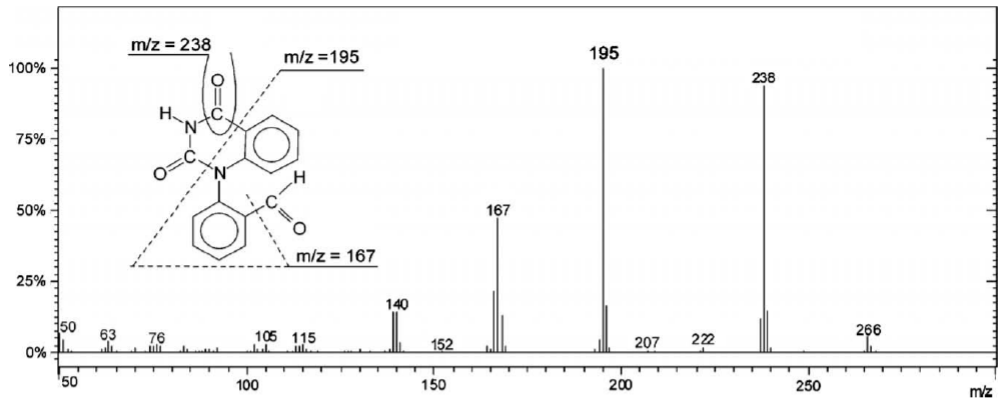


Figure 1. Fragment ions observed in the GC/EI-MS spectrum of the degradation product of carbamazepine (reproduced from [3] with permission of ACS Publications).

structurally-related caffeine and another quinazoline compound, which was identified as 1-(2-benzaldehyde)-(*1H, 3H*)-quinazoline-2,4-dione. Also, the CID spectrum of its $[M+H]^+$ molecule produced by LC-APCI-MS^2 revealed all the major fragments seen in the GC-MS spectrum. Finally, ^{1}H-NMR was applied, and a significant change to the aromatic hydrogens was observed between carbamazepine and its oxidation products. Similarly, the other two compounds were identified; however, due to insufficient volatility and polarity, structural elucidation was based on MS data obtained by LC-MS^2 [3].

The intermediates and products of carbamazepine oxidation by H_2O_2/UV mechanism were investigated by Vogna et al. [11]. Structural elucidation was based on mass fragmentation using an IT mass analyzer after GC separation. This study highlighted one of the main drawbacks of GC-MS (i.e. degradation of thermally-unstable carbamazepine in the GC, thus forming acridone and iminostilbene), so, to avoid any misleading conclusions regarding the mechanism of their production, GC-MS analyses needed to be performed only after the complete removal of the parent compound, thus missing the intermediates in the early oxidation stages [11]. Of the carbamazepine TPs found, the majority had the acridine structural fragment, while O_3 treatment resulted in quinazoline-type compounds [3,11] (Table 2). The formation of distinct degradation products suggested that the type of transformation reaction largely depended on the oxidation technique. Also, in view of the mutagenic properties of acridine, an important issue is raised concerning the generation of intermediates more hazardous than the parent compound. This supports the need for prolonged or upgraded oxidation treatments to ensure the complete degradation of any toxic intermediates [11].

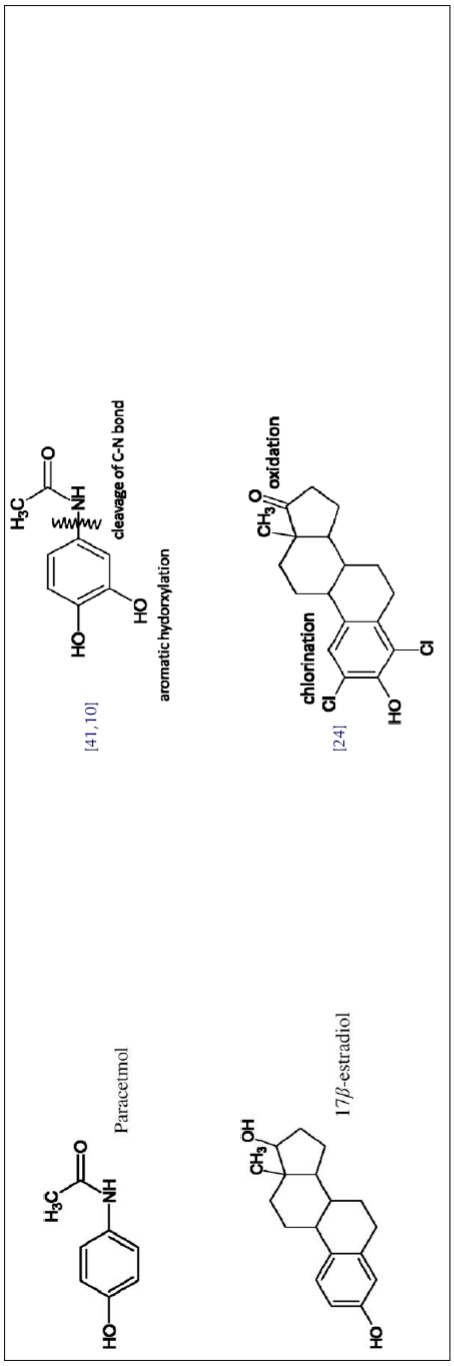
Acridine-like TPs, as well as hydroxylated carbamazepine and carbamazepine-epoxide (Table 2), were identified as products of TiO_2 photocatalysis in a study by Doll and Frimmel [23]. Structural characterization was carried out by interpreting mass spectra obtained with HPLC-(+)ESI-MS^2 using a QqQ mass spectrometer. Where authentic standards were available, degradation products were assigned according to retention times.

Chiron et al. [32] showed that, by direct photolysis, carbamazepine underwent slow degradation with acridine as the major TP. Intermediates, generated under different conditions of UV irradiation or photocatalysis with $OH^{\cdot}$ and $Cl_2^{\cdot-}$, were detected by HPLC-(+)APCI-MS^n and their structures were identified from mass fragmentation spectra. The molecular weight of the TPs was assigned on the basis of pseudomolecular ions $[M+H]^+$ and $[M+Na]^+$, while their structures were assigned according to their MS^2 or MS^3 mass fragmentation [32].

3.1.2. Bezafibrate. Lambropoulou et al. [19] investigated the breakdown of lipid-regulating agent bezafibrate during TiO_2-mediated photocatalysis. Three analytical techniques (LC-ToF-MS, GC-MS and HPLC) with diode-array detection (HPLC-DAD) were applied and produced complementary information that enabled them to identify 21 TPs. LC-ToF-MS was shown to be ideal for screening purposes, allowing up to 17 of the TPs to be detected. A combination of full-scan spectra, together with high sensitivity and good resolving power, provided the elemental formulae, which were used to confirm or to refute suggested structures. The following approaches were taken for the unequivocal identification of TPs:

Table 2. Parent Pharmaceuticals, their mass fragmentation and main structural transformations

Parent compound(s)	[Reference]/Main transformation pathways
Sulfonamides	[35] hydroxylation; ring opening, release of SO_2, NH_2, NO_2; cleavage of S-N bond
Bezafibrate	[M+H]+ 362; DI: m/z 139; [19] cleavage of amide bond; hydroxylation $(OH)_x$; ring opening; cleavage of ether bond
Clofibric acid	[25] aromatic hydroxylation; cleavage of ether bond; cleavage of chlorine / OH attack → hydroxylation
β-blocker (atenolol, metoprolol)	[1] hydroxylation; cleavage of ether bond; -OH substitution
Carbamazepine	[3] R^2: =O; R^1: -COOH or -COH; [11] R_1: -COH



(a) identification program embedded into the ToF software with a mass-accuracy threshold;
(b) the typical chlorine isotopic pattern;
(c) characteristic fragment ion used as a diagnostic ion (DI: m/z 139, Table 2); and,
(d) the presence of the sodium adduct along with the protonated molecule to provide the molecular weight of the TP.

Thus, during the first stages of treatment, multiple hydroxylations of bezafibrate were observed as a consequence of ·OH radical attack on the aromatic moiety. Further transformation steps involved ring opening and the cleavage of amide or ether bonds (Table 2). With the exception of 4-chlorobenzamide, the other TPs identified by LC-ToF-MS were not detected by GC-MS. This is most probably due to low ionization efficiency, the polar character and/or the thermal instability of the analytes, and low volatility. The abundant full-scan-mode fragmentation obtained by EI ionization in GC-MS enabled comparison of the mass spectra obtained with those in the NIST library at a fit value of > 70% [19].

Dantas et al. [26] investigated the effect of ozonization on bezafibrate using HPLC hyphenated to a single quadrupole mass detector. Despite LC-MS not allowing the identification of all the numerous peaks recorded, they were able to resolve the structures of five TPs. The parent compound and four TPs were identified in negative ESI mode, where in-source CID provided 1–3 ion fragments in addition to the deprotonated molecule. Further, enhanced positive ESI allowed identification of one TP, the structure of which was assigned based on four ion fragments and the $[M+1]^+$. Structural transformation of bezafibrate with O_3 was similar to those resulting from photocatalytic TiO_2 treatment [19] (see Table 2). Ring opening was observed in three TPs, while the other two species revealed the introduction of three oxygen atoms in the molecule as hydroxyl groups [26].

3.1.3. Sulfonamides. Sulfonamides are a group of synthetic antibacterial agents widely used for the treatment of bacterial infections. In veterinary practice, they are also administered as growth promoters in food-producing animals, causing hazard from persistence of their residues in food. There are fears that the presence of such antibacterials in food and in the aquatic environment may stimulate dissemination of antibacterial resistance among native bacterial populations.

Calza et al. [35] studied the TiO_2/UV breakdown of veterinary sulfonamides (sulfamerazine, sulfadiazine, sulfadimethoxine and sulfathiazole) using HPLC-MS. The mass detector was a 3D-IT operated in (+)APCI mode. The sulfonamides investigated showed similar MS behavior and followed the same type of transformation pathway, despite differences in the stability of the intermediates (Table 2). Thus, the TiO_2/photolytic

transformation of sulfonamide begins with an •OH attack on the aromatic ring(s) to form hydroxysulfonamide(s), which are intermediate(s) with a protonated molecule of mass 16 Da higher than the parent molecule. The hydroxyl intermediate as well as the parent compound were subsequently subjected to oxido/reductive cleavage of the S-N bond, ring opening and the release of N-containing compounds (Table 2). Complementary to LC-MS analysis, ion chromatography was employed to observe the release of ammonium ions, nitrate, nitrite and sulfate during degradation [35].

3.1.4. Diclofenac. Several investigations have been conducted on the breakdown pathways of diclofenac, involving different treatment processes, including photo-Fenton [20], ozonation and H_2O_2/UV [42], heterogeneous catalytic oxidation with H_2O_2 [43] and photolysis [12,39]. Distinct degradation routes were proposed and the production of intermediates differed, depending on the treatment applied.

Pérez-Estrada et al. [20] accomplished separation and identification of photo-Fenton TPs using GC-MS and LC-MS, and accumulated sufficient complementary information to identify 18 TPs. Screening and identification of apolar TPs of diclofenac used GC-EI-MS Q mass analyzer and comparing full-scan mass spectra of the peaks with those held in the spectral libraries or identification from the fragmentation patterns. Analysis with positive CI gave the molecular ions. Semi-polar and polar TPs were analyzed by an LC instrument connected to a ToF mass detector with an ESI interface operated in both positive and negative modes. Identification of TPs of diclofenac was based on accurate-mass measurements (Fig. 2), which allowed empirical formulae to be calculated using an elemental-composition tool. The method of using a dual sprayer for electrospray – alternately introducing a reference and the sample – produced mass measurements with an enhanced accuracy (i.e. with an error smaller than 2.5 ppm). This enabled internal ''in-time'' autocalibration [20], thus avoiding instrumental drifts over the course of measurements. The latter was a crucial development in ToF mass analyzers, which now provide measurements with much higher accuracy.

3.1.5. Other pharmaceuticals. Andreozzi et al. [41] studied the oxidation of analgesic and antipyretic drug paracetamol (4-(*N*-acetyl)-aminophenol) by means of ozonation and H_2O_2 photolysis. The transformations that occur during ozonation are caused by either direct O_3 or by •OH radical reactions and the TPs generated are similar those formed during H_2O_2/UV treatment. In general, aromatic degradation products are formed by •OH radical attack on the C-2 and/or at the *ipso* position of the phenyl ring, with the latter leading to cleavage of $-NHCOCH_3$ and hydroquinone formation. The subsequent opening of the hydroquinone ring gives rise to several dicarboxylic acids. The major transformation pathways are given in Table 2 [41]. To identify the TPs formed by the H_2O_2/UV treatment, Vogna et al. [10] used an integrated GC-MS approach with ^{15}N labeling that enabled them to track the fate of nitrogenous breakdown products. For analyses, an IT mass detector with an EI ionization source was used to provide the authors with abundant MS^n fragments [10]. Silylation to allow for analysis of the highly polar polyhydroxylated and carboxylated species prior to injection into gas chromatograph was essential in both studies [10,41]. To confirm the results of their GC-MS analyses, the authors used HPLC-DAD, where the UV spectra of the breakdown products were compared to spectra of authentic compounds. Additionally, product characterization was performed using NMR techniques and produced results consistent with those of GC-MS analyses [10,41].

β-blockers target specific β-adrenergic receptors in humans and, by blocking the action of catecholamines (i.e. noradrenalin and adrenalin), reduce elevated blood pressure and stabilize heart rate. Many of the β-adrenergic receptors also occur in other mammals, vertebrates and invertebrates, thus suggesting a potential for their β-blocking activity in these species [52,53]. Additionally, it is possible that β-blockers and other pharmaceuticals show specific non-target effects that are unrelated to their therapeutic effects [54]. Song et al. [1] studied the breakdown pathway of three β-blockers (atenolol, metoprolol and propranolol). Production of the active oxygen species in the degradation experiments (i.e. hydroxyl radicals) was induced by γ-radiolysis, where TPs were observed at multiple radiation doses. Analyses were performed by HPLC-ToF-MS using ESI in positive mode. Comparison of each protonized parent molecule $[M+H]^+$ with its breakdown product revealed the addition of 16 mass units, corresponding to hydroxylation of the aromatic ring. Another typical reaction was the addition of electrophilic hydroxyl radical at the *ipso* position of the aromatic ring, resulting in the cleavage of the ether bond. This yielded a phenolic and an *ipso*-adducted side-chain product. Furthermore, the side chain at *p*-position of the atenolol and metoprolol phenyl ring was substituted by a hydroxyl group (Table 2). In the same work, the authors [1] presented a time–concentration profile of the TPs identified, which showed an initial increase, followed by a decline. This again implied that AOP treatment has to be sufficiently long to achieve mineralization with concurrent toxicity reductions that are the ultimate aims of the water treatment [1].

The kinetic behavior, reaction pathways and intermediates of clofibric acid during TiO_2-mediated photocatalysis were investigated by Doll and Frimmel [25]. For both kinetic studies and confirmation of TP structures by comparison with authentic standards, HPLC-DAD and HPLC with fluorescence detection (HPLC-FLD) were

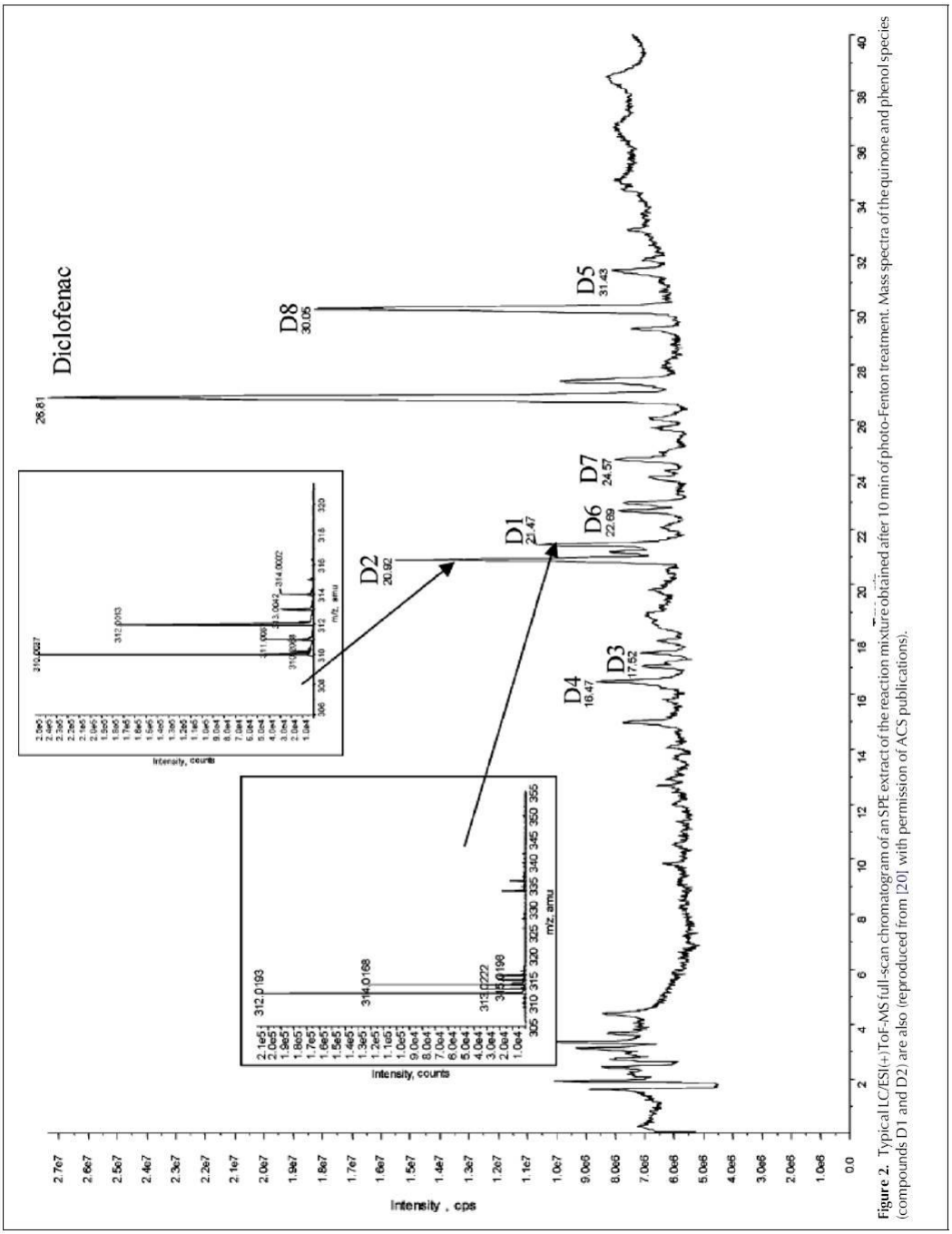


Figure 2. Typical LC/ESI(+)ToF-MS full-scan chromatogram of an SPE extract of the reaction mixture obtained after 10 min of photo-Fenton treatment. Mass spectra of the quinone and phenol species (compounds D1 and D2) are also (reproduced from [20] with permission of ACS publications).

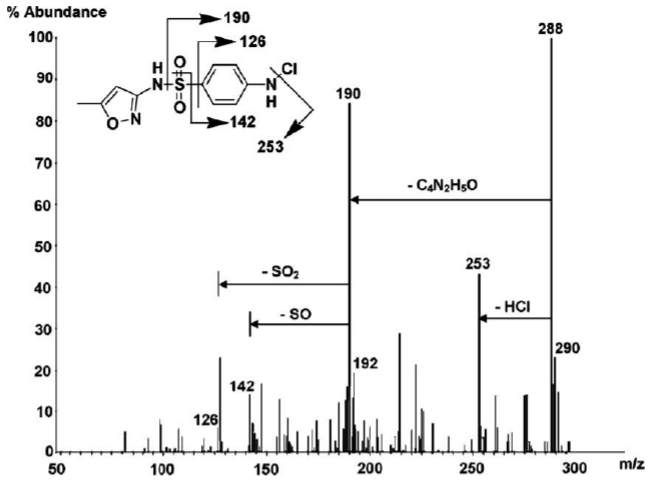


Figure 3. Transformation product of sulfamethoxazole with halogenated aniline moiety, a product of free chlorine reaction with sulfamethoxazole [(+)ESI-LC-MS spectrum obtained at 120-eV fragmentation voltage] (reproduced from [27] with permission of ACS Publications).

used. The remaining TPs were determined by HPLC coupled by means of ESI ion source to a QqQ mass spectrometer. The fragmentation in a negative-ion spray voltage allowed identification of three TPs containing a hydroxyl and a carboxyl moiety (Table 2) [25].

3.2. Chlorinating-agent disinfection

The effect of chlorine as a disinfecting agent on sulfamethoxazole was investigated by Dodd and Huang [27]. Two chlorinated sulfamethoxazole products were identified using LC coupled to a single quadrupole mass spectrometer. The instrument was operated in the (+)ESI mode, where, to obtain additional in-source fragmentation, the authors increased the fragmentation voltage from the standard 80 eV to 120 eV. An example of a structure elucidation of N-chlorinated sulfamethoxazole is shown in Fig. 3, where the protonated molecule ($[M+H]^+$ 288) exhibits the monochlorine isotopic pattern. In the same study, a third TP was observed by UV detection, but yielded no signal in positive APCI or ESI modes during LC-MS analyses. Instead, GC-EI-MS was successfully applied for its detection as well as structural characterization, while ^{1}H-NMR was employed to support the proposed structural assignments [27].

Hu et al. [24] studied the breakdown of 17β-estradiol in drinking water disinfected with sodium hypochlorite. Seven TPs, including three chlorine-substituted products (2,4-dichloro-17β-estradiol, monochloroestrone and 2,4-dichloroestrone) and four by-products, were detected in a chlorinated 17β-estradiol solution. The Cl-substituted products were determined by LC-(-)ESI-Q-MS and their structures revealed by (i) recognising the deprotonated molecule to assign a molecular weight, and (ii) the typical chlorine isotopic pattern to determine the number of chlorine atoms. The position of the Cl moiety in E2 and estrone molecule was postulated by considering the principles of the chlorination-substitution reaction and partial atomic charges in the 17β-estradiol molecule [24] (Table 2). The remaining four chlorination by-products were produced by a further reaction with HOCl, involving Cl-substitution followed by dehydration or cleavage of the C10-C9 bond in 17β-estradiol. These compounds were identified by monitoring the daughter ions produced by in-source CID of the analyte ions. This confirmed their identity, albeit with some loss of sensitivity [24].

4. Conclusions

While MS is routinely used in quantitative and confirmatory analysis of pharmaceuticals, its applications to the structural characterization of unknown PTPs remains limited. Given the large gap in our knowledge to be bridged in elucidating breakdown pathways of pharmaceuticals in the environment, a significant increase in research efforts is necessary, where advances in modern MS instrumentation will play an important role.

Along with the increasing sensitivity and selectivity of the established mass analyzers (i.e. oaToF-MS and IT-MS), ''hybridization'' is now driving new instrument development, by allowing the addition (or replacement) of sections of conventional MS^2 instruments with devices that can provide superior performance characteristics. Besides the already reputable Qq-ToF, Qq-LIT and LTQ-

Orbitrap mass analyzers, QIT-ToF and ToF-ToF are promising tools to improve combinatory MS^n and high-resolution analysis of unknown compounds [55].

Along with hybrids, advances in ionization techniques promise to increase the sensitivity of the novel MS instruments. APPI has recently been introduced, proving to be efficient in allowing the ionization of compounds previously non-ionizable (or poorly ionizable) with either ESI or APCI [21]. Whereas Robb et al. [56] stated that ''the range of compounds that can be sufficiently ionized by APPI closely follows that of APCI'', implementing APPI instead of APCI or ESI often results in improved sensitivity, due to the lower susceptibility of the APPI source to ion suppression [57]. Examples of such pharmaceuticals are anticancer-drug idoxifene and its metabolites [58] and steroid drugs (e.g., cortisol, cortisone [59] and lanosterol [60]). However, obviously there is a substantial delay between the ongoing development of MS and its implementation in environmental laboratories, most probably because of the lack of MS expertise and the cost.

Identification of PTPs is justified for clarifying the reactions and the processes in water treatment, and consequently for minimizing pharmaceutical residues in the waste, environmental and drinking water. We have indicated that the breakdown pathways and the production of intermediates depend on water-treatment methods, even though the processes are driven by the same reactive species (predominantly hydroxyl radicals), so different intermediates and reaction products may be formed.

Breakdown during AOPs depends on the physicochemical characteristics of compounds, but typically involves hydroxylation of the aromatic ring by an electrophilic attack from ·OH radicals, cleavage of C—O, C—N or S—N bond, cleavage on α-position from the aromatic moiety and ring opening, while chlorine disinfection merely yields chlorinated products.

Furthermore, comparison of the PTPs determined by LC-MS and GC-MS yields distinctly different compounds, and that implies a need to combine both separation methods in order to obtain a comprehensive view of pharmaceutical transformations. Also, the possible generation of toxic intermediates or TP mixtures along the mineralization pathway more hazardous than parent compounds should not be neglected. To evaluate and to improve existing treatment methodologies, as well as to develop comprehensive environmental risk assessment, PTP identification and quantification studies should be combined with toxicity studies.

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3.4.2 Scientific paper: “Fate of carbamazepine during water treatment”

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Fate of Carbamazepine during Water Treatment

TINA KOSJEK,[†] HENRIK R. ANDERSEN,[‡] BORIS KOMPARE,[§] ANNA LEDIN,[‡] AND ESTER HEATH*,[†]

Department of Environmental Sciences, Jožef Stefan Institute, Jamova 39, Ljubljana, Slovenia, Department of Environmental Engineering, Technical University of Denmark, Miljoevej, Building 113 DK-2800 Kgs. Lyngby, Denmark, and Institute of Sanitary Engineering, Faculty of Civil and Geodetic Engineering, University of Ljubljana, Hajdrihova 28, Ljubljana, Slovenia

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Seven transformation products of carbamazepine generated by at least one of three common water treatment technologies (UV-radiation, oxidation with chlorine dioxide (ClO_2), and biological treatment with activated sludge) were identified by complementary use of ion trap, single quadrupole, and quadrupole-time-of-flight mass spectrometers. Acridine was formed during all of the three treatment processes, while acridine 9-carbaldehyde was identified as an intermediate during ClO_2 oxidation. Further treatment of acridine with ClO_2 produced 9-hydroxy-acridine. UV-treatment resulted in the formation of acridone, hydroxy-(9*H*,10*H*)-acridine-9-carbaldehyde, acridone-*N*-carbaldehyde, and 1-(2-benzaldehyde)-(1*H*,3*H*)-quinazoline-2,4-dione, while biological breakdown of acridine yielded acridone. In parallel, the transformation product iminostilbene was observed during sample analysis. In addition, this study compared the treatment technologies according to the removal of carbamazepine and the production and decay of its transformation products. The most successful method for the removal of carbamazepine was UV treatment, while acridine and acridone were more susceptible to biological treatment. Therefore, based on the enhanced biodegradability of carbamazepine residues achieved by UV irradiation, we propose a coupled treatment technology involving an initial UV treatment step followed by biological treatment, which may satisfactorily remove the parent compound and its transformation products.

Introduction

Carbamazepine (CBZ) is a dibenzadiazepine derivative used for its antiepileptic and psychotropic activity, for severe pain syndromes associated with neurological disorders, and is a global pharmaceutical. It is administered chronically and usually in high dosages (100−2000 mg daily) and hence its annual production is high (*1*−*3*). The drug is excreted with <3% remaining in its unaltered form, with the pharmacologically active 10,11-epoxycarbamazepine as the major metabolite, which is then hydrolyzed to dihydroxy derivatives and excreted principally as glucuronide conjugates. Additionally, CBZ is inactivated by hydroxylation of the aromatic ring and N-glucuronidation of the carbamyl moiety (*4*). It is a relatively polar pharmaceutical and lacks sites for specific interactions with soils and sediments, which leads to its nonappreciable sorption properties (*5*−*7*). It is persistent to biodegradation and shows almost no elimination during wastewater treatment (*1, 7, 8*), which makes CBZ a pharmaceutical of high environmental relevance. Studies have reported its presence in wastewaters (up to 6.3 $\mu g\ L^{-1}$) (*1, 9*), in surface waters (up to 1.1 $\mu g\ L^{-1}$) (*1, 10, 11*), and in drinking water (30 $ng\ L^{-1}$) (*12*). In contrast with conventional biological wastewater treatment (*8*) and ClO_2 treatment (*13*), CBZ is effectively removed by ozonation (*5*), by UV/H_2O_2 induced photolytic degradation (*14*), photocatalytic degradation with TiO_2 (*15*), or by direct photolysis (*16*). Although these technologies remove CBZ, the result is not always complete mineralization and the disappearance of CBZ provides only a partial indication of treatment efficiency, since transformation products (TPs) more resilient to degradation may form. In addition, the overall toxicity of treated water arising from a mixture of stable TPs may worsen the environmental and health impact (*17, 18*). Thus, it is important not to overlook TPs when comparing the efficiency of different water treatment technologies. Albeit, abiotic transformation studies of CBZ have been made (*14*−*16, 19*), to our knowledge, the formation of TPs by biological treatment and more generally the degradation of CBZ's TPs is yet to be investigated (*20*).

In this paper, we study the efficiency of three water treatment technologies: UV treatment, chlorine dioxide treatment, and biodegradation with activated sludge, with respect to the removal of CBZ and its TPs. At the core of the present study is a comparison of these approaches, where the importance of biological treatment of TPs is underlined. Finally, solutions for the efficient removal of both CBZ and its TPs are proposed.

Experimental Section

Standards, Solvents, and Other Chemicals. Carbamazepine (99%, CAS 298-46-4) was purchased from Acros Organics (New Jersey), while acridine (ACIN: 97%, CAS 260-94-6) and 9(10*H*)-acridone (ACON: 99%, CAS 578-95-0) were both obtained from Sigma-Aldrich (St. Louis, MO). *N*-Methyl-*N*-[tert-butyldimethyl-silyl]trifluoroacetimide (MTBSTFA) was purchased at Acros Organics.

Treatment Experiments. *UV Treatment.* UV treatment was performed in an UV reactor with a circulating flow system setup. The apparatus consisted of a steel container (8 L), a water pump (85 $L\ h^{-1}$), and a medium pressure metal−halogen UV lamp (690 W). The UV lamp (Bau 42, Scan Research A/S, Denmark) emitted a polychromatic light at 400−185 nm, with an enhanced emission within the photochemical relevant wavelength range of 250−190 nm. The emission spectrum is given in the Supporting Information. The fate and behavior of CBZ and its TPs were studied during a 30 min irradiation time. Spiked tap water solutions containing 100 $\mu g\ L^{-1}$ of the test substance were sampled at set time-intervals. Photodegradation experiments were also performed separately with ACIN and ACON as respective starting compounds. Finally, to determine the impact that a radical scavenger has 1.2%, 2.5%, and 6.2% (V/V) of methanol was added to the starting solution.

Chlorine Dioxide Treatment. A ClO_2 solution was prepared by adding 25 mL of 9% (w/V) HCl and 25 mL of $NaClO_2$ (7.5% w/V) to 400 mL of deionized water. After 12 h the volume was made up to 1000 mL with deionized water giving an approximately 1 $g\ L^{-1}$ ClO_2 stock solution. The stock solution

* Corresponding author e-mail: ester.heath@ijs.si; tel: +38614773584; fax: +38612519385.
[†] Jožef Stefan Institute.
[‡] Technical University of Denmark.
[§] University of Ljubljana.

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TABLE 1. Bioreactor Test Parameters

	bioreactor	test compound	c_X ($\mu g\ L^{-1}$) removal	c_X ($\mu g\ L^{-1}$) TPs	$c(O_2)$ mg L^{-1}
	R0	control: NO			9.0 ± 1.4
	R1	CBZ	50	200	9.5 ± 0.7
	R2	CBZ	50	500	9.3 ± 1.5
	R3	ACIN	50	200	9.5 ± 1.4
	R4	ACIN	50	500	9.5 ± 1.6
	R5	ACON	50	200	7.6 ± 1.7
aerobic	R6	ACON	50	500	9.5 ± 1.4
	R7	control: NO			0.12 ± 0.04
	R8	CBZ	50	200	0.12 ± 0.04
	R9	ACIN	50	200	0.12 ± 0.04
anoxic	R10	ACON	50	200	0.12 ± 0.04

was then normalized using the "DPD-method" and an Allcon spectrophotometer (Alldos GmbH Germany). A neutral pH was achieved by adding 0.11 M Na_2HPO_4 and the test solutions were exposed to concentrations of ClO_2, ranging from 0.7 to 13.5 mg L^{-1}. The experiments were made in distilled water spiked with 100 $\mu g\ L^{-1}$ of either CBZ or ACIN. The test mixtures were left to react for 2 h, whereupon the reaction was interrupted by the addition of 20 mg of Na_2SO_3.

Biological Treatment. Experiments were performed in laboratory bench scale flow-through bioreactors (*21*−*23*) containing activated sludge, which were operated in parallel under identical conditions: nutrient, hydraulic retention time, and biomass concentration. Table 1 gives the different test parameters.

Seven aerobic and four anoxic bioreactors were operated. Prior to sampling, the bioreactors were kept under test conditions for a period sufficient to allow for adaptation. Temperature was maintained at 23.3 ± 1.3 °C.

Control Studies. In the biodegradation experiments, the R0 (aerobic) and R7 (anoxic) outlets were used to distinguish between the products of matrix biodegradation or bacteria lyses and actual pharmaceutical degradation. As an additional control, the spiked inlet samples R1, R2, or R8 (for CBZ); R3, R4, or R9 (for ACIN), and R5, R6, or R10 (for ACON) were also analyzed to exclude the possibility of other potential breakdown mechanisms such as light, thermal, or hydrolysis. For the photodegradation and ClO_2 experiments, untreated spiked solutions were used as controls.

Sample Preparation. Sample preparation was based on solid phase extraction, followed by gas chromatography−mass spectrometry (GC-MS) or liquid chromatography−mass spectrometry (LC-MS). For quantitative purposes, the sample preparation involved an additional derivatization step prior to GC-MS analysis (see Supporting Information).

Instrumental Analysis. *GC-MS.* Qualitative analyses were made using a Varian 3800 GC hyphenated with an ion trap

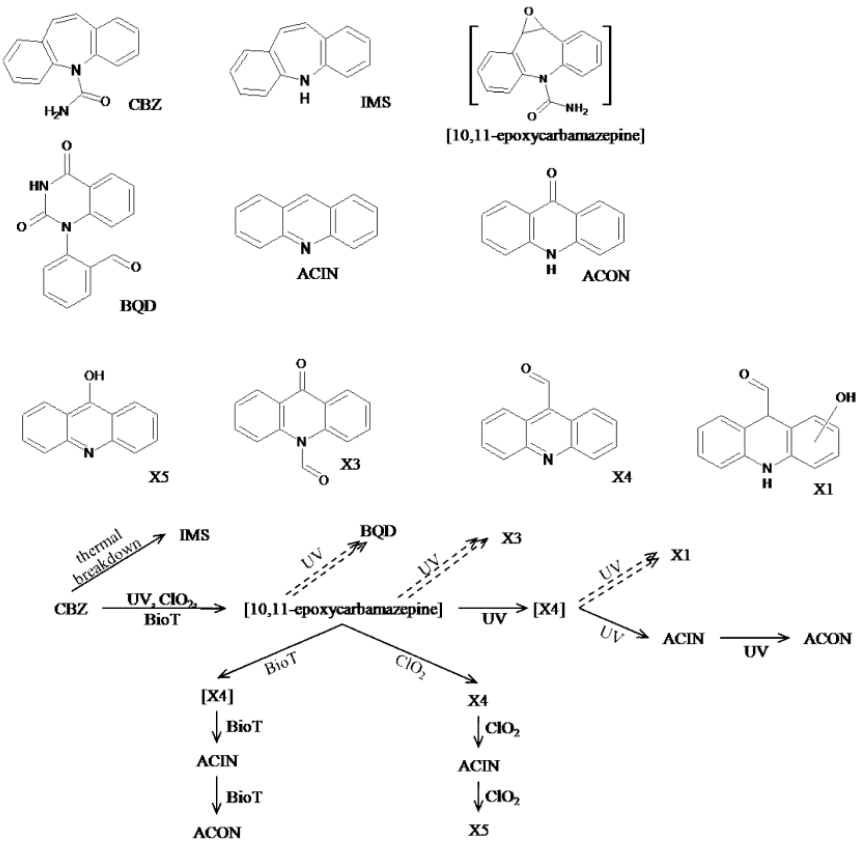

FIGURE 1. Proposed chemical structures of TPs and the proposed breakdown pathway of CBZ. BioT: biotransformation; ClO_2: chlorine dioxide treatment; UV: UV irradiation.

TABLE 2. Carbamazepine and Identified TPs

compound	abb.	elemental formula	molecular weight[a]	breakdown mechanism	identification/ confirmation method
carbamazepine	CBZ	$C_{15}H_{12}N_2O$	236 ($[M + H]^+ = 237.1030$)	parent compound	LC-QqTOF, GC-IT, GC-MSD, NIST library, authentic standard
iminostilbene	IMS	$C_{14}H_{11}N$	193	thermal degradation in GC liner	GC-IT, GC-MSD, NIST library
acridine	ACIN	$C_{13}H_9N$	179 ($[M + H]^+ = 180.0814$)	photolysis, ClO_2 oxidation, biodegradation	LC-QqTOF, GC-IT, GC-MSD, NIST library, authentic standard
acridone	ACON	$C_{13}H_9NO$	195 ($[M + H]^+ = 196.0765$)	photolysis, biodegradation of ACIN	LC-QqTOF, GC-IT, GC-MSD, NIST library, authentic standard
hydroxy-(9*H*,10*H*)-acridine-9-carbaldehyde	X1	$C_{14}H_{11}NO_2$	225	photolysis	GC-IT
acridone-*N*-carbaldehyde	X3	$C_{14}H_9NO_2$	223	photolysis	GC-IT
1-(2-benzaldehyde)-(1*H*,3*H*)-quinazoline-2,4-dione	BQD	$C_{15}H_{10}N_2O_3$	266	photolysis	GC-IT, McDowell et al. (*19*)
acridine-9-carbaldehyde	X4	$C_{14}H_9NO$	207	ClO_2 oxidation	GC-IT
9-hydroxy-acridine	X5	$C_{13}H_9NO$	195 ($[M + H]^+ = 196.0767$)	ClO_2 treatment of ACIN	LC-QqTOF

[a] The accurate mass of the protonated molecule is provided in brackets, when determined by a high resolution instrument (i.e. QqTOF).

Saturn 2000 mass spectrometer (GC-IT). Quantitation was achieved using a HP6890 series gas chromatograph fitted with a mass selective detector (GC-MSD). Both spectrometers were operated in the electron impact ionization mode (EI). For further mass fragmentation the capabilities of an IT mass analyzer, i.e., multiple reaction monitoring (MRM), tandem MS (MS/MS), and multiple MS (MS^n) ion preparation modes in the resonant waveform, were utilized.

LC-MS. LC-MS analyses were performed using a Waters Acquity ultra-performance liquid chromatograph (Waters Acquity UPLC, Waters Corp., Milford, MA) hyphenated to a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (QqTOF Premier, Waters). The instrument was equipped with an electrospray ionization interface operating in the positive electrospray ionization mode (ESI(+)). Further details about the GC-MS and LC-MS methods are given in the Supporting Information.

Results and Discussion

Methodology of Detection and Practical Issues Encountered in the Determination of CBZ and its TPs. To include the broadest range of TPs, screening was made by operating the GC-MS and LC-MS analyses on parallel samples. The detection of TPs in the GC samples was made by comparing the total ion chromatograms (TIC) of the treated samples with those of the control. Any new peaks appearing in the treated samples were then investigated further. In the LC analysis the transformation products were detected by applying the spectral and chromatographic algorithm MetaboLynx, a software package embedded in MassLynx v4.1 (Waters Corp.).

While LC-QqTOF and GC-IT were used to identify new compounds, GC-MSD was used for quantitative purposes. Optimization and validation of the GC-MSD method was made using CBZ, ACIN, and ACON, for which authentic standards are available. Optimization took into account chromatographic separation, peak shape and response, possible matrix effects, and the linearity of the mass spectrometer's response. A crucial step in optimizing this procedure was the use of the derivatization reagent MTBSTFA, whereas samples analyzed by GC-IT were not derivatized as this could affect the screening and identification of TPs. Both ACON and CBZ were transformed into their *tert*-butyl-dimethylsilyl ethers (ACON-MTBS and CBZ-MTBS), while ACIN was left underivatized. The use of MTBSTFA improved both separation and peak shape; moreover, the chromatographic response increased by 58% and 74% for ACON-MTBS and CBZ-MTBS, respectively. It also avoided the artifactual formation of iminostilbene (IMS, Figure 1), believed to form from the thermal breakdown of CBZ in the injector. In the underivatized GC samples, the amount of IMS corresponds to the amount of CBZ; furthermore, in LC its formation is not observed, which supports the hypothesis of it being a thermal breakdown product.

When extracting samples after chlorine dioxide treatment, there is the potential for the salt-effect to give misleading results. To evaluate this, two calibration curves were prepared, one in deionized water and the other in a 1.8 mM NaCl solution containing the relevant analyte in the concentration range 15−150 $\mu g\ L^{-1}$. The results show an almost complete overlap of the curves for each of the investigated compounds in both solutions and thus any salt-effect can be discarded. In addition, despite not using an internal standard, a satisfactory linearity with r^2 0.992, 0.989, and 0.985 was achieved for CBZ, ACIN, and ACON, respectively.

Identification of Transformation Products. By utilizing the capabilities of the QqTOF mass spectrometer: tandem mass fragmentation and accurate mass measurement, in combination with the IT mass analyzer enabling MS^n fragmentation, eight TPs were identified. In support to the proposed chemical structures, confirmatory methods were also used including matching the TP fragmentation patterns with those held in the NIST mass spectral library or published mass spectra and, where possible, a comparison of the compound's chromatographic and mass spectrometric behavior with that of a commercially available authentic compound. Table 2 summarizes all the applied identification and confirmation techniques.

Under UV irradiation of CBZ, the following TPs became evident (see Supporting Information, Figure S3): ACIN, ACON, X1, X2, X3, and BQD. According to its EI-MS and EI-MS/MS fragmentation pattern and a match with the NIST library (Table 2) the most abundant peak was acridine (ACIN). Further, its identity was confirmed by comparing its retention time and mass fragmentation pattern to that of the authentic compound. In parallel, ACIN was detected using MetaboLynx processing, while for its structural elucidation QqTOF tandem mass fragmentation and accurate mass measurement were performed. The latter gave a 0.6 ppm deviation from the theoretical mass of the

protonated acridine. For a detailed description of the identification process for ACIN see the Supporting Information.

The second TP, which was formed during UV irradiation, (see Supporting Information, Figures S3 and S5) shows an increase in mass of 16 Da in its EI mass spectrum, which corresponds to ACIN with an additional oxygen atom. A search of the NIST library and a comparison with the authentic compound confirms this to be 9(10*H*)-acridinone (ACON, Table 2). During derivatization the ether forms on the 9-hydroxy group of the ACON "enol" tautomer, yielding a fragmentation pattern with the corresponding-type fragmentation as CBZ-MTBS. Complementarily, the ESI(+)TOF acquisition and collision-induced dissociation of the protonated ACON molecule at $[M + H]^+$ 196 were performed, where its elemental composition was confirmed with a mass error of 1.5 ppm.

The chemical structure of X1, another UV breakdown product eluting at 10.2 min, was resolved based on its EI-MS and EI-MS/MS fragmentation (see Supporting Information). X1 was identified as hydroxy-(9*H*,10*H*)-acridine-9-aldehyde, which confirms the existence of this TP, previously proposed to be a product of CBZ photocatalytic breakdown (*15*). Other structural isomers are also possible and further investigation is needed to confirm—with certainty—its structure.

The structural elucidation of X2 was, despite its relatively prominent chromatographic peak, unsuccessful. Since the highest ion fragment yielded an even mass (see Supporting Information, Figures S7 and S8), it is supposed that X2 was subjected to the loss of the molecular ion, which is not uncommon for a "hard" ionization method, such as EI. One solution would be to use chemical ionization (CI) to observe the molecular ion, which is enabled by modern GC-IT instruments. Another option would be to employ LC-MS using milder ionization techniques. Unfortunately, an inspection of the ESI(+) mass spectra revealed no compound having the related mass fragmentation pattern. Alternatively, NMR studies would be the way ahead, but this requires obtaining a sufficient amount of X2 by adapting the dosage of UV irradiation, optimizing the enrichment by solid phase extraction, and developing an appropriate purification method.

The fragmentation pattern of X3 resembles closely that of ACON, with the exception of the molecular ion at m/z 223. The molecular ion shows a mass increase of 28 Da, i.e., a carbonyl group, which is likely positioned on the heterocyclic nitrogen as the residual from the carbamyl side chain of CBZ (see Supporting Information, Figure S9). Thus, X3 is tentatively assigned as acridone-*N*-carbaldehyde, which to the authors' knowledge is the first identification of this TP.

The last UV-breakdown product, sufficiently volatile to be analyzed by GC-IT (Figures S3 and S10) was identified according to its EI-MS, EI-MS/MS, and EI-MS^3 fragmentation. McDowell et al. (*19*), who determined three TPs during the ozonation of CBZ all containing the quinazoline structural fragment, published an identical mass fragmentation pattern. Hence, based on a match to the mass spectra published by McDowell et al. (*19*) one can assume that this compound is 1-(2-benzaldehyde)-(1*H*,3*H*)-quinazoline-2,4-dione (BQD).

The inspection of the GC-IT extracted mass chromatogram of the ClO_2-treated sample (Supporting Information, Figures S3 and S11) yielded a new compound X4, the concentration of which increases with increasing ClO_2 dose. Its mass spectrum suggests it is an acridine structural fragment with the mass increased by 28 Da, which corresponds to a carbonyl-bearing compound, i.e., acridine-carbaldehyde.

X5 was identified during ClO_2 treatment of ACIN. The compound was highlighted by MetaboLynx and its mass spectra (ESI(+)TOF and ESI(+)TOF-MS/MS) matched that of ACON (see Figure S5). Taking into account the similar mass fragmentation and that oxidation at position 9 is favorable due to a low electron density at this carbon on the acridine ring (*24*), X5 is assigned as the 9-hydroxy-derivative of acridine.

Fate during UV, Chlorine Dioxide, and Biological Treatment. Based on the identified TPs, a possible degradation pathway is proposed (Figure 1). During UV degradation six TPs were detected and structures of five were tentatively assigned (ACIN, ACON, X1, X3, BQD), chlorine dioxide treatment produced three TPs (ACIN, X4, X5), and biodegradation produced two TPs (ACIN, ACON). One compound (IMS) was formed during sample analysis. The high number of TPs is indicative of the complexity of the reactions involved, however regardless of the nature of the transformation, i.e. abiotic or biological, ACIN is involved as an important intermediate.

In case of chlorine dioxide treatment, abiotic breakdown is the consequence of chemical oxidation, while during UV treatment an organic molecule may undergo structural alteration during the electronic transition from an excited state to the ground state (direct/primary photolysis) or by photolytic transformation induced by reactive oxygen species generated by the UV-photons (advanced oxidation/secondary photolysis) (*25*). As derived from Figure 1, the most reactive site on the CBZ molecule is at the 10,11-double bond, which is commonly attacked by hydroxyl radicals or oxidation reagents to yield an intermediate that evolves into 10,11-epoxycarbamazepine (*15*). Subsequent opening of the epoxide ring will give a labile species that forms a quinazoline derivative (BQD) (*19*) or suffers a facile ring contraction to give acridine-9-carbaldehyde (X4) (*14*) or acridone-*N*-carbaldehyde (X3). This ring contraction takes place via a pinacol-type rearrangement (*26*) and is reasoned by the tendency of an azepine ring to yield an aromatic structure. Further conversion of X4 leads to the cleavage of an aldehyde group to form ACIN and further ACON (Figure 1). This is supported by a rapid increase in X4 during the first phase of ClO_2 treatment and the fact that it reaches a peak concentration before the secondary breakdown product ACIN does (see Supporting Information). Another reactive site is the carbamyl side chain, the cleavage of which is possible by thermal degradation and by various oxidizing species.

To get an insight into the reaction mechanism, methanol was added to the test solution during the UV experiment to inhibit any radical mediated breakdown processes and enable the detection of short-lived intermediates. In contrast to expectations, no significant difference in the decay of CBZ was observed nor any additional intermediates detected in the presence of 1.2%, 2.5%, or 6.2% of methanol. However, increasing the content of methanol in the test solution did cause a notable decrease in the formation of ACON, suggesting that the predominant mechanism responsible for transformation of ACIN to ACON is indirect photolysis.

Under UV experimental conditions, CBZ shows a steady decrease following first order kinetics. This results in 99.8% elimination after 30 min of UV irradiation (Figure 2 left). The amount of TPs formed increases during the first 5 min, and then reaches a steady state before decreasing slowly after 15 min. The exception is X2 (see Supporting Information, Figure S12), which does not reach its maximum concentration within the 30 min. It is important to emphasize that the TPs (except for X1) are not eliminated within 30 min of irradiation, which supports the need for prolonged treatment to ensure their complete oxidation. During UV treatment of ACIN, the concentration of ACON steadily increases with the irradiation time. This confirms the transformation step from ACIN to ACON in the proposed breakdown pathway (Figure 1).

With increasing dosage of ClO_2 the decay of CBZ and, simultaneously, an exponential increase of ACIN are observed (Figure 2 right). The latter is thought to be a result of the

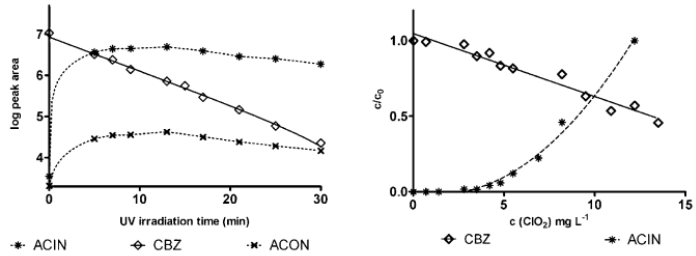

FIGURE 2. Left: the fate of carbamazepine during the UV treatment. Right: ClO_2 treatment of CBZ.

TABLE 3. Removal of CBZ, ACIN, and ACON during Biological, UV, and ClO_2 Treatment

	percent removal			
compound	aerobic	anoxic	UV treatment (10 min UV)	ClO_2 treatment (13.5 mg L^{-1} ClO_2)
CBZ	16%	16%	93%	54%
ACIN	92%	90%	76%	38%
ACON	40%	23%	<10%	not tested[a]

[a] ACON was not treated with ClO_2.

higher resilience of ACIN to oxidation by ClO_2. This hypothesis is confirmed by treating CBZ and ACIN individually, where at the highest concentration of ClO_2 (13.5 mg L^{-1}) 54% of CBZ was removed; whereas only 38% of ACIN was eliminated (Table 3). The ClO_2 oxidation tests were not made using ACON as the starting compound, since it is not a transformation product of CBZ when treated with ClO_2.

In general, ClO_2 treatment proved to be less efficient in comparison to UV for both CBZ and ACIN (Table 3). Also, the number of the TPs detected under ClO_2 oxidation of CBZ was lower, with only acridine-9-carbaldehyde (X4) and ACIN being detected (see Supporting Information, Figure S3). Further, the ClO_2 oxidation of ACIN alone resulted in the formation of a single TP, 9-hydroxy-acridine (X5).

As an alternative method of removing TPs, biological treatment was investigated. Table 3 shows the removal efficiencies of CBZ, ACIN, and ACON determined in the benchtop activated-sludge bioreactors under aerobic and anoxic conditions. The most important outcome of this experiment was the efficient removal of ACIN, which was 90% and 92% under anoxic and aerobic conditions, respectively. Further, the elimination of CBZ is poor with only 16% removal irrespective of the RedOx conditions (Table 3), while the removal of ACON is more efficient under aerobic conditions (40%) comparing to anoxic removal (23%). Biodegradation products were also screened for in the bioreactor outlets, where only trace levels of ACIN were found in the reactors fed with CBZ (Table 1: R1, R2, and R8). The absence of TPs in these reactors is attributed to the low biodegradability of CBZ, while, agreeing with expectations, substantial amounts of ACON were formed in the bioreactors fed with the readily biodegradable ACIN (R3, R4, and R9). According to Figure 1, the biological transformation reactions take place on a moiety with the most readily available electrons, which in the case of CBZ is the nonaromatic double bond and the nonbonded nitrogen electrons. Thus the 10,11-double bond is first transformed into the epoxide by a monooxygenase enzyme system (*25*). In addition, ring contraction and the cleavage of the carbamyl bond both occur in vivo to form X4 (*27*), which is further converted into ACIN and ACON. The latter did not show formation of any additional TPs.

Taking into account the poor biological removal efficiency of CBZ together with the enhanced biodegradability of carbamazepine residues by UV irradiation (Table 3), prolonging the treatment time to achieve complete mineralization may not be necessary. Instead, one could apply a combination of UV treatment with a second biological treatment step (sandfilter) for advanced water treatment. In this respect, the proposed coupled treatment technology for removing carbamazepine residues may be relevant for treating raw water for potable water production. Furthermore, the data presented in Table 3 show that the chlorine dioxide treatment of CBZ produces the readily biodegradable ACIN. The system involving ClO_2 oxidation with a subsequent biological treatment is then potentially useful for wastewater treatment, since together with an improved elimination of CBZ residues, there will be a decrease in the BOD (biological oxygen demand), which had been created from COD (chemical oxygen demand) during the ClO_2 oxidation. Naturally, the two systems proposed above will require scale-up and further evaluation, both from a scientific and economic perspective.

Most notably, the major intermediates arising from ClO_2, UV, and biological treatment of CBZ belong to the azaarenes, an established class of air and water pollutants, known for their photoenhanced toxicity, mutagenic, and carcinogenic activity (*16, 18, 28*). This raises an important issue concerning the possible environmental impact of pharmaceutical residues in either domestic wastewaters or drinking waters.

Acknowledgments

The Danish Research council project DanED, the Swedish Foundation for Strategic Environmental Research project MistraPharma, EU Structural Funds to the Centre of Excellence in Environmental Technologies (Contract 3211-05-000184), Slovenian Research Agency (Program groups P1-0143 and P2-0180), Ad futura fellowship (Science and Education Foundation of the Republic of Slovenia, Public Fund), and Slovenian-Danish bilateral collaboration (BI-DK/07-09-003) are acknowledged for their economic support. For helpful and valuable advice the authors are grateful to Dr. Bogdan Kralj Dušan Žigon and Jernej Iskra from Jožef Stefan Institute, Ljubljana, Slovenia. Also, thanks to Prof. Jure Svete (Faculty of Chemistry and Chemical Engineering, University of Ljubljana) for his attempts to synthesise 9-acridone-*N*-carboxaldehyde.

Supporting Information Available

Mass spectra of the transformation products and a graph showing the behavior of carbamazepine and its transformation products during chlorine dioxide treatment. The information is available free of charge via the Internet at http://pubs.acs.org.

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Fate of carbamazepine during water treatment: identification of transformation products

Tina Kosjek, Henrik R. Andersen, Boris Kompare, Anna Ledin, Ester Heath

SUPPORTING INFORMATION

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Sample preparation

Wastewater samples were filtered through Machery-Nagel GF-2 microfibre glass and 1.2 µm cellulose nitrate filters (Sartorius, Goettingen, Germany). Solid phase extraction (SPE) was performed at a neutral pH using an Oasis® HLB reversed-phase sorbent (Waters, Corp., Milford, MA, USA). For preconcentration and clean-up of 200 mL samples, the SPE cartridges were first conditioned with 3 mL of ethyl acetate, 3 mL methanol, equilibrated with 3 mL of tap water and enriched at a flow-rate of 4–5 mL min^{-1}. Each cartridge was then washed with water (3 mL), dried for 30 min under vacuum and eluted with 1 mL acetone, 1 mL of 7/3 ethylacetate/acetone mixture and 1 mL of ethylacetate. The combined eluant was evaporated to dryness with nitrogen. The extracts were then dissolved in either 0.5 mL ethylacetate for analysis by gas chromatography – mass spectrometry (GC-MS) or in 0.5 mL of 2/8 methanol/water for liquid chromatography – mass spectrometry (LC-MS) analysis. For derivatisation, 30 µL MTBSTFA was added to 0.5 mL of the ethylacetate sample and left to react at 60°C for 12 hours.

Instrumental analysis

GC-MS. GC-MS analyses were made using a Varian 3800 GC hyphenated with an Ion trap Saturn 2000 mass spectrometer (GC-IT). 10 µL samples were injected (split-splitless) using a PTV injector at 80 °C for 0.30 min before being increased at 200°C/min to 300°C and held for 5 min. For separation a Zebron ZB-5 HT INFERNO 30 m × 0.25 mm × 0.25 µm (Phenomenex) column was used. The GC temperature programme is as follows: 1 min at 80°C, increased to 225°C at 25°C/min and held for 1 min, increased to 231°C at 1°C/min, then to 280°C at 10°C/min and finally to 320°C at 45°C/min and held for 3 min. The total runtime was 22.59 min. The IT mass analyser was operated in electron ionisation (EI) mode, and the masses scanned between *m/z* 40 to 650. For the further mass fragmentation multiple reaction monitoring (MRM), tandem MS (MS/MS) and multiple MS (MS^n) ion preparation modes in the resonant waveform were used.

Quantitation was achieved using a HP6890 series gas chromatograph with a mass selective detector (GC-MSD). The GC oven was programmed as follows: an initial temperature of 65 °C was held for 2 min, then ramped at 30 °C/min to 180 °C, at 10 °C/min to 210 °C, at 20 °C/min to 240 °C, held for 1 min and then at 30 °C/min to 300 °C (5 min). The total runtime was 18.33 min. A DB-5MS 30 m × 0.25 mm × 0.25 µm (J&W) capillary column was used, with He as the carrier gas (37 cm s^{-1}). One µL samples were injected at 250 °C in splitless mode, and the transfer line was maintained at 280 °C. The MSD was operated in EI mode with selected ion monitoring. The following fragment ions were monitored: 179 for ACIN, *m/z* 252 and 193 for ACON-MTBS and *m/z* 293 and 195 for CBZ-MTBS derivative.

LC-MS. The LC-MS analyses were performed using a Waters Acquity ultra performance liquid chromatograph (Waters Acquity UPLC®, Waters Corp., Milford, MA, USA), coupled to a quadrupole – time-of-flight mass spectrometer (LC-QqTOF). The UPLC system was equipped with a binary solvent delivery system and an autosampler. The injection volume was 2 µL. Separation was achieved using a 5 cm long Waters Acquity UPLC® BEH Shield RP18 1.7 µm column with a 2.1 mm internal diameter. Compounds were analysed under positive ion conditions and were eluted from the column using water (A) and methanol (B) as mobile phases. The elution gradient was linearly increased from 20 % to 100 % B in 8 min, and kept isocratic for 1 min, decreased back to 20 % in 1 min and then finally kept isocratic for 1 min. The total runtime was 11.00 min. Flow rate was 0.2 mL min^{-1} and the column temperature was 35 ºC. The UPLC system was interfaced to a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (QqTOF Premier, Waters, Milford, Massachusetts, USA). The instrument was equipped with an electrospray ionisation interface operating in the positive ion mode (ESI(+)). The capillary voltage was 2.8 kV, while the sampling cone voltage was 40 V. Source and desolvation temperatures were set to 100 and 280 °C, respectively. The nitrogen desolvation gas flow rate was 620 L/h. ESI(+)TOF-MS spectra were acquired over an *m/z* range of 50 - 1000 in two parallel experiments performed at collision energies of 5 and 25 eV. The ESI(+)TOF-MS/MS experiments were performed at different collision energies between 10 to 30 eV. Data were collected in the centroid mode, with a scan accumulation time set at 0.2 s and an interscan delay of 0.025 s. The data station operating software was MassLynx v4.1. The instrument was calibrated over a mass range of 50 – 1000 Da using a sodium formate calibration solution. Reproducible and accurate mass measurements, at a mass resolution of 10000, were obtained using an electrospray dual sprayer

with leucine enkephalin ($[M+H]^+$ = 556.2771) as the reference compound. The latter was introduced into the mass spectrometer alternating with the sample via a Waters LockSpray device.

Emission spectrum of the UV lamp

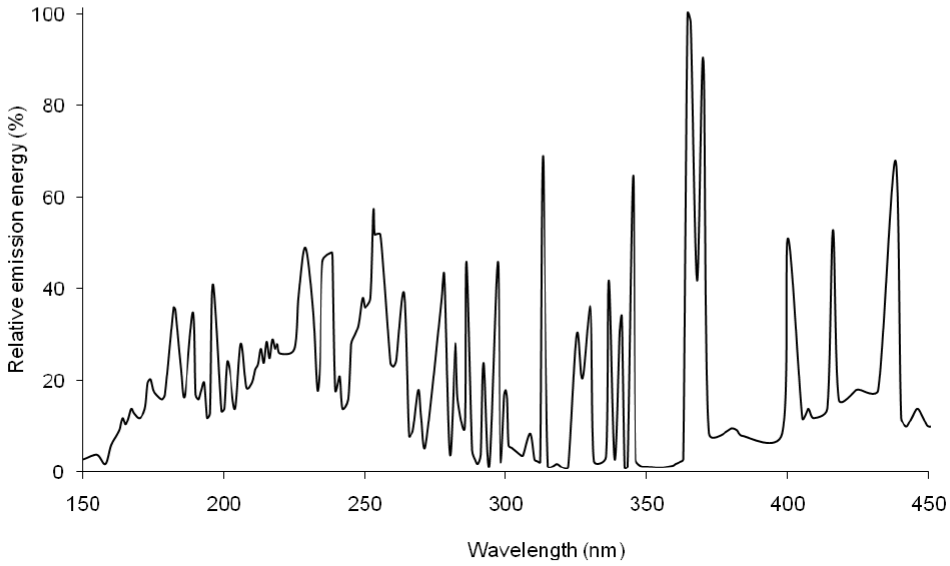

Figure S 1: Emission spectrum of a 690 W medium pressure metal-halogen UV lamp (Bau 42, Scan Research A/S, Denmark) emitting polychromatic light down to 185 nm, with an enhanced output compared to standard medium pressure mercury lamps in the 190-250 nm range.

Identification of CARBAMAZEPINE (CBZ)

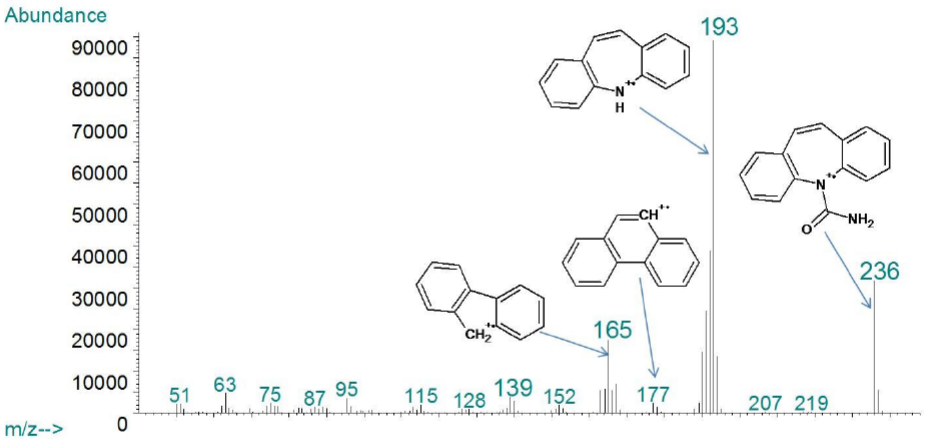

Figure S 2: EI-MS spectrum of underivatised CBZ with the proposed ion fragments

In the EI mass spectrum of CBZ (Figure S2) in its underivatised state we observe a molecular ion at *m/z* 236, which is subjected to a cleavage of the NHCO side chain to produce a base peak at *m/z* 193, corresponding to an iminostilbene structural fragment. Further loss of NH forms a phenantrene ring at *m/z* 178 and is followed by the loss of CH to give a 9H-fluoren-9-yl fragment at *m/z* 165. During derivatisation, the carbonyl group of CBZ is transformed into the enol-*tert*-butyl-dimethylsilyl ether, the EI mass spectrum of which shows a molecular ion at *m/z* 350, the typical loss of a *tert*–butyl group at *m/z* 293 and a base peak at *m/z* 193. Correspondingly, the ESI(+)ToF of CBZ shows a protonated molecule at *m/z* 237, a sodium adduct $[M+Na]^+$ at *m/z* 259 and a fragment ion at *m/z* 194, attributed to the protonated iminostilbene fragment. The collision induced fragmentation of *m/z* 237, produces fragment ions at *m/z* 194, *m/z* 179 and *m/z* 165. The accurate mass of the protonated CBZ molecule was 237.1030, equivalent to $C_{15}H_{13}N_2O$ (mass error ± 0.8 ppm).

Identification of IMINOSTILBENE (IMS)

IMS: This is an artefact created by thermal degradation in the GC. The compound was identified based on its EI spectra and has an abundant molecular ion at m/z 193, while the remaining two fragment ions m/z 180 and 165 are just noticeable. Its mass spectrum also matches that of IMS in the National Institute of Standards and Technology (NIST) mass spectral library.

GC-IT extracted mass chromatograms of UV and ClO_2 treated samples

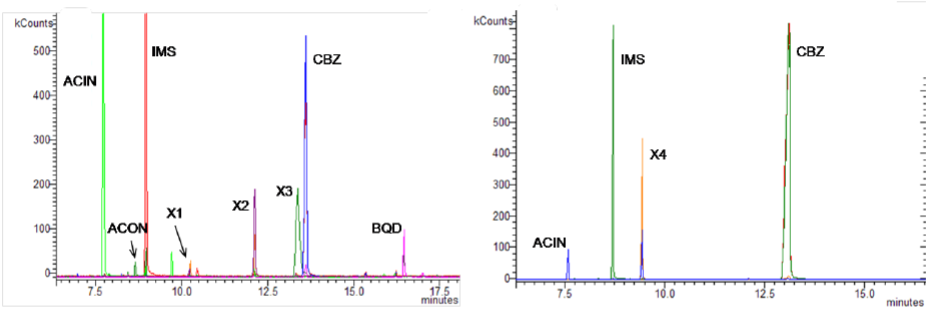

Figure S 3:Extracted mass chromatogram showing the residual carbamazepine (underivatised) and its degradation products after 15 minutes of UV treatment (left) and after addition of 13.5 mg L^{-1} ClO_2 (right)

Identification of ACRIDINE (ACIN)

ACIN: Figure S3 is a GC-IT extracted mass chromatogram of a CBZ sample subjected to UV treatment, where CBZ and its UV transformation products are evident. Among the most abundant peaks, is the one eluting at t_R 7.8 min, the EI mass spectrum of this peak shows a base peak corresponding to the molecular ion at *m/z* 179 and fragment ions at *m/z* 151 and 89. The EI-MS/MS produced the same ion fragments. The NIST library matched it to acridine and we confirmed its identity by comparing its retention time and mass fragmentation pattern to that of the authentic compound. In parallel, using MetaboLynx to process the LC-QqTOF data revealed a peak at t_R 5.9 min (Figure S3). Its ESI(+) TOF mass spectrum shows a protonated molecule at $[M+H]^+$ 180, while the ESI(+)-MS/MS fragmentation (collision energy: 30 eV) gives two additional fragments: *m/z* 152 and *m/z* 128. The accurate mass measurement of the protonated molecule at $[M+H]^+$ 180.0814 is only 0.6 ppm from the theoretical mass of the protonated acridine ($C_{13}H_{10}N$: 180.0813), confirming its identity.

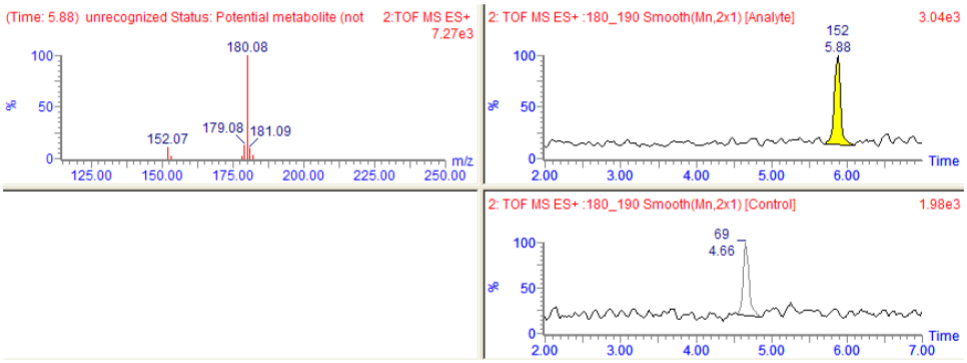

Figure S 4: A report segment from MeatboLynx data processing: a chromatogram of a newly-formed compound (top / right), its ESI(+) mass spectrum (top / left) and its absence in the chromatogram of a control sample (bottom / right)

Identification of ACRIDONE (ACON) and X5

Figure S3 shows a second TP at t_R 8.7 min. The base peak is attributable to the molecular ion at *m/z* 195 and fragment ions at *m/z* 167 and 139. The mass increase of 16 Da corresponds to ACIN with one added oxygen atom and is the result of either replacing a hydrogen atom with a hydroxyl group or replacing a double bond with a ketone functional group. A search of the NIST library supports this assumption yielding a good match to 9(10H)-acridinone. This was confirmed using the authentic compound. During derivatisation the ether forms on the 9-hydroxy group of the ACON 'enol' tautomer. The EI mass spectrum of ACON-MTBS shows a molecular ion at *m/z* 309 and, likewise CBZ-MTBS, the loss of a *tert*-butyl fragment to give a base peak at *m/z* 252. In addition, the subsequent losses of methyl groups at *m/z* 236 and 222 were evident. In the LC-MS chromatogram ACON eluted at t_R 5.1 min. Its ESI(+)TOF spectrum shows the protonated molecule at $[M+H]^+$ 196, a sodium adduct $[M+Na]^+$ 218 and a potassium adduct at $[M+K]^+$ 234, while ESI(+)-MS/MS (collision energy: 30 eV) produces an abundant fragments ion at *m/z* 167 and m/z 178 (Figure S4, bottom). The mass of the protonated molecule at $[M+H]^+$ 196.0765 (± 1.5 ppm) confirms it as being acridone: $C_{13}H_{10}NO$.

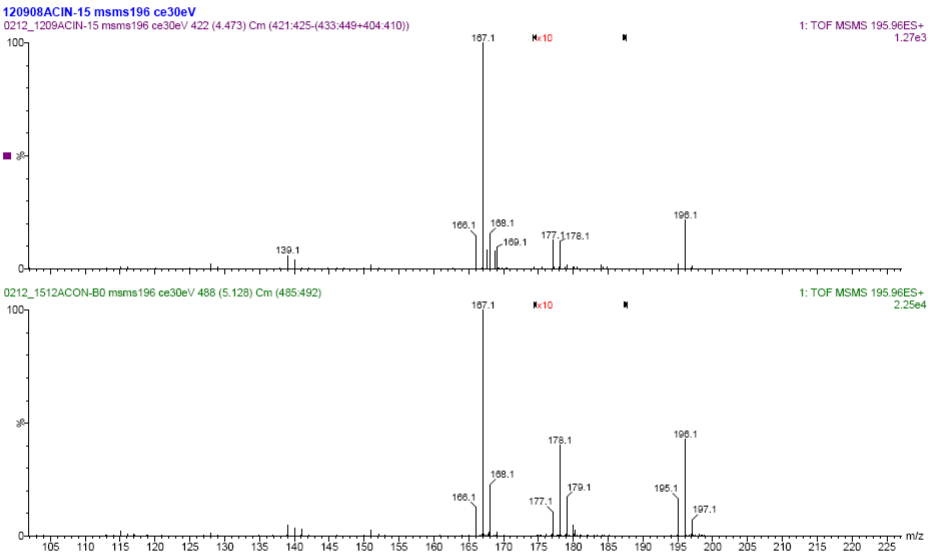

Figure S 5: Comparison of ESI(+) TOF-MSMS spectra of hydroxyacridine, X5 (top) and acridone (bottom)

X5: The peak X5, highlighted by MetaboLynx, elutes at t_R 4.5 min, where its ESI(+)TOF spectrum shows a protonated molecule at $[M+H]^+$ 196. The elemental composition gives the formula $C_{13}H_{10}NO$ (mass error 2.6 ppm). From the identical ESI(+)TOF-MS/MS fragmentation of ACON and X5, we can assign the latter, tentatively, as its structural isomer hydroxy-acridine. The position of the –OH group was not determined. However, taking into account similar mass fragmentation and that oxidation at position 9 is favourable due to the low electron density at this carbon on the acridine ring, we assume that X5 is the 9-hydroxy-derivative of acridine.

Identification of X1

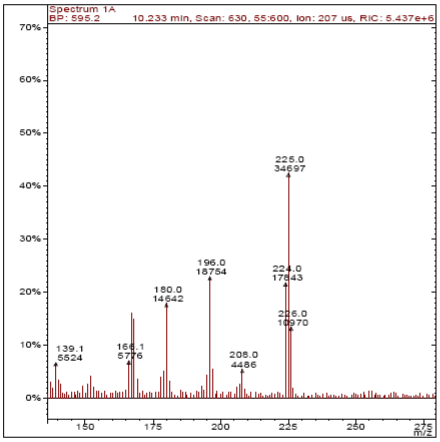

Figure S 6: GC-IT-MS spectrum of X1, postulated chemical structure: hydroxy (9H,10H)-acridine-9-carbadehyde

X1: X1 (Figure S3) elutes at 10.2 min. Its EI mass spectrum shows a molecular ion at *m/z* 225 (100 %), *m/z* 208 (12 %) corresponding to the loss of a hydroxyl group and *m/z* 196 (53 %) - a mass loss generated by the cleavage of an aldehyde group from the molecular ion. The lower ion fragments, *m/z* 180 and 167 arise from the cleavage of an oxygen atom and a 9-CH group from the acridine structure. The EI-MS/MS fragmentation also confirms this, allowing us to tentatively assign its chemical structure as hydroxy-(9*H*,10*H*)-acridine-9-aldehyde. Other structural isomers are also possible and further investigation is needed to confirm with certainty its structure.

Identification of X2

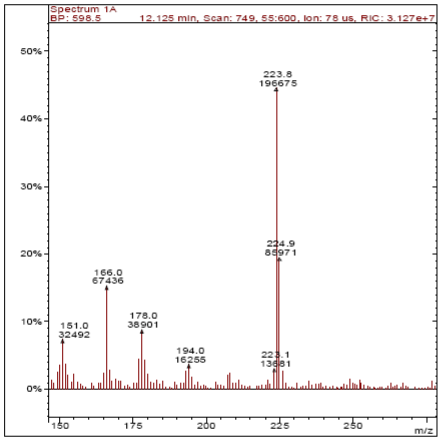

Figure S 7: GC-IT-MS spectrum of X2

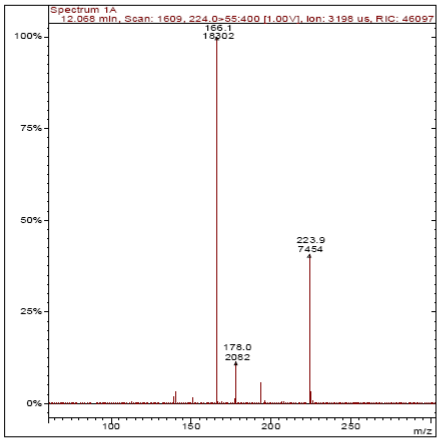

Figure S 8: GC-IT-MS/MS spectrum of X2

X2: Its EI-MS and MS/MS mass spectra reveal the fragmentation patterns, suggesting that X2 is a direct CBZ derivative. However, because the highest ion fragment has an even mass, it is likely that that the molecular ion of X2 does not appear in the spectra, which is not uncommon for a "hard" ionisation method, such as EI. A solution would be to use chemical ionisation (CI) to observe the molecular ion. Unfortunately, the inspection of the ESI(+) mass spectra revealed no compound having the related mass fragmentation pattern.

Identification of X3

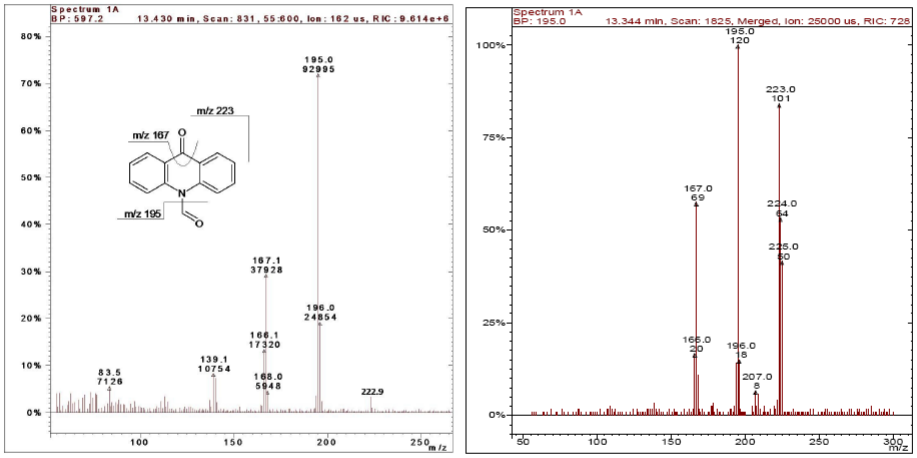

Figure S 9: GC-IT-MS (left) and GC-IT-MS^2 (right) spectra of X3, postulated chemical structure: acridone-N-carbaldehyde

X3: X3 elutes at 13.4 min (Figure S3). Its EI mass spectrum shows the molecular ion at *m/z* 223 (4 %), a base peak at *m/z* 195 and fragments at *m/z* 167 (40 %), 139 (11 %) and 84 (7 %). The fragmentation is confirmed with EI-MS/MS using an ion trap mass spectrometer. The fragmentation pattern of X3 (Figure S8, left) resembles closely that of ACON, with the exception of the X3 molecular ion at *m/z* 223. The molecular ion shows a mass increase of 28 Da, i.e. a carbonyl group, which is likely positioned on the heterocyclic nitrogen as the residual from the carbamyl side chain of CBZ. Thus X3 is tentatively assigned as acridone-N-carbaldehyde, which has to our knowledge not yet been identified as a TP of CBZ.

Identification of BQD

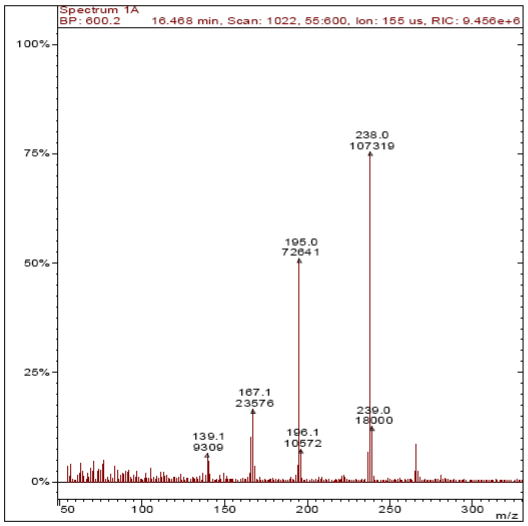

Figure S 10: GC-IT-MS spectrum of BQD (1-(2-benzaldehyde)-(1H,3H)-quinazoline-2,4-dione)

BQD: The last UV-breakdown product, sufficiently volatile to be analysed by GC-IT (Figure S3), appears at 16.5 min. The prominent mass fragments are at *m/z* 266 (molecular ion), 238 (base peak), 195, 167 and 140 (Figure S9). The EI-MS^3 acquisition (*m/z* 238 > 195) again resulted in the same ion fragments at *m/z* 167 and *m/z* 140. An identical mass fragmentation was published by McDowell *et al.*[1], who determined three TPs during ozonation of CBZ that contained the quinazoline structural fragment. Hence, based on the matching mass spectra with McDowell *et al.* we assume that this compound is 1-(2-benzaldehyde)-(1*H*,3*H*)-quinazoline-2,4-dione (BQD).

[1] McDowell, D.C.; Huber, M.M.; Wagner, M.; Von Gunten, U.; Ternes, T.A. Ozonation of Carbamazepine in Drinking Water: Identification and Kinetic Study of Major Oxidation Products. *Environ. Sci. Technol.* **2005**, 39, 8014-8022.

Identification of X4

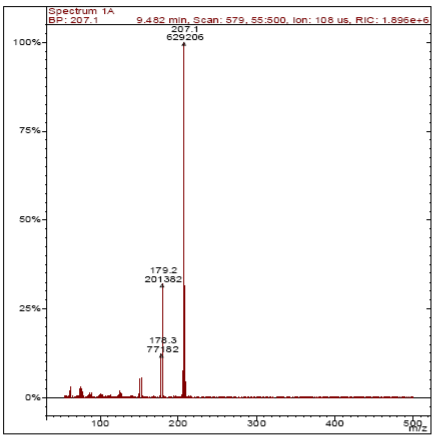

Figure S 11: Mass spectrum of the X4, the compound proposed to be acridine-9-carbaldehyde

X4: A comparison between the extracted mass chromatogram (*m/z* 179) of the ClO_2-treated sample (Figure S3) and the control reveals a new peak at t_R 9.5 min. It shows a steady increase with increasing ClO_2 dose. Its mass spectrum has a molecular ion at *m/z* 207 (M^+ = 100%), while the pattern of the remaining ion fragments is identical to ACIN (Figure S10). Although the MS^2 fragmentation of *m/z* 207 does not give any additional structural information, we conclude, that X4 contains the acridine structural fragment, the mass of which is increased by 28 Da, corresponding to a carbonyl bearing compound, i.e. acridine-carbaldehyde.

Formation of CBZ breakdown products during UV treatment

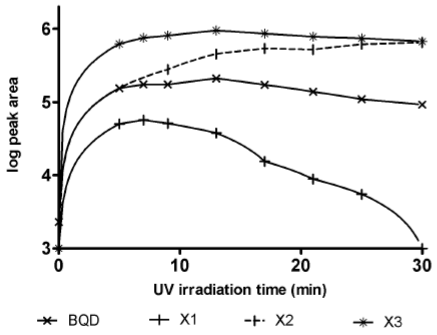

Figure S 12: Formation of carbamazepine TPs (BQD, X1, X2, X3) during UV treatment

ClO_2 treatment of CBZ and formation of breakdown products

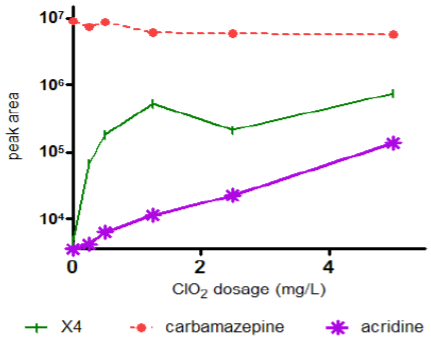

Figure S 13: The graph shows the behaviour of CBZ and breakdown products during the ClO_2 treatment of CBZ.

Removal of CBZ, ACIN and ACON in the bench-top bioreactors

Table S1: Removal of CBZ, ACIN and ACON in the bench-top bioreactors

compound	Aerobic			Anoxic		
	% removal	STDEV	n	% removal	STDEV	n
carbamazepine	16	± 6	4	16	± 10	2
acridine	92	± 6	4	90	± 6	2
acridone	40	± 12	3	23	± 1	2

4 Conclusions

In conclusion, the findings of this study support my hypothesis that by being able to detect and recognise the behaviour of both pharmaceuticals and their transformation products, it is possible to enhance the efficiency of water treatment and so limit their discharge into the either receiving or potable water. Previously, only a few studies have attempted to investigate this and there has been a significant gap in our understanding of the fate and behaviour of these compounds in the aquatic environment and only a few pharmaceutical transformation products have been identified. However, the identification of these new emerging compounds is essential, not only to provide a comprehensive risk assessment on drug resides in the environment, but also for designing improved treatment technologies.

Testing this hypothesis has involved developing sensitive and robust analytical methods capable of detecting sub ng L^{-1} levels of pharmaceutical residues in various aqueous matrices. The method was then applied to environmental samples and to studies on their behaviour during water treatment using laboratory scale bioreactors. The novel contributions to the science made by this study have resulted in eight published papers and one accepted for publication in SCI international journals, one book chapter and one paper submitted for publication. The work has been presented at twenty five international conferences with nineteen oral and nine poster presentations (Appendix 1). The overall conclusions of the present doctoral thesis are listed as follows:

1. To measure accurately the individual pharmaceuticals in aquatic matrices required the development, optimisation and validation of suitable analytical procedure both for intra and interlaboratory determination of NSAIDs:
   - Within this thesis I developed the analytical procedure for determining NSAIDs and involved the use of solid phase extraction followed by derivatisation and GC-MS analysis. The outcome of a robust intralaboratory validation study (good linearity, sensitivity, low limits of detection, high recovery and precision) is a proof that the developed method is suitable for determining NSAIDs in aqueous environmental samples.
   - The interlaboratory validation involved two separate round robin studies. It was revealed that the GC-MS protocol for the analysis of ibuprofen, ketoprofen and naproxen in more complex environmental matrices was superior to LC-MS/MS analysis, which was attributed to the matrix suppression effect found. In addition, the process of filtration and the filter material had no affect on the determination of NSAIDs.
   - When the method was applied to the determination of NSAIDs in Slovenian aquatic matrices revealed concentrations in surface water comparable to those found elsewhere in Europe.

2. Experiments were performed to ascertain both the behaviour and effects that pharmaceuticals have on activated sludge treatment. The experiments involve the use of laboratory-scale bioreactors, which were fed with an artificial wastewater containing various concentrations of pharmaceuticals.
   - An immediate conclusion was that in terms of biological removal it was possible to divide the test compounds in two groups, one group consisting of those pharmaceuticals readily degraded: ibuprofen, ketoprofen and naproxen, and a second group containing recalcitrant compounds: diclofenac, clofibric acid and carbamazepine.
   - An investigation into the effects that pharmaceuticals have on the microbial community revealed that concentrations ≥ 50 μg L^{-1} cause changes in the microbial composition of the activated sludge, an effect that becomes exaggerated with increasing concentration.
   - Another important conclusion is that bioreactor hydraulic retention time, the presence of the nutrient and substrate (pharmaceutical) concentration impact the extent of biotransformation in an activated sludge system.

3. One of the original aims of the thesis was to identify transformation products formed during water treatment. This was achieved by using complementary chromatographic and mass spectrometric techniques. In addition, the detection of unknown transformation products hidden in the background noise is enabled by applying a spectral and chromatographic search algorithm. For their identification, LC-QqTOF was found to be the most powerful among the available mass spectrometric techniques due to its ability to perform MS/MS fragmentation, high resolution and good mass assignment accuracy.

4. Only the pharmaceuticals persistent to biological treatment with activated sludge yielded detectable biotransformation products. Among a number of diclofenac's biotransformation products that were detected, 1-(2,6-dichlorophenyl)-1,3-dihydro-2H-indol-2-one, hydroxy-diclofenac, a benzoquinone imine derivative and a nitro-analogue of diclofenac were identified. Further, 4-chlorophenol was identified in the bioreactor feed with clofibric acid, and acridine and 9-acridone were formed during the biotransformation of carbamazepine. Of the seven compounds identified in biological treatment, five were detected for the first time as transformation products in this process.

5. The study method was able to identify seven abiotic transformation products of carbamazepine generated by UV-radiation and oxidation with chlorine dioxide. Acridine was formed during both treatment processes, while acridine 9-carbaldehyde was identified as an intermediate during chlorine dioxide oxidation. Further treatment of acridine with chlorine dioxide produced 9-hydroxy-acridine. UV-treatment resulted in the formation of acridone, hydroxy-(9*H*,10*H*)-acridine-9-carbaldehyde, acridone-N-carbaldehyde and 1-(2-benzaldehyde)-(1*H*,3*H*)-quinazoline-2,4-dione. In parallel, the transformation product iminostilbene was observed during sample analysis. Of these transformation products, not only was one compound novel, but it allowed for the first time an overall degradation pathway to be constructed involving both biological and abiotic treatments.

6. Another conclusion is that abiotic transformation products are more persistent to advanced oxidation or disinfection treatment than the parent compound, but they show enhanced biodegradability. Such examples are 4-chlorophenol, the biotransformation product of clofibric acid, and two transformation products of carbamazepine, acridine and 9-acridone. This finding suggests that in order to achieve optimal elimination of both parent compound and its transformation products a possible solution would be to apply a sequential abiotic and biological treatment.

7. An important finding is that at least three (bio)transformation products exhibited a higher toxicity than the parent compounds: 4-chlorophenol, acridine and 9-acridone. This supports my original thesis that when performing a risk assessment of the effect of pharmaceuticals in the aqueous environment the formation of metabolites during water treatment and in the environment, needs to be taken into account. In response to this I have already begun to compare the ecotoxicity of parent pharmaceuticals, the transformation products and their mixtures, and the results will be published in two SCI publications.

With my findings I confirmed that transformation products are formed during water treatment and that it is possible to minimise their discharge by implementing the appropriate treatment strategies. These findings would be also applicable to other persistent micro-pollutants.

In any future work, I believe that the range of model pharmaceuticals should be extended particularly focusing the persistent, genotoxic and widespread used pharmaceuticals, such as substances with psychotropic, anticancer and cardiovascular activity. Similarly to the study presented herein, the analytical methods should be further developed and applied to assess their environmental occurrence. Elimination efficiency should be assessed for each compound subjected to specific treatment technologies. Further attempts should be made to identify transformation products by applying alternative analytical techniques, such as complementary mass spectrometric detection and NMR. To confirm the proposed chemical structures a comparison with authentic standards should be made, where some of them will require in-house synthesis. Further, the ecotoxicity, genotoxicity and cytotoxicity of the identified transformation products and treated mixtures should be studied. Based on the toxicity results and taking into account their environmental occurrence a risk assessment may be prepared. Along with these studies, alternative treatment technologies should be developed aiming to achieve a complete mineralisation of both parent

compounds and the transformation products. Finally, the proposed treatment technologies will require a scale-up and evaluation from the scientific and economic perspective.

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Appendix 1

International work experience

1. IDAEA, CSIC (Consejo Superior de Investigaciones Científicas), Barcelona: April – August 2007
2. University of Almeria, Department of Hidrogeology and Analytical Chemistry, Almeria, Spain: May 2007 (1 week)
3. Danish Technical University, DTU Environment, Lyngby, Denmark: September – December 2007
4. Danish Technical University, DTU Environment, Lyngby, Denmark: August 2008 (1 month)
5. Danish Technical University, DTU Environment, Lyngby, Denmark: February 2009 (3 weeks)

Additional courses

1. Aseptic Preparation (University of Leeds, Organised by Sanolabor),
2. Advanced Wastewater Treatment (Technical University of Denmark);
3. Q-ToF Training Course (Waters Corporation);
4. Interpretation of MS and MS-MS Mass Spectra (Hyphen MassSpec, Organised by Instrumentalia);
5. Management Workshops (Organised by LUI and DMRS);
6. GC-Ion trap mass spectrometer Training course (Analytical Instruments, Denmark);
7. Principles of toxicology with risk assessment (STox 2008).

Personal bibliography for 2004-2009

TINA KOSJEK [27733]

ARTICLES AND OTHER COMPONENT PARTS

1.01 Original scientific article

1. Kosjek, T.; Heath, E.; Krbavčič, A. Determination of non-steroidal anti-inflammatory drug (NSAIDs) residues in water samples. *Environment International* **31**, 679 (2005).
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1.04 Professional article

11. Heath, E.; Kosjek, T.; Kompare, B. Zdravilne učinkovine pod lupo. *Embalaža, okolje, logistika* **39**, 40 (2008).

1.06 Published scientific conference contribution (invited lecture)

12. Heath, E.; Kosjek, T. Ostanki zdravilnih učinkovin v okolju. In: *Pomen biotehnologije in mikrobiologije za prihodnost: voda*. 157-167 (Biotehniška fakulteta, Oddelek za živilstvo, Ljubljana, 2007).

1.08 Published scientific conference contribution

13. Kosjek, T.; Heath, E.; Krbavčič, A. Development of an analytical procedure for the determination of non-steroidal anti-inflammatory drug residues in waters. In: *Jubilejni 10. Slovenski kemijski dnevi 2004.* 1-6 (FKKT, Maribor, 2004).
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20. Kosjek, T.; Heath, E.; Kompare, B.; Kovačič, J. Določanje učinkovitosti odstranjevanja ostankov zdravilnih učinkovin iz odpadnih vod. In: *Vodni dnevi 2006, Portorož* (Slovensko društvo za zaščito voda, Ljubljana, 2006).

1.12 Published scientific conference contribution abstract

21. Heath, E.; Kompare, B.; Kosjek, T. Pharmaceutical residues in a waste water treatment plant. In: *1st EMCO Workshop, Dubrovnik, Croatia* (Analytical and bioanalytical chemistry 385, 2005).
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MONOGRAPHS AND OTHER COMPLETED WORKS

2.13 Treatise, preliminary study, study

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PERFORMED WORKS (EVENTS)

3.14 Invited lecture at foreign university

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