Organic micropollutants in freshwater ecosystems

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Pollution dynamic and adverse effects at population genetic level in a model freshwater population

Von der Fakultät für Mathematik, Informatik und
Naturwissenschaften der RWTH Aachen University
zur Erlangung des akademischen Grades
eines Doktors der Naturwissenschaften genehmigte Dissertation vorgelegt
von

Diplom-Meeresbiologe
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Tag der mündlichen Prüfung: 20 Oktober 2016

Diese Dissertation ist auf den Internetseiten der Universitätsbibliothek online verfügbar

Erklärung

Die vorliegende Dissertation wurde im Department Wirkungsorientierte Analytik am Helmholtz-Zentrum für Umweltforschung-UFZ in Zusammenarbeit mit dem Lehr- und Forschungsgebiet für Ökosystemanalyse des Instituts für Umweltforschung (Biologie V) der RWTH Aachen unter Betreuung von Herrn PD. Dr. Werner Brack und Herrn Prof. Dr. Henner Hollert angefertigt.

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Dipl. Pedro Antonio Inostroza Bustos Aachen, den 03.August 2016

"For the things we have to learn before we can do them, we learn by doing them"

– Aristotle, The Nicomachean Ethics

ABSTRACT

The environment, and particularly freshwater ecosystems, is permanently under anthropogenic pressure, mainly due to the need of mankind to satisfy the ongoing demand of goods and services in order to support our society. However, continuous requests of ecosystem services undoubtedly evoke environmental consequences. Chemical contaminations are widely known for their harmful impacts on aquatic organisms and are today discussed as being responsible for increasing global impairments of ecological balance. In addition to direct effects, sublethal effects on the genetic level are increasingly suggested to provide versatile indicators for the assessment of hazardous chemicals. Such genetic effects of chemical stressors on aquatic organisms have so far been poorly addressed.

The aim of this thesis is to contribute to our understanding how anthropogenic pressures, particularly chemical and non-chemical stressors, may impair aquatic ecosystem functioning. The novel approach presented here is based on the analytical and thematic combination of evolutionary ecotoxicology and body burden analysis of organic micropollutants.

The CHAPTER 1 offers an overview of the state-of-the-art regarding the occurrence and potential ecological effects of organic micropollutants in aquatic environments. Furthermore, a concept regarding the likely value of including evolutionary ecotoxicology in future assessments is presented.

In CHAPTER 2, a multi-target screening method based on pulverised liquid extraction and a modified QuEChERS approach with additional hexane phase was developed and optimised. This method allows the extraction and measurement of a wide range of organic micropollutants, acknowledging the emerging relevance of biological environmental tissues in environmental chemistry and ecotoxicology. The new method developed here was successfully applied in different freshwater ecosystems, including the River Danube along its watercourse and the River Holtemme in Central Germany. The method exhibited particularly robust performance compared to other published analytical methods. In essence, low quantification limits and high recovery rates make this method suitable to detect pesticides, such as insecticides, herbicides and fungicides and wastewater-derived pollutants such as industrial chemicals and pharmaceuticals, in tissues of biological samples. The results obtained with this method were combined with other environmental matrices in order to examine the environmental dynamics of emerging organic micropollutants in the River Holtemme.

In CHAPTER 3, a multi-compartment approach based on chemical activity, equilibrium and predicted baseline toxicity was developed. A direct injection, pressurised liquid extraction methods, and the multi-target screening method developed in CHAPTER 2 were used in order to quantify emerging organic micropollutants in water, sediment and biota, respectively. Freely dissolved concentrations of compounds quantified in the River Holtemme and their corresponding chemical activities were calculated in the water, sediment

and biota (*Gammarus pulex* tissues) compartments. The bioavailable fraction of pollutants and thus the fate and distribution of emerging compounds were assessed. According to equilibrium partitioning theory, the chemical activity of an organic compound is equal in sediment organic carbon, in exposed biota and in pore water, if equilibrium is reached between these phases. Sediments showed highest chemical activities and significant differences were quantified between water and biota compartments. The findings obtained suggest that the system studied here was in disequilibrium based on the equilibrium partitioning theory. Additionally, sediment samples exhibited the highest potential toxicity. Hazard assessment of the quantified contaminants showed a strong dependency on which compartment is analysed.

CHAPTER 4 demonstrates the biological effects of long-term exposure to pollution on a model freshwater invertebrate population. Briefly, the adverse effects of global and emerging anthropogenic pressures were assessed using a novel approach based on evolutionary ecotoxicology and body burden analysis of organic micropollutants. This approach was then successfully applied to *G. pulex* populations occurring along the River Holtemme. The results provide empirical evidence of both direct and indirect effects due to chemical and non-chemical stressors. The analyses revealed pollutant-induced changes in the genetic structure as well as higher mutation rates downstream of a wastewater treatment plant. Furthermore, hindered gene flow due to physical barriers (i.e. weirs) separating upstream and downstream waters in the River Holtemme was detected. Although, these findings offer new insights into the field of ecotoxicology in general, and allows for new interpretation of the role of wastewater treatment plants as sources of chemical stress in the environment.

ZUSAMMENFASSUNG

Die Umwelt und insbesondere aquatische Ökosysteme stehen permanent unter anthropogener Belastung beruhend auf der stetigen Nachfrage nach Waren und Dienstleistungen zum Erhalt unserer Gesellschaft. Die kontinuierliche Bereitstellung von Ökosystemdienstleistungen bringt zweifelsohne auch Konsequenzen für die Umwelt mit sich. Chemische Kontaminationen sind für ihre schädlichen Wirkung auf aquatische Organismen bekannt und werden heute als mitverantwortlich für globale Veränderungen im ökologischen Gleichgewicht diskutiert. Neben direkten Effekten auf aquatische Organismen werden heute auch sublethale Effekte auf genetischer Ebene als wichtige Kenngrößen zur Gefahreneinschätzung von Kontaminanten diskutiert. Die genetischen Auswirkungen von chemischen Stressoren auf Süßwasserorganismen sind bis dato jedoch sehr wenig untersucht.

Ziel dieser Dissertation ist es zum wissenschaftlichen Verständnis beizutragen, inwiefern anthropogene Belastungen, inbesondere chemische und nicht-chemische Stressoren, aquatische Ökosysteme beeinträchtigen können. Der hier verwendete neue Ansatz basiert auf der analytischen und inhaltlichen Verbindung von evolutionärer Ökotoxikologie und der Belastungsanalyse von organischen Mikroschadstoffen.

Kapitel 1 liefert einen Überblick über den aktuellen Wissensstand bezüglich des Auftretens und der möglichen ökologischen Effekte von Mikroschadstoffen in aquatischen Systemen. Des Weiteren wird ein Konzept zum Wert Evolutionsökologisch-toxikologischer Ansätze bei zukünftigen Gefahreneinschätzungen von Mikroschadstoffen präsentiert.

In Kapitel 2 wurde ein mehrzieliges Screening-Verfahren entwickelt und optimiert, dass auf einer Flüssigextraktion sowie einem modifizierten QuEChERS Ansatz mit zusätzlicher Hexan-Phase beruht. Diese Methode erlaubt es, eine große Bandbreite von organischen Mikroschadstoffen zu extrahieren und zu messen. Die Analyse solcher Stoffe aus biologischen Geweben in Umweltproben gewinnt in der Umweltchemie und Ökotoxikologie zunehmend an Bedeutung. Die hier entwickelte Methode wurde erfolgreich in mehreren Süßwasser-Ökosystemen angewandt, wie in dem bedeutendem Donauflusssystem und der Holtemme in Sachsen-Anhalt in Mitteldeutschland. Die Methode ist im Vergleich zu anderen, in der Litertaur bekannten, analytischen Methoden deutlich robuster. Sie eignet sich aufgrund ihrer niedrigen Bestimmungsgrenze sowie einer hohen Ausbeute besonders, um Pestizide wie Insektizide, Herbizide, Fungizide und Abwasserschmutzstoffe wie z.B. industrielle Chemikalien und Pharmazeutika in dem biologischen Geweben zu detektieren. Die mit dieser Methode erzielten Ergebnisse wurden mit weiteren Umweltvariablen verschnitten um die Dynamik von organischen Mikroschadstoffen am Beispiel der Holtemme zu untersuchen.

In Kapitel 3 wurde ein Multi-Kompartment-Ansatz basierend auf chemischer Aktivität, chemischem Equilibrium sowie Vorhersagen zur Basis-Toxizität entwickelt. Eine Direktinjektionsmethode, eine Flüssigdruckextraktionsmethode und das in Kapitel 2 entwickelte mehrzielige Screening wurde angewandt, um neuartig organische

Mikroschadstoffe in Wasser, Sediment und Organismen zu quantifizieren. Frei gelöste Stoffkonzentrationen und ihre entsprechenden chemischen Aktivitäten wurden für Wasser, Sedimente und Organismen (Gewebe von *Gammarus pulex*) berechnet. Ziel war es, die biologisch wirksame Fraktion von Schadstoffen und somit der Verbleib sowie die Verteilung aufkommender Schadstoffe in Süßwasserökosystemen zu analysieren. Laut der Gleichgewichts-Verteilungs-Theorie ist die chemische Aktivität einer organischen Substanz in Sediment, Biota und Porenwasser gleich, wenn ein Equilibrium zwischen diesen drei Phasen erreicht ist. Sedimente wiesen dabei die höchsten chemischen Aktivitäten, während es gab signifikanten Unterschiede zwischen Wasser und Organismen. Die in dieser Studie erzielten Ergebnisse weisen darauf hin, dass sich das System gemäß der Gleichgewichts-Verteilungs-Theorie im Disäquilibrium befindet. Darüber hinaus zeigten die Sedimentproben die höchste potentielle Toxizität. Gefahrenanalysen zeigten, dass die spezifische Toxizität davon abhängig ist, welches Kompartiment (Wasser, Sediment, Organismen) genau analysiert wird.

Kapitel 4 demonstriert die ökologischen Effekte einer Langzeitbelastung mit organischen Schadstoffen durch die Analyse einer aquatischen Modellpopulation (*Gammarus pulex*, Amphipoda) auf sublethaler, genetischer Ebene. In diesem Kapitel werden die schädlichen Auswirkungen globaler und neu-aufkommender anthropogener Belastungen beurteilt. Dies geschieht mithilfe eines neuartigen Ansatzes, der auf evolutionärer Ökotoxikologie und der Körperbelastung mit organischen Mikroschadstoffen beruht und mit *Gammarus pulex* als Modellorganismus entwickelt und erfolgreich in der Holtemme angewandt wurde. Die Ergebnisse liefern den empirischen Nachweis sowohl direkter als auch indirekter Effekte von chemische und nicht-chemische Stressoren auf Populationsebene. Die Auswertungen zeigten unter anderem eine deutliche, schadstoff-induzierte Veränderung in der genetischen Struktur sowie erhöhte Mutationsraten durch chemische Stressoren unterhalb von Kläranlagen. Ebenso wurde festgestellt, dass der genetische Austausch durch physikalische Barrieren (Dämme) im Fließgewässer eingeschränkt wird. Diese Erkenntnisse liefern neue Einblicke in die Ökotoxikologie und ermöglicht neue Interpretationsmöglichkeiten hinsichtlich der Rolle von Kläranlagen als Quelle chemischer Stressoren in der Umwelt.

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ABBREVIATIONS AND SYMBOLS

Abbreviations

2ABA	2-Aminobenzimidazole
5MBT	4-/5-Methyl-1H-benzotriazole
AChE	Acetylcholinesterase
AFT	Ames Fluctuation Test
ANOVA	Analysis Of Variance
ASE	Accelerated Solvent Extraction
BCF	Bioconcentration Factor
BQE	Biological Quality Element
BT_P	Predicted Baseline Toxicity
CAS	Chemical Abstract Service
CBZ-diol	10,11-Dihydroxy-10,11-dihydrocarbamazepine
CI	Confident Interval
D. magna	*Daphnia magna*
DEET	N,N-Diethyl-meta-toluamide
DesAtr	Desethylatrazine
DesTer	Desethylterbuthylazine
DF	Distribution Factor
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DOC	Dissolved Organic Carbon
DOM	Dissolved Organic Matter
dSPE	dispersive Solid Phase Extraction
EqP	Equilibrium Partitioning
EQS	Environmental Quality Standards
ESI	Electrospray Ionization
EU	European Union
FDR	False Discovery Rate
FP	FastPrep
G. fossarum	*Gammarus fossarum*
G. pulex	*Gammarus pulex*
GC-MS	Gas Chromatography Mass Spectrometry
HPLC	High Performance Liquid Chromatography
HR	High Resolution
HRMS/MS	High Resolution Tandem Mass Spectrometry
HWE	Hardy-Weinberg Equilibrium
IBD	Isolation by Distance
IDL	Instrument Detection Limit
JDS3	Joint Danube Survey 3
JDS	Joint Danube Survey
LC	Liquid Chromatography
LC-HRMS	Liquid Chromatography High Resolution Mass Spectrometry
LC-HRMS/MS	Liquid Chromatography High Resolution Tandem Mass Spectrometry
LC-MS	Liquid Chromatography Mass Spectrometry
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LD	Linkage Disequilibrium

LLE	Liquid Liquid Extraction
LSER	Linear Solvation Energy Relationship
LVSPE	Large Volume Solid Phase Device
M04	Prothioconazole-desthio
MCI	Molecular Connectivity Index
MCMC	Markov Chain Monte Carlo
MCPA	2-methyl-4-chlorophenoxyacetic acid
MDS	Non-metric Multidimensional Scaling
ME	Matrix Effect
MQL	Method Detection Limit
MLR	Multiple Linear Regression
MRM	Multiple Reactions Monitoring
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MT13	Terbuthylazine-2-hydroxy
MW	Molecular Weight
N-Ac-SMX	N-Aceytylsulfamethoxazole
NAAP	n-Acetyl-4-aminoantipyrine
nAChR	nicotinic Acetylcholine Receptor
OC	Organic Carbon
PCR	Polymerase Chain Reaction
pH	potential Hydrogen
PLE	Pressurised Liquid Extraction
PN	Phosphorus-Nitrogen
POC	Particulate Organic Carbon
POM	Particulate Organic Matter
ppLFERs	polyparameter linear free energy relationships
PSA	Primary Secondary Amine
PTFE	Polytetrafluoroethylene
PTSA	*p*-toluene-sulfoamide
PuLE	Pulverised Liquid Extraction
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
RDA	Redundancy Analysis
RSD	Repeatability of the Method
SIMPER	Similarity Percentage
sMRM	scheduled Multiple Reactions Monitoring
SMX	Sulfamethoxazole
SPE	Solid Phase Extraction
TK	Toxicokinetic
TOC	Total Organic Carbon
TP	Transformation Product
TU	Toxic Unit
WFD	Water Framework Directive
WWTP	Wastewater Treatment Plant

Symbols

+	Pollutants Detected Under the MQL
$\Pi_{B,Lip}$	Biota-Sediment Concentration Quotient
Π_{SOC}	Sediment-Water Concentration Quotient
°C	Celsius Degree
α	Chemical Activity
AcFA	Acetone + 1% Formic Acid
A_r	Allelic Richness
CO_2	Carbon Dioxide
D_d	Directional D-values
DF_{BS}	Biota-Sediment Distribution Factor
DF_{BW}	Biota-Water Distribution Factor
DF_{SW}	Sediment-Water Distribution Factor
C_{di}	Measured Internal Concentration
C_e	Effective Concentration
C^{fd}	Freely dissolved concentration
C_m	Concentration in Sediment
C_n	Nominal Concentration
C_p	Pore Water Concentration
C_{SOC}	Sediment Concentration Normalised To Sediment Organic Carbon
C_w	Water Concentration
D	Partitioning Coefficient
DT_{50}	Half-Life
EC_{50i}	Median Effect Concentration
EtAc	Ethyl acetate: Acetone
F_{IS}	Inbreeding Coefficient
f_{LIPID}	Fraction of Lipids
F_{ST}	Wright's Fixation Index
f_{OC}	Fraction of Organic Carbon
H_o	Observed Heterozygosity
K_d	Distribution Coefficient
K_{DOC}	Dissolved Organic Carbon Partitioning Coefficient
K_{OC}	Soil Organic Carbon-Water Partitioning Coefficient
K_{OW}	Octanol-Water Partitioning Coefficient
K_{POC}	Particulate Organic Carbon Partitioning Coefficient
kV	kilo Volt
m^2	Square meter
$MgSO_4$	Magnesium Sulphate
mTU	Maximum Toxic Unit
N	Number of Alelles
NaCl	Sodium Chlorine
N_{PA}	Number of Private Allele
La_{50}	Lethal Chemical Activity
LC_{50}	Lethal Concentration
Q-values	Assignment Probability Values
R^2	Regression Coefficient

S_L	Subcooled Liquid Solubility
*s*TU	Summed Toxic Unit
uH_E	Unbiased Expected Heterozygosity
v/v	volume: volume

CHAPTER 1

Introduction to the effect of organic micropollutants in freshwater biota: insight at population genetic level

1.1 Mankind influences on the environment

A decade ago Paul Crutzen coined the term "Anthropocene" epoch that has begun in late eighteenth century, when analyses of air trapped in polar ice showed the beginning of growing concentrations of carbon dioxide and methane (Crutzen, 2002). This term refers to effects of human activities and their outcomes at global scale. The nimble expansion of mankind in numbers and over all possible ecosystems has continued apace. Human population has increased tenfold during the past three hundred years and is expected to reach 10 billion in this century. About 50% of the planet's land surface is already exploited by humans (Vitousek et al., 1997). Tropical rainforests are disappearing at a quick pace, releasing carbon dioxide and strongly enhancing species extinction. More than half of all accessible fresh water is used by mankind. Over the last fifty years, humans have changed the world's ecosystems more rapidly and extensively than in any other comparable period in human history (Millennium Ecosystem Assessment, 2005). Hence, the earth is rapidly turning into a less biologically diverse, less forested, much warmer and probably much chemically polluted due to the permanent anthropogenic pressures (Steffen et al., 2007). The main role of chemicals for mankind is to satisfy the services required to maintain viable human civilizations. For instance, pesticides have been used to control undesired plague that exerted adverse effects in crops and additionally that one producing adverse health problem in human populations. This latter is the case of the malaria, a mosquito-borne infectious disease, where vector control methods are used to reduce the levels of transmission by *Anopheles* mosquito. Their consequences are widely debated; nevertheless the recognition as a global issue is undeniable.

Pollution by chemicals is already widespread to all known environments by human, for example, microplastics (<1 mm) were measured in marine sediments up to 4,900 metre depth in the deep sea (Van Cauwenberghe et al., 2013). Moreover, the occurrence of aerosols in the atmosphere can lead to weaker hydrological cycle and then affect directly the quality of fresh water (Ramanathan et al., 2001). We are reaching planetary boundaries and we became one of the chief environmental drivers on the Earth. Rockström and co-workers (2009) identified nine main earth-system processes responsible of alter the earth stability. Interestingly, three of them are touched in this investigation, one straightaway such as chemical pollution and two more tangentially; rate of biodiversity loss and freshwater use.

1.2 Chemical pollution in freshwater ecosystems

The increasing chemical pollution of aquatic ecosystems is a pivotal matter in environmental science as a result of the largely unknown long-term effects on aquatic life and ultimately on human health. Currently, more than 300 million tons of synthetic compounds are used annually and eventually they and/or their metabolites will be release into the aquatic environment (Schwarzenbach et al., 2006). Particularly, in the European Union (EU) there are more than 100,000 registered chemicals, where around 30% to 70% are in daily use (EINECS, European Inventory of Existing Chemical Substances).

The source, dynamic and treatment of the relatively small number of macropollutants such as acids, salt, nutrients occurring at μg/litre to mg/litre concentrations are rather well understood (Mengis et al., 1997). Nevertheless, it is challenging to assess the occurrence, fate and ecological risk on aquatic environment of synthetic chemicals present in the environment at trace concentrations (ng/litre or even pg/litre) (Jackson et al., 2001). These micropollutants are ubiquitous in aquatic ecosystems not only in industrialised areas but far-off environments. They enter surface water through several pathways including point sources (Reemtsma et al., 2006) and nonpoint sources (e.g. land runoff and precipitations) (Liess and von der Ohe, 2005), and depending on their physical-chemicals properties, these chemicals distribute amongst the different environmental compartments. In this study it was understand sediment, water, and biota as environmental compartments in a freshwater ecosystem.

Chemical discharges from point sources such wastewater treatment plants (WWTPs) tend to be continuous, with a narrow variability over time. Usually, they can be monitored by measuring discharge and chemical concentrations regularly at a single place. Therefore, point sources are comparatively simple to measure and regulate, and can be often be controlled by treatment at the source. On the other hand, nonpoint sources can be continuous as well, but they are often more intermittent and linked to seasonal or periodical agricultural activities or irregular events, for example heavy precipitation. Usually nonpoint inputs derive from extensive areas of land and can be transported overland, underground, or through the atmosphere where the final destination are receiving surface waters. Thus, nonpoint sources, antagonistically to point sources, are difficult to measure and regulate (Carpenter et al., 1998; Jackson et al., 2001).

Independently of the source, many of these chemicals may persist in the environment or may show a permanent exposure if losses by environmental transformation and degradation are continuously replaced by new emissions. If these chemicals are taken up by aquatic organisms and bind to biological receptors they may cause adverse effects and pose a risk to freshwater ecosystems (Beketov et al., 2013; Malaj et al., 2014). Organisms in the environment are exposed not only to isolated micropollutants but to complex chemical mixtures (e.g. pesticides and wastewater-derived chemicals) (Jobling et al., 1995). Then, additive or even synergistic effects can depict such mixtures harmfully consequences. Altenburger *et al*., (2004) investigated the joint effect of a mixture of 10 compounds concluding that the responses based on mode of action addition play a more pivotal role compare to the concentration-response model to explain the effect data. Therefore, only some

of the mixture components may be expected to contribute to a combined effect. Summarising, there are certain matters about when we must raise our concern regarding the chemical pollution in freshwater. For example, production of drinking water relies on clean surface water. Hence, protecting the integrity of our natural waters resources against chemical pollution can safeguard aquatic life and thus, directly and indirectly, human health.

1.3 Pesticides in the environment

There is little doubt that pesticides play a significant role reducing regional biodiversity in freshwater ecosystems (Beketov et al., 2013), due to the significant amount of global terrestrial surface domesticated (about 25%-30% in the fifteens) for agriculture (Lambin and Geist, 2006). Pesticides are amongst the best ecotoxicologically characterised and regulated groups of pollutants. However, it is still not clear to what extent pesticides affect aquatic life, from genome to community structure in the wildlife. Not only target pests are exposed to pesticides but these compounds may also affect non-target organisms through specific events and long-term exposure in agricultural landscapes. In fact, organisms occupying habitats near to agricultural areas are prone to be exposed to high concentration of pesticides, especially when their whole or part of their life cycle occurs into the water.

In EU, the Water Framework Directive (WFD) aims to achieve good ecological and good chemicals status of surface waters. The assessment of chemical status relies on compliance with Environmental Quality Standards (EQS) for priority substances. Pesticides are amongst these priority substances (EC 2008) and the assessment of these chemicals in surface waters became an essential need in the EU in order to accomplish WFD aims.

Pesticides pollution of surface running water in each particular region depends on several factors, such as distance of crop field to surface water, riparian drainage canal features (surface area, depth, and flow), surrounding fields (kind of soil, grassland, slope, and distance to the water bodies) and climate conditions (temperature, humidity, wind, and precipitation) (Capel et al., 2001). Indeed, agricultural pesticides are primarily transported to surface waters via runoff and tile drains at heavy precipitation events (Kronvang et al., 2004). In some countries, the load of pesticides coming from urban use, thus primarily discharge from WWTP, is estimated to be responsible for at least 30%-50% of the total annual concentrations at the water phase (Blanchoud et al., 2004). Therefore, based on their widespread distribution, agriculture pesticides, are suggested to pose a threat to all living biota in aquatic ecosystems (Liess and von der Ohe, 2005; Malaj et al., 2014) including microbial organisms (Maltby et al., 2009; Schäfer et al., 2011).

The presence of pesticides in freshwater systems triggers several ecological processes. Downstream drift of macroinvertebrates is a direct outcome of pesticide exposure (Brittain and Eikeland, 1988). Basically, downstream drift is a common response of lotic macroinvertebrates to a disturbance, an environmental factor and including as well chemical pollution. This process has been reported to occur in asymmetric systems (e.g. streams and rivers) exposed to pesticide pollution (Beketov and Liess, 2008). However, it has been suggested that not all pesticides can trigger downstream process. Laboratory studies observed

that principally neurotoxic insecticides, such as pyrethroids, organochlorines, and organophosphates, pronounced drift-initiation potential (Beketov and Liess, 2008; Lauridsen and Friberg, 2005). The status of the drift process in the agricultural landscapes is unknown and even more its ecological consequences. For instance, drift can reduce population densities of non-target organisms with unclear ecological outcomes (Brittain and Eikeland, 1988).

1.4 Wastewater-derived chemicals

In recent years, the occurrence and fate of wastewater-derived chemicals in the aquatic environment has been recognised as one of the emerging issues in environmental chemistry. Wastewater-derived chemicals are defined as personal care products, hormones, pharmaceuticals and industrial chemicals; in general, all kinds of chemicals derived from WWTPs. Many of these anthropogenic pollutants are polar, non-volatile, and poorly bio-degradable chemicals (Paxéus, 2004). As a result of their permanent use by current societies and the fact that selected wastewater-derived chemicals are not effectively removed along the wastewater treatment (Reemtsma et al., 2006), a considerable range of wastewater-derived chemicals and their metabolites and/or transformations products have been widely detected in aquatic ecosystems (Löffler et al., 2005; Loos et al., 2009; Miller et al., 2015; Radović et al., 2014). Unexpectedly, little is known about the extent of environmental occurrence, dynamic and ultimate fate of many wastewater-derived chemicals becoming an environmental issue.

In general, pharmaceuticals are absorbed by the organism after intake and undergo metabolic reactions, such as hydroxylation, cleavage or glucuronidation. Nevertheless, a significant amount will not be metabolised and will leave via the urine or faeces and will hence enter the wastewater treatment system. Once pharmaceuticals are in the treatment plant, they can associate to the sewage sludge or remain in the aqueous phase (Carballa et al., 2004). To give an example, the antibiotic amoxicillin can be removed by up to 75%-100% in the WWTP (Castiglioni et al., 2006). By contrast, carbamazepine, a seizure disorders and neuropathic pain drug, and diclofenac, a nonsteroidal anti-inflammatory drug, are poorly removed (<10% and 17%, respectively) (Heberer, 2002). An alternative pathway into the aquatic environment, via runoff, derives from the dispersion of manure on fields as fertilizer (Boxall et al., 2004). Even groundwater can be exposed to antibiotic residues leaching from farmland fertilised with manure or through sewage disposal by spray and broad irrigation in agricultural areas. To date, pollution with pharmaceuticals is reported worldwide in different environments such as marine (Bertin et al., 2011) and river sediments (Radović et al., 2014), in surface water (Loos et al., 2010) and biota (Huerta et al., 2013; Miller et al., 2015).

Some studies highlighted that species at different trophic level may be at risk due to the exposure to pharmaceuticals especially via dietary uptake, and thus biomagnification (Carlsson et al., 2006; Markman et al., 2008). However, while the toxicity of some pharmaceuticals is well accepted, effects at population and/or community level are poorly understood in the environment. Indeed, integrative investigations should consider different families of pollutants in the field, and not just one or two classes, in a more integrative perspective, consider involved mechanism and/or outcomes of transformations products are

missing in order to have a wider comprehension of the role of organic micropollutants in the aquatic environment.

1.5 Adverse effects: from DNA to ecosystem level

Once the organisms are exposed to the organic micropollutants many specific effects may produce a response (e.g. triggered by binding to nuclear receptor and enzyme inhibitors). The interplay amongst the chemical and the biological receptor sometimes may exert a specific toxic outcome reaction with biomolecules and non-specific effects by uptake into and perturbation of membranes (Escher and Hermens, 2002). At cellular level, the main biological target entities are membranes, proteins and genetic material. Depending on the reactivity of the pollutant, the target site, dose and duration of the exposure, the effects can range from DNA damage, constraints related to protein function, with subsequent toxicity, carcinogenicity and ultimately mutagenicity effects (Escher and Hermens, 2002). Concurrently, same effects can be caused by intermediate products, metabolites and transformation products (Smital et al., 2004). For instance, already in the seventies investigation reported that metabolites of the pesticides trichlorfon can apply higher mutagenic activity than their parent compound (Fischer et al., 1977).

Many wastewater-derived chemicals, especially pharmaceuticals, are biologically active molecules that once in the environment rise concern about the potential impacts not just in surface water but in the whole aquatic system (Fent et al., 2006). Primarily, several studies reported adverse effects on aquatic organisms at low concentrations. For instance, the oestrogen ethinyl estradiol showed reproduction impairments at individual and population level (Jobling et al., 2006; Nash et al., 2004). Exposure to diclofenac, showed disruption of internal organs (kidney) and generated necrosis in the gills in fish (Schwaiger et al., 2004) and when invertebrates, *G. pulex*, were exposed to fluoxetine, a selective serotonin reuptake inhibitor used in the treatment of depression, behaviours changed (De Lange et al., 2006). Adverse consequences from DNA to ecosystem level should not be underestimated, even more when invertebrates like *G. pulex* are able to biotransformate and bioaccumulate xenobiotics reaching high enrichment factors (Ashauer et al., 2012). Basically, adverse effects are extensively reported for freshwater organisms from invertebrates to fishes.

At higher organisation level and wider spatial scale, the impacts of organic micropollutants are challenging and our understanding of the consequences remains limited. Beketov and co-workers (2013) analysed the effects of pesticides in invertebrates at region scale in Europe and they found out that pesticides caused statistically significant effects, reducing the biodiversity with losses in taxa up to 42% of the recorded pool. Moreover, the effects were determined at concentrations currently considered environmentally protective to the biota. Whereas, Schäfer and co-workers (2007) determined a decrease in relative abundance and number of sensitive species associated to pesticide stress in streams in France. These examples support the finding by Malaj and co-workers (2014) of clear evidences that organic micropollutants threaten the ecological integrity of freshwater ecosystems at continental scale.

1.6 Evolutionary ecotoxicology

Earth is in its sixth great extinction event, with rates of species loss growing rapidly for both terrestrial and marine ecosystems (Pimm et al., 1995). Hence, a better understanding of the biodiversity at all level of biological organization is primordial in order to assess anthropogenic pressures. Analysis of population genetic variability may help to unravel the role of the chemical pollution on the diversity and structure of freshwater population at genetic level.

Here, evolutionary ecotoxicology may play an important role. Evolutionary ecotoxicology is an emerging scientific field aiming to elucidate microevolutionary processes caused by the environmental pollution in natural populations (Bickham, 2011; Medina et al., 2007). Shugart and co-workers (2010) described this emerging approach in experimental designs typical of ecotoxicology, including the selection of appropriate model species, comparisons of matched references sites and impacted sites, correlation of effects with gradient exposures, and empirical studies using controlled experiments. Therefore, the conceptual basis for evolutionary ecotoxicology derives from both evolutionary theory and conservation biology, and the analytical and laboratory methods are those of molecular population genetics (Bickham, 2011).

Along with the environmental exposure, organisms may be in contact with mutagenic pollutants, which cause direct DNA damage throughout base substitutions, deletions or duplications, or structural modifications of the chromosomes with subsequent adverse somatic effects. Chemical pollutants that are not mutagenic do not cause direct structural modifications or alterations to DNA. However, exposure to both group of chemicals result in an evident stress to organisms and hence on the genetics of populations (Rose and Anderson, 2005). All of the four main evolutionary forces involved in shaping genetic patterns (mutation, selection, genetic drift and gene flow) of every population can be extremely modified by pollution pressure. Regarding population genetic diversity, several outcomes of pollution effects are therefore hypothesized (Bickham, 2011). The most frequently expected response is genetic erosion (van Straalen and Timmermans, 2002), a genome-wide loss of genetic variability due to a diminishing of effective population size of an exposed population and thus a subsequent genetic drift. For instance, Matson and co-workers (2006) observed reduced haplotype and nucleotide diversity in marsh frog (*Rana ridibunda*) populations in highly polluted industrial areas compared to unpolluted reference areas. However, not always changes in diversity need to be related to constrain in diversity (Bach and Dahllöf, 2012; McMillan et al., 2006; Whitehead et al., 2003). Enhanced genetic diversity were reported in several investigations (Shugart et al., 2010; Theodorakis and Shugart, 1997).

It is well established that reduction in genetic diversity, henceforth genetic erosion, may have physiological implications for wildlife populations, in part due to a higher relative incidence of deleterious alleles (Brown et al., 2009). Genetic erosion can alter the responsiveness of individuals to chemical pollution (Brown et al., 2011) and potentially constrain the ability of those populations to adapt to environmental change (Brown et al., 2009). Genetic structure, can also be altered through selection of certain genes that increase

the tolerance to adverse effects and indicate at population scale whether the individuals are long-term exposed to chemical pollution intense enough to randomly decimate almost the entire population, so that the population may suffer bottlenecks (Ribeiro and Lopes, 2013).

Due to the complexity and simultaneity of several evolutionary processes, it can be quite challenging to distinguish the causal link between the exposure to pollution and its outcomes on the genetic level in natural populations (Bickham, 2011; Medina et al., 2007). Hence, for more comprehensive investigations, integrative approaches must be developed in order to overcome these shortcomings. Some recommendations are to compare multiple populations on unpolluted and polluted areas (Belfiore and Anderson, 1998), to measure the presence and levels of pollutants in the environment, body burden of pollutants in appropriate sentinel species, and to deduce the emergent population effects by molecular genetics (Bickham, 2011).

Genetic markers such as allozymes, microsatellites and mitochondrial and nuclear DNA sequences can be used to investigate microevolutionary processes caused by chemical pollution. Amongst them, microsatellites emerged as one of the most popular choices because they have the potential to provide contemporary estimates of migration, and have enough resolving power to distinguish relatedness of individuals (Selkoe and Toonen, 2006). Microsatellites are basically tandem repeats of 1-6 nucleotides found in high frequency in nuclear genomes of most taxa. They can be amplified throughout polymerase chain reaction (PCR) and they are one of the few molecular markers that allow researchers insights into fine-scale questions (Selkoe and Toonen, 2006). Therefore, they are a promising molecular tool in order to investigate fine-scale changes due to chemical pollution in freshwater invertebrates.

1.7 *Gammarus pulex* as field model invertebrate population

Gammarus pulex is a widespread benthic freshwater macroinvertebrates (Ward, 1986), and an important species in European surface waters (Jażdżewski, 1980). *G. pulex* inhabits streams by lying beneath loose stones, rocks, leaves or wood. The organism is capable to swim or crawl on the ground and it is considered as a primary herbivore amphipod with a diet consisting of decomposed organic matter (Gee, 1988). Amphipods play a milestone role to litter breakdown and are an important source of food for higher trophic levels (e.g. fish) (Friberg et al., 1994). The lifespan is between 17 and 23 months for females and between 2 and 5 years for males (Welton and Clarke, 1980). Maturity is reached at a body length of approximately 6 mm, and adults can reach a size of about 12 mm (females) to 16 mm (males) (Welton and Clarke, 1980). In the laboratory, female *G. pulex* can produce between two to five and up to seven egg clusters during their life cycle; each cluster containing an average of 16 eggs (range 10-26) (Welton and Clarke, 1980), leading to fast population growth under favourable conditions. Furthermore, *G. pulex* can represent the dominant macroinvertebrate in terms of biomass (28%-38%) of a whole freshwater community (MacNeil et al., 1997).

The reproductive behaviour is characterised by a precopulatory guarding phase which plays a key role in the reproductive cycle. The female is carried beneath the male, and the pair swims together for few days until the female moults, and copulation takes place (Malbouisson et al., 1995). Following fertilization the pair separates and the fertilised eggs are carried brood pouch on the ventral side of the female until hatching. Therefore, eggs are in permanent contact with the water phase along their development. Previous studies showed that both precopulatory pairing (Malbouisson et al., 1995) and offspring production (Maltby and Naylor, 1990) may be disrupted by exposure to toxic chemicals.

Because of their feeding behaviour, *G. pulex* incorporates organic macropollutants that were not degraded by abiotic or biotic conditions (Ashauer et al., 2012; Gross-Sorokin et al., 2003; MacNeil et al., 1997). This may lead to adverse effects and consequently to populations constrains in the freshwater systems posing the risk that their ecological function is replaced in a long-term by another organism.

In comparison to other macroinvertebrates, *G. pulex* is known to be more sensitive to chemicals stressors. Therefore, it is frequently used in biomonitoring studies (Maltby et al., 2002), in laboratory toxicity studies (Ashauer et al., 2011), in microcosms experiments (van den Brink et al., 1995), molecular studies (Gergs et al., 2010; Xuereb et al., 2007) and toxicokinetic studies (Ashauer et al., 2006). Previous studies reported behaviour impairment due to exposure to organic micropollutants, for instance, changes in their feeding rate, locomotor activities, and increasing of their ventilation rates (De Lange et al., 2009). Furthermore, recovery and mortality tests performed in laboratory conditions determined that once *G. pulex* is exposed to a cocktail of pollutants their response decreased compared to single exposure (Ashauer et al., 2006). This can be expected in field conditions where macroinvertebrates are exposed to complex mixtures of chemicals together with others stressors.

1.8 Objectives and outline of the thesis

The overarching goal of environmental risk assessment is to quantify potential threats exerted by chemical and non-chemicals sources of stress on the physical surroundings (e.g. air, water, land, plants and wildlife) in order to protect the environment. The general scope of this thesis was to investigate whether anthropogenic pressures such as chemical and non-chemical stress affect the genetic variability of invertebrate populations. In order to carry out this main scope, a novel approach was developed based on evolutionary ecotoxicology and body burden analysis of organic micropollutants. Several questions were addressed in this study, which are mentioned as follow:

i. To which extent does a freshwater macroinvertebrate population, exposed to wastewater effluents and agricultural chemical, bioaccumulate organic micropollutants?
ii. To which extent do organic micropollutants alter primordial population genetics parameters such as genetic diversity and the structure of an exposed population of invertebrates?

iii. Are populations genetically eroded when they are exposed to micropollutants in a long-term scenario?
iv. Do mutagenic compounds, in a long-term scenario, increase the presence of unique alleles in the aquatic ecosystem?
v. Do evolutionary ecotoxicological approaches help as tools for environmental risk assessment?

In order to answer these questions, a multi-target screening method based on pulverised liquid extraction and a modified QuEChERS approach with an additional hexane phase was developed and optimized (CHAPTER 2). This method is capable to extract and quantify organic micropollutants of diverse chemical classes in freshwater invertebrates. The method was developed using *G. pulex* inhabiting the National Park Harz in Central Germany (Sachsen-Anhalt) and its applicability was tested in *Dikerogammarus spp.* inhabiting the highly anthropogenised River Danube. The River Danube is the second longest European river (after the River Volga). It originates in Germany and flows south-eastward for a distance of some 2,850 km passing through several Central and Eastern European capitals, before emptying into the Black Sea. The River Danube Basin is the second Europe's largest river basin, with a total area of 801,463 km^2. It is the world's most international river basin as it includes the territory of 10 countries.

Once the method was satisfactorily applied to the River Danube within the Joint Danube Survey 3 (Inostroza et al., 2016), the body burden analysis of organic micropollutants was applied in the River Holtemme (CHAPTER 3) in Central Germany (Sachsen-Anhalt). The River Holtemme is characterised principally by forest, semi-natural areas, agricultural landscapes and medium size cities. Hence, it was considered as a model river with a strong land use gradient on a short distance. A holistic multi-compartment approach was made, considering surface water, sediments and body burden in *G. pulex*, in order to investigate the occurrence, fate, chemical activity and baseline toxicity of organic micropollutants in this model freshwater system (CHAPTER 3).

Using molecular analysis, targeting microsatellites markers, several population genetics responses were assessed in the context of long-term exposure to organic micropollutants in the River Holtemme (CHAPTER 4). Combining traditional and Bayesian analysis plus the incorporation of body burden analysis, developed in CHAPTER 2, erosion hypothesis and population genetic structure were tested along the present land use gradient in the river system.

The CHAPTER 2 is published in the international peer-reviewed journal Environmental Pollution. The CHAPTER 3 is under preparation to be submitted in an international peer-reviewed journal and the CHAPTER 4 is published in the international peer-reviewed journal Environmental Science & Technology. Moreover, this thesis provides a synthesis of the main results related to the settled up research questions and, at last, potential further research challenges are addressed (CHAPTER 5).

CHAPTER 2

Internal concentration of pesticides and wastewater-derived pollutants on freshwater invertebrates: method development and application in the River Danube

ABSTRACT

While environmental risk assessment is typically based on exposure toxicant concentrations in water and/or sediment, awareness is increasing that internal concentrations or body burdens are the key to understand adverse effects in organisms. In order to link environmental micropollutants as causes to observed effects, there is an increasing demand for methods to analyse these chemicals in organisms. Here, a multi-target screening method based on pulverised liquid extraction (PuLE) and a modified QuEChERS approach with an additional hexane phase was developed. It is capable to extract and quantify organic micropollutants of diverse chemical classes in freshwater invertebrates. The method was tested on gammarids from the River Danube (within the Joint Danube Survey 3) and target compounds were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Furthermore, a nontarget screening using high resolution-tandem mass spectrometry (LC-HRMS/MS) was conducted. A total of 17 pollutants were detected and/or quantified in gammarids at low concentration. Pesticide concentrations ranged from 0.1 to 6.52 ng g^{-1} (wet weight) and wastewater-derived pollutants from 0.1 to 2.83 ng g^{-1} (wet weight). The presence of wastewater-derived pollutants was prominent along all spots sampled. Using non-target screening, it was successfully identify several chlorinated compounds. These results demonstrate for the first time the presence of pesticides and wastewater-derived pollutants in invertebrates of the River Danube.

Submitted in a slightly modified form as:

Inostroza, P.A., Anna-Jorina Wicht, Thomas Huber, Claudia Nagy, Werner Brack and Martin Krauss. Body burden of pesticides and wastewater-derived pollutants on freshwater invertebrates: Method development and applicability in the Danube River. Environmental Pollution 214(2016):77-85.

2.1 INTRODUCTION

Organic micropollutants such as pesticides, biocides, pharmaceuticals, personal-care products, or industrial chemicals are ubiquitous in the aquatic environment (Schwarzenbach et al., 2006). These synthetic compounds enter surface water bodies through various pathways including wastewater treatment plant effluents, untreated wastewater, urban runoff and leaching from agricultural lands. Depending on their hydrophobicity and volatility, these compounds partition between sediments, water and the atmosphere. Many of these chemicals may persist in the environment or may show a permanent exposure if losses by environmental transformation and degradation are continuously replaced by new emissions. If these chemicals are taken up by aquatic organisms and bind to biological receptors they may cause adverse effects and pose a risk to freshwater ecosystems (Beketov et al., 2013; Malaj et al., 2014).

While risk assessment is typically based on external toxicant concentrations in water and sediment, awareness is increasing that the internal chemical environment is the key to adverse effects in organisms (Escher and Hermens, 2002). The concept of the internal exposome has been set up for humans (Rappaport and Smith, 2010) but may be easily transferred to other organisms (Simon et al., 2013). In aquatic ecosystems, invertebrates play a key role in food webs and for ecosystem functions (e.g., litter degradation). They have relatively long life cycles and may integrate over environmental conditions, including contamination, for a longer time. Invertebrate communities represent one of the Biological Quality Elements (BQEs) according to the European Union Water Framework Directive (EU WFD) and they are extensively used as biological indicators to assess water quality (Birk and Hering, 2006; Metcalfe-Schmith, 1994). Macroinvertebrates are known to be highly sensitive to insecticides but may be also affected by a large range of contaminants. For linking environmental micropollutants as causes to observed effects, there is an increasing demand for methods to analyse these chemicals in the organism focusing on bioavailable and bio-accumulating pollutants. Hydrophobic organic chemicals (log K_{OW}>3) are typically accumulated in lipids in organisms. However, also interaction of less hydrophobic chemicals with proteins and other biomolecules and thus accumulation in biota tissues have been observed (Berlioz-Barbier et al., 2014). Thus, multi-target screening tools with a broad chemical domain are required to determine body burdens in macroinvertebrates.

Gammarids, a family of amphipods, are ubiquitous benthic macroinvertebrates in European inland water courses (Jażdżewski, 1980). They play a prominent function in the freshwater ecosystems breaking down coarse particulate organic matter and linking lower trophic levels to higher-level consumers as prey to fish (Friberg et al., 1994). They spend much of their life in contact with sediments, providing a continuous exposure to both hydrophilic water- and hydrophobic sediment contaminants (Ashauer et al., 2012; Tlili et al., 2012). Gammarids are expected to be optimal model organisms for body burden monitoring. They have already been used as model organisms for assessing both adverse effects (Cold and Forbes, 2004; Rasmussen et al., 2012) and uptake of organic micropollutants under

laboratory conditions (Ashauer et al., 2012, 2006; Gross-Sorokin et al., 2003; Miller et al., 2015).

Although numerous multi-target screening tools based on GC-MS and LC-MS are available for water and sediment samples (Hernández et al., 2011; Hug et al., 2014; Krauss et al., 2010) only few methods are available for screening in macroinvertebrates (Huerta et al., 2015; Miller et al., 2015; Tlili et al., 2012, Berlioz-Barbier et al., 2014, Tixier et al., 2003). A major challenge is sample preparation with sufficient recovery of a broad range of chemicals. Sample preparation is required not just to extract the desired substance from the tissue but also to remove the complex mixture of biological matrix compounds that might interfere with the analysis of the targeted pollutants in order to improve the sensitivity and accuracy of the analysis (Pan et al., 2014; Ribeiro et al., 2014). Liquid chromatography-high resolution mass spectrometry (LC-HRMS) offers the possibility to detect hundreds of polar contaminants in targeted approaches without pre-selection in full scan analysis. Furthermore, it allows the detection of known compounds suspected of being present in environmental samples (suspect screening) without reference standards, ever after measurement (post-target screening) and the screening for yet unknown non-target chemicals (Hernández et al., 2012, 2005; Krauss et al., 2010).

For biological environmental samples previous studies have utilized pressurized liquid extraction, soxhlet extraction and microwave-assisted extraction often followed by additional steps to remove matrix interferences prior to instrumental analysis. The vast majority of the analytical methods are based on time and/or solvent consuming procedures and they only targeted selected compounds or compound groups. It is imperative to develop more versatile methodological procedures, easily modifiable and able to overcome the shortcomings of the traditional methods. A new approach may be the use of QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) which presents several advantages such as higher recoveries for a wide polarity and volatility range of analytes, reduces the amount of sample used and may significantly save solvents, waste and time required for the analysis. QuEChERS has been successfully applied for preparation of a wide variety of samples, including food, plants, vegetables, fruits, soils and water samples. This method is based on a salting-out extraction with an organic solvent followed by dispersive solid phase extraction (dSPE) clean-up step. Since the development of this method, subsequent studies have adjusted and optimised the procedure according to the substance classes targeted and the complexity or characteristics of the matrices (Jia et al., 2012; Johnson, 2012; Lehotay et al., 2010; Norli et al., 2011; Plassmann et al., 2015). In the present study QuEChERS is adapted to and validated for invertebrate samples for a broad range of compounds.

Rigorous evaluation of novel analytical procedures under real world conditions, characterised by complex mixtures at often low internal concentrations, is key to propose them for monitoring purposes. The River Danube appears to be an optimal case to test tools for multi- and non-target screening of invertebrates since this river receives chemicals from a large range of pollution sources. Overall, concentrations may be seen as typical for large rivers instead of reflecting hot spots of contamination. In the Joint Danube Survey 3 (JDS3),

macroinvertebrates have been collected from several sites from the upper course of the River Danube in Austria down to the delta in Romania for analysing body burdens as one parameter that might explain changes in invertebrate communities. This allowed for testing the new analytical method for applicability under routine monitoring conditions.

Therefore, the objectives of this study were (i) to develop a method which allows to extract and quantify organic micropollutants of diverse chemical classes and physicochemical properties, (ii) to compare extraction and clean-up procedures for a subsequent analysis in liquid chromatography-tandem mass spectrometry (LC-MS/MS), (iii) to provide a suspect and non-target screening tool based on high-resolution (HR) MS full scan analysis for invertebrate analysis, and (iv) to apply the method on environmental samples from the Joint Danube Survey 3 (JDS3) to detect and quantify organic micropollutants in benthic macroinvertebrates.

2.2 METHODOLOGY

2.2.1 Reagents, chemicals and consumables

A list of 74 analytes with a wide range of properties (log *D* at pH 7 from -2.89 to 5.36) was selected for method development based on their occurrence in water samples and sediments (see Appendix Table A.1). Representatives belonged to different pollutant families such as pesticides, pharmaceuticals and other wastewater-derived pollutants and some of their main metabolites.

Methanol (gradient grade), acetonitrile (HPLC grade), acetone (HPLC grade), ethyl acetate (HPLC grade), sodium hydroxide (analytical grade), formic acid (analytical reagent grade, 98%), and sodium chloride (NaCl) were supplied by Sigma-Aldrich and primary secondary amine (PSA) by Agilent. Stock solutions (1 mg mL^{-1}) were prepared in methanol (MeOH) and stored in amber vials (40 mL) at -20°C in the dark.

2.2.2 Sample collection

Due to logistical reasons method development and validation were performed with the species *G. pulex*, which was obtained in frozen state from FiMö Aquaristik GmbH (Bünde, Germany) and stored at -20°C while method application in the River Danube used *Dikerogammarus* spp., an invasive species replacing *Gammarus* species in this river ecosystem (Dick and Platvoet, 2000). Both species are closely related and coexist in similar niche in freshwater ecosystems (Truhlar and Aldridge, 2015). Thus, no influence of the consideration of different species on body burden analysis is expected.

Gammarids of the species *Dikerogammarus* spp. for method application and evaluation were collected in 18 sampling spots from the River Danube along its watercourse as part of JDS3 in 2013 (Liška et al., 2015). *Dikerogammarus* spp. is an ubiquitous benthic macroinvertebrate in the Ponto-Caspian region of eastern Europe/Ukraine, particularly in the Danube River system (Jażdżewski, 1980; Nesemann et al., 1995), which has become invasive across the western part of the continent (Dick and Platvoet, 2000). Sampling techniques,

transportation and storing of benthic invertebrates are described in Umlauf et al., (2015). Samples were taken at sites JDS17, JDS19, JDS20, JDS24, JDS25, JDS31, JDS34, JDS36, JDS39, JDS43, JDS44, JDS45, JDS50, JDS52, JDS53, JDS59, JDS60 and JDS 61 (see Appendix Figure A.1).

Lipid content varies seasonally in gammarids (Gee, 1988) and body burden is expected to be correlated with lipid content. Therefore, individuals of different size structure were collected in order to avoid any bias related with higher bioaccumulation and exposure time. This approach was expected to provide a representative picture of the body burden of *Dikerogammarus* spp in the River Danube.

2.2.3 Gammarids spiking

Recovery experiments were performed by spiking 50 μL of a mixed standard solution of 1 μg mL^{-1} of each analyte in MeOH before homogenisation to obtain a final concentration of 100 ng mL^{-1} in vial (in the case of gammarids: 250 ng g^{-1} dry weight). For assessment of matrix effects, gammarids were spiked after clean-up but before analysis by LC-MS/MS with a final concentration of 250 ng g^{-1}. The calibration was carried out with samples spiked with the respective concentration in four replicates before homogenisation.

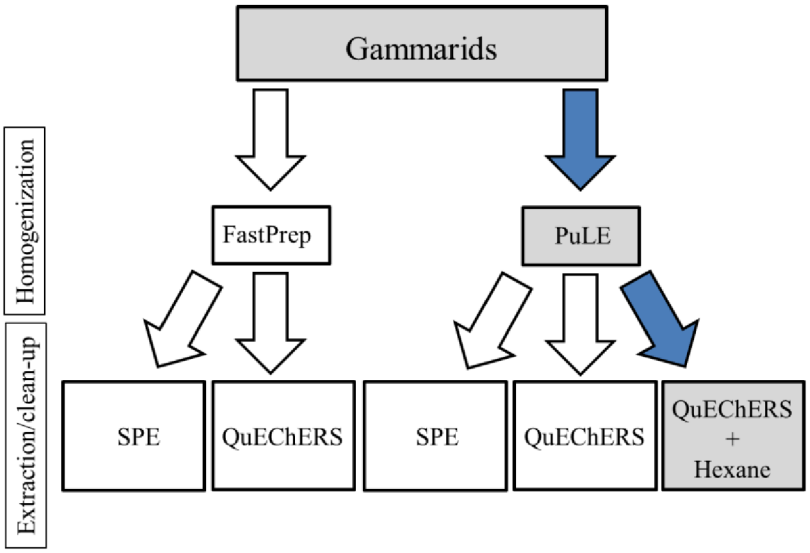

Figure 2. 1: Sample preparation scheme during method optimization. In grey colour the final procedure used both for method validation and application.

2.2.4 Sample extraction and clean-up

Homogenisation and clean-up procedures tested for gammarid tissue samples are summarized in Figure 2.1. Homogenisation by FastPrep®-24 (MP Biomedicals) was used as described by Grabicova et al. (2015). Briefly, 200 mg of freeze-dried gammarids were homogenised in 2 mL tubes with 1 mL of acetonitrile for 10 minutes. Samples were then centrifuged (4,000×*g* for 10 minutes) and filtered with a PTFE syringe filter (pore size 0.45

μm, Chromafil®, Macherey-Nagel). The supernatant was frozen at -20°C for 24 hours and then centrifuged again to remove precipitated proteins and other solid particles from the samples. A 100 μL aliquot was transferred to an autosampler vial for analysis. Pulverised liquid extraction (PuLE) (Miller et al., 2015) was performed using an Ultra-Turrax® T-25 (IKA, Staufen). A mass of 900 mg of thawed gammarids was placed in glass tubes containing 4 mL of acetonitrile: water (1:1 *v/v*), homogenised for 60 seconds and vortexed for another 60 seconds.

Solid phase extraction (SPE) was performed with a vacuum manifold (J.T. Baker) with Oasis HLB 6cc (200 mg) cartridges. Prior to use, cartridges were conditioned with 6 mL of MeOH and 6 mL of bidistilled water. Both FastPrep and PuLE extracts were dissolved in 5 mL of acetonitrile and 4.5 mL of the supernatant were transferred into a glass flask containing 95.5 mL of ammonium acetate (10 mM) and passed through the cartridge at a flow rate of 1 mL min^{-1}. Cartridges were dried for 30 minutes by a nitrogen stream. The analytes were eluted with 10 mL of ethyl acetate: acetone (1:1 *v/v*). All purified extracts were evaporated to dryness under a nitrogen stream at room temperature and the residue was reconstituted in 500 μL of MeOH and filtered with a PTFE syringe filter (pore size 0.45 μm, Chromafil®).

The clean-up using QuEChERS followed in general the method developed by Anastassiades et al. (2003). Briefly, 4 mL of gammarids homogenate in acetonitrile were thoroughly mixed with 800 mg of anhydrous $MgSO_4$ and 200 mg of NaCl. To avoid agglomerations of salts, the mixture was immediately shaken for 1 minute using a vortex mixer and centrifuged at 4,000×*g* per 5 minutes. An additionally clean-up step by dispersive SPE (dSPE) was tested for possible improvement of analytical performance. To this end, aliquots of 3.5 mL of the supernatant were transferred to glass centrifuge tubes containing 50 mg of primary-secondary amine (PSA) and 400 mg of anhydrous $MgSO_4$. The tubes were vortexed for 60 seconds, centrifuged at 4,000×*g* for 5 minutes and the supernatant was concentrated under a nitrogen stream at room temperature to dryness. Finally, the residues were reconstituted in 500 μL of MeOH and filtered with a PTFE syringe filter (pore size 0.45 μm, Chromafil®). The second clean-up step tested was the addition of 1 mL of hexane during the QuEChERS LLE step for the removal of lipids. The hexane fraction was located above the acetonitrile fraction due to its lower density and it was separated using a glass pipette.

2.2.5 LC-MS/MS analysis

For LC-MS/MS analysis, a 1260 Infinity LC system (Agilent) coupled to a QTrap 6500 MS (ABSciex) with a Turbo V ion source was used. A Poroshell 120 C18 column (50×4.6 mm, 2.7 μm particle size, Agilent) was installed between the pump and the auto sampler in order to trap background contaminants from the eluents, degasser or pump. Aliquots of 10 μL of extract were injected onto a Kinetex C18 column (50×3.0 mm, 2.6 μm particle size, Phenomenex). Analytes were separated by gradient elution at a flow rate of 0.4 mL/min using LC-MS grade water (A) and LC-MS grade MeOH (B), both containing 0.1% of formic acid. The initial content of 5% B was held for 1 minute and increased to 95% B over 5.2 minutes. After 11.4 minutes with 95% B, the column was re-equilibrated for 5

minutes to the initial composition. Following electrospray ionization (ESI), the QTrap instrument was operated in scheduled multiple reactions monitoring (sMRM) mode switching between positive and negative ionization. MRM transitions for the analytes are given in Appendix Table A.1.

For quantification of samples, matrix matched calibration standards were prepared from the commercially obtained *G. pulex* by adding a mixed analyte standard to final concentrations levels of 0.1, 0.2, 2, 4, 10 and 20 ng ml^{-1} in vial to the homogenate and processing these with the same procedure as the samples. For peak integration, compound calibration and quantification the software MultiQuant 3.0 (ABSciex) was used.

2.2.6 LC-HRMS non-target screening

To test the applicability of the sample preparation method for subsequent non-target screening analysis, a small-scale pilot study was conducted with the JDS3 samples aiming at the detection of chlorinated or brominated compounds, as these are likely contaminants of anthropogenic origin and an automated detection based on the characteristic isotope patterns is feasible. To this end, extracts were analysed by LC-HRMS using a Thermo Ultimate 3000 LC system (consisting of a ternary pump, autosampler and column oven) coupled to a quadrupole-orbitrap instrument (Thermo QExactive Plus) via a heated electrospray ionisation source. Samples were analysed in full scan mode (100-1000 m/z) at a nominal resolving power of 140,000 (referenced to m/z 200) in positive ion mode. Additional analytical runs were made to obtain HRMS/MS spectra for peaks of interest from selected samples using data-dependent MS/MS acquisition.

LC separation was done on a Kinetex C18 EVO core-se column (50×2.1 mm, 2.6 μm particle size) using a gradient elution with 0.1% of formic acid (eluent A) and methanol containing 0.1% of formic acid (eluent B) at a flow rate of 300 μL/min. After 1 minute of 5% B, the fraction of B was linearly increased to 100% in 12 minutes and 100% of B were kept for 11 minutes. The eluent flow was diverted to waste and the column was rinsed for 2 minutes using a mixture of isopropanol+acetone 50:50 / eluent B / eluent A (85% / 10% / 5%) to remove hydrophobic matrix constituents from the column. Finally, the column was re-equilibrated to initial conditions for 5.7 minutes. The injection volume was 5 μL and the column was operated at 40°C. The heated ESI source and the transfer capillary were both operated at 300°C, the spray voltage was 3.8 *k*V (pos mode) or 3.5 *k*V (neg. mode), the sheath gas flow rate was 45 a.u. and the auxiliary gas flow rate 1 a.u. To obtain HRMS/MS spectra of peaks of interests, full scan acquisition (resolving power 70,000) was combined with data-dependent acquisition (resolving power 35,000) after HCD fragmentation at different collision energies for the six most intense ions from an inclusion mass list containing the ion masses of the peaks of interest. A precursor isolation window of 1.3 m/z was used. LC and other MS settings were the same as described above.

For non-targeted data evaluation, HRMS full scan chromatograms were converted from profile to centroid mode using ProteoWizard (Kessner et al., 2008) and peak detection was done using MZmine 2.17 (Pluskal et al., 2010). Details on the workflow and settings are

given in the Appendix (Table A.2). From the peak lists peaks with an intensity lower than 10 times that of a blank sample were removed using an R script and further processed using the R package "nontarget" (Loos, 2012; Schymanski et al., 2014). Peaks were finally grouped into components, i.e., the monoisotopic peak and its associated isotope or adduct peaks representing an individual chemical compound. Details on the settings are given in the Appendix Table A.3.

For peaks the non-target package assigned the possibility of a Cl or Br isotope pattern, the original raw data file was re-visited using the Xcalibur QualBrowser software for a visual inspection of the isotope pattern and calculation molecular formulas and simulation of the theoretical isotope pattern for plausible molecular formulas. For molecular formulas showing a good match (measured isotopologue intensities within 10% of theoretically predicted ones), the Chemspider compound database (Royal Society of Chemistry) was searched for possible candidate structures.

2.2.7 Method validation

Validation of the selected method combining homogenisation with Ultra-Turrax and QuEChERS with an additional hexane phase clean-up was focused on the assessment of method quantification limits and repeatability. Linearity of calibration curves were determined by measuring the peak area at six concentrations levels from 0.1 to 20 ng mL^{-1} (four replicates for each compound).

For estimating method quantification limits (MQLs), extracts of spiked gammarids were four times injected measuring peak areas as described previously (Wells et al., 2011). MQL was calculated using the following equation:

$$MQL_i = \frac{IDL_i \quad C_n}{\overline{X}_i} \tag{2.1}$$

Where IDL_i is the instrument detection limit for compound i, C_n is the nominal concentration, and $\overline{X}_i$ is mean peak areas of i. With $S_{\bar{x}_i}$ representing the standard deviation of the mean of peak areas of i and $t_{(n-1,1-\alpha)}$ being the value chosen from t-table for four replicates at a 99% confidence interval and n indicating the number of replicates.

$$IDL_i = t_{(n-1,1-\alpha)} S_{\bar{x}_i} \tag{2.2}$$

Absolute recoveries were calculated by comparing spiked *G. pulex* before homogenisation at 1 μg mL^{-1} (equivalent to 4.4 mg g^{-1} wet weight; four replicates) in relation to the signal of the analyte in solvent. The absolute recovery involves losses during extraction and clean-up as well as the effect of ion suppression in the ESI source. In addition, relative recoveries of the clean-up procedure were determined by comparing peak areas of gammarid matrix spiked before and after extraction/clean-up (four replicates).

Repeatability of the measurement (expressed as RSD, %) was calculated from four samples (three environmental samples and one concentration of the calibration curve at 10 ng mL^{-1}). Because the assessment of matrix effect (ME) has become imperative for the favourable outcome of many analytical methods, this was calculated through signal intensity measurement.

$$\mathrm{ME} = \left(\frac{\text{peak area}_{(\text{fortified extract})}}{\text{peak area}_{(\text{solvent standard})}} - 1 \right) \times 100\% \quad (2.3)$$

A value of ME=0% means that no matrix effect occurred. Negative values represent suppressions of the analyte signal, and positive values stand for enhancements induced by matrix.

Statistical analyses were conducted using R (R Development Core Team, 2008). For calculation of compound properties, we used the Calculator Plugins of JChem, version 15.8 (Chemaxon, Budapest, Hungary).

2.3 RESULTS

2.3.1 Optimisation of the extraction and clean-up method

Comparing the different homogenisation and clean-up procedures involving FastPrepP, PuLE, QuEChERS, SPE and hexane clean up, the combination of PuLE and QuEChERS with an additional hexane phase clean-up (PuLE+QuEChERS+Hexane) offered the best and most robust performance among the different tested procedures in this study. The selected method showed significantly higher absolute recoveries amongst the tested procedures (Appendix Figure A.2, and Table A.4 for multiple comparisons). With regarding to relative recoveries, the selected method showed higher performance only compared to PuLE+SPE (Figure 2.2A, for details see Appendix Table A.5). Moreover, the selected method presented the lowest matrix effect (Figure 2.2B, for details see Appendix Table A.5). Comparisons at class level were carried out (e.g. insecticides, herbicides, fungicides and wastewater-derived chemicals) and in general significant higher performance were achieved for fungicide, herbicide and wastewater-derived pollutants. With respect to insecticides the selected method presented significant higher performance only over FastPrep, PuLE+SPE in this study.

The available sample size is usually a limiting factor for analysis. Thus, the impact of sample size on the analysis of invertebrates was evaluated using samples of 10, 30, 50, 75 and 100 individuals (corresponding to 0.3, 0.9, 1.5, 2.25 and 3 grams wet weight, respectively). A compromise avoiding extensive matrix effects, safeguarding sufficiently large recovery and detecting most compounds with acceptable detection limits was reached in spiked gammarids with 30 individuals (~900 mg wet weight per 500 μL of final extract, Figure 2.3, see Appendix Table A.6). Depending on the amount of extract required for

analysis, a downscaling for a smaller number of individuals is still possible (e.g., 100 μL extract from 6 individuals).

In summary, the investigation of different extraction procedures led to the selection of an extraction protocol based on pulverised liquid extraction using an Ultra-Turrax dispenser (60 seconds) of 900 mg (wet weight) of thawed sample in 4 mL acetonitrile: water (1:1 *v/v*) and 1 mL hexane. Extracts (4 mL) were mixed with 800 mg of anhydrous $MgSO_4$ and 200 mg of NaCl; pH was adjusted to 7 and samples were centrifuged at 4,000×*g* for 60 seconds. Aliquot of 3.5 mL of acetonitrile phase were transferred to dSPE which contained 50 mg of PSA and 400 mg of anhydrous $MgSO_4$ were added and further mixture was vortexed for 60 seconds and afterwards centrifuged at 4,000×*g* for 5 minutes. Finally, the residues were reconstituted in 500 μL of MeOH and filtered with a PTFE syringe filter (pores 0.45 μm, Chromafil®). Purified extracted were analysed by LC-MS/MS and additionally by LC-HRMS.

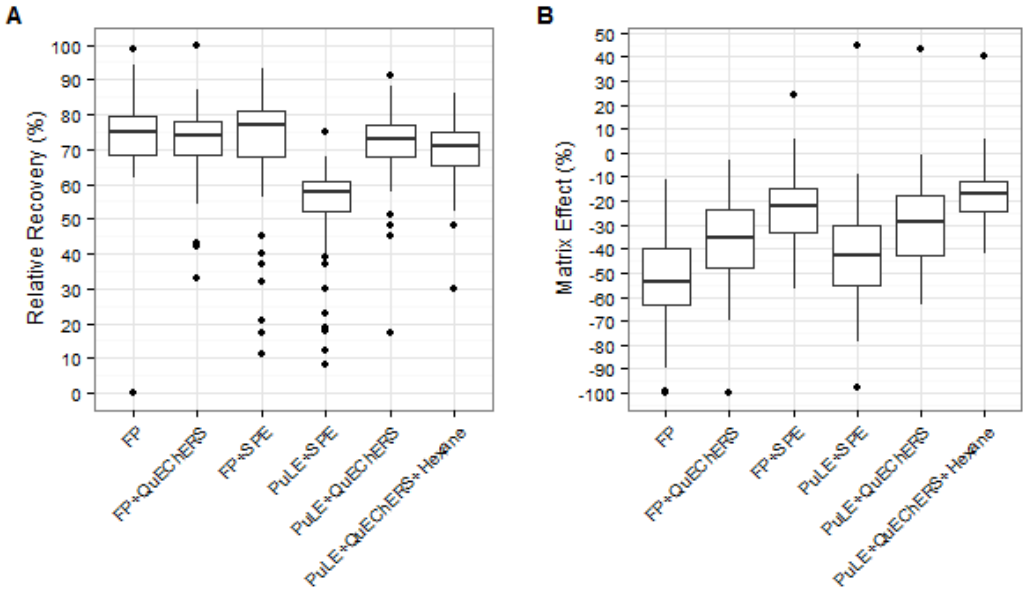

Figure 2.2: (A) Relative recoveries including matrix effect of the different procedures tested. Range of recoveries represented through box-and-whisker plot for all compounds. (B) Matrix effect for different extraction procedures. Different homogenisation, extraction and clean-up procedures are summarised as follow: FP represents FastPrep; QuEChERS represents Quick, Easy, Cheap, Effective, Rugged and Safe; and PuLE represents pulverised liquid extraction.

Method quantification limits (MQLs) for organic micropollutants in *G. pulex* as well as recoveries are presented in Table 2.1. MQLs were in the range of 0.01-2.13 ng g^{-1} wet weight. Calibration curves were generated using linear regression analysis ($R^2 \geq 0.92$) in the concentration range of 0.1-20 ng mL^{-1}. The overall method repeatability indicated by RSD ranged from 1.3% to 21% with an average of 5.8% (±5.2% of standard deviation), indicating rather good repeatability. Contamination of the purchased matrix used for matrix-matched calibration was assessed. In general the use of this kind of presumably uncontaminated matrix is suitable nevertheless concentrations of caffeine carbamazepine and 1H-benzotriazole were detected and ranged from 0.13 to 5.83 ng g^{-1} wet weigh (Appendix Table A.7).

2.3.2 Internal concentration in gammarids from the Danube River

A total of 17 chemicals out of the 74 targeted analytes could be detected or quantified in *Dikerogammarus* spp. samples collected along the River Danube (Table 2.2). Concentration ranges were 0.1-0.53 ng g^{-1} wet weight for insecticides, 0.21-6.52 ng g^{-1} wet weight for fungicides, 0.19-4.17 ng g^{-1} wet weight for herbicides and 0.1-2.83 ng g^{-1} wet weight for wastewater-derived pollutants. The lowest concentrations were calculated for thiacloprid and 5-methyl-1H-benzotriazole (5MBT) at 0.1 ng g^{-1} wet weight and the highest for the herbicide fenuron at 6.52 ng g^{-1} wet weight.

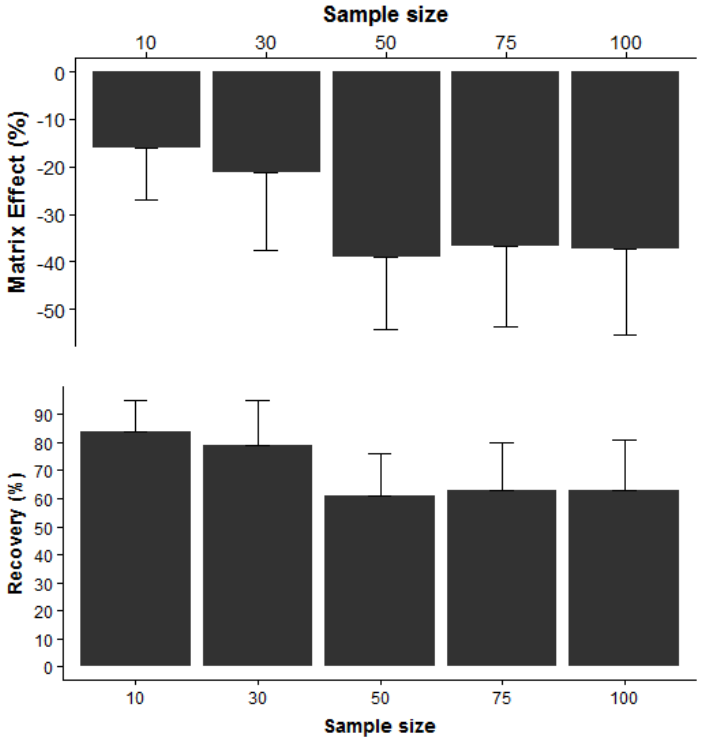

Figure 2.3: Top bar plot represents mean matrix effects values per sample size (number of individuals). Bottom bar plot represents mean absolute recovery per sample size (number of individuals) for PuLE+QuEChERS+Hexane procedure.

Wastewater-derived pollutants were most frequently detected in sixteen out of eighteen sampling sites, but only quantifiable at twelve sites (Table 2.2). The internal concentrations of these compounds in gammarids samples were below 10 ng g^{-1} wet weight for 1H-benzotriazole and ranged from 0.10 to 0.58 ng g^{-1} wet weight for 5MBT. Carbamazepine (CBZ), a seizure disorders and neuropathic pain drug, was detected below the quantification limit (<0.15 ng g^{-1}) in gammarids from the River Danube. N,N-diethyl-m-toluamide (DEET) was detected in a concentration range of 0.20 to 2.82 ng g^{-1} in gammarids from the River Danube (Table 2.2).

Regarding pesticides, in this study, herbicides fenuron and metolachlor were determined at 0.21-6.52 ng g^{-1} wet weight and at 0.29 ng g^{-1} wet weight in gammarids samples, respectively (Table 2.2). The organophosphate diazinon and the neocicotinoid

thiacloprid were detected in *Dikerogammarus* spp. tissues in the range of 0.10 to 0.53 ng g^{-1} wet weight (see Table 2.2 for details). Few triazole fungicides were detected in *Dikerogammarus* spp (Table 2.2). Most of them were below the quantification limits with the exception of difenoconazole which occurred in one site (JDS36) at low concentration (0.19 ng g^{-1} wet weight), flusilazole (0.57 and 0.67 ng g^{-1} wet weight) in JDS34 and JDS17 respectively, and propiconazole at 1.05-4.17 ng g^{-1} wet weight in three sites of the River Danube.

Table 2. 1: Method performance features for *G. pulex* analysis. Absolute recoveries (%), method quantification limit (MQL) in ng g^{-1} wet weight, log K_{OW} values and log *D* at pH 7 for each compound.

Chemicals	**Recovery**	**MQL**	**log K_{OW}**	**log *D***
1H-Benzotriazole	56 (±18)	2.13	1.44	1.49
5-Methyl-1H-benzotriazole	50 (±11)	0.01	1.70	1.96
10,11-Dihydroxydihydrocarbamazepine	71 (±26)	0.63	-0.21	0.85
Acetamiprid	56 (±10)	0.58	0.80	1.65
Atrazine	72 (±11)	0.62	2.61	2.06
Azoxystrobin	127 (±13)	0.06	2.50	4.64
Bentazone	93 (±13)	0.04	2.80	-0.27
Boscalid	55 (±9)	0.68	2.96	4.88
Caffeine	69 (±12)	0.01	-0.07	-0.79
Carbamazepine	69 (±9)	0.15	2.45	3.22
Chloridazone	48 (±6)	0.78	1.14	1.46
Chlorotoluron	69 (±8)	0.04	2.41	2.58
Chloroxuron	88 (±8)	0.02	3.70	3.54
Clomazone	78 (±9)	0.40	2.50	3.14
Clothianidin	62 (±8)	0,56	0.70	-2.75
DEET	77 (±10)	0.03	2.02	2.34
Deisopropylatrazin	54 (±18)	0.46	1.36	0.95
Desethylatrazine	57 (±12)	0.04	1.51	1.36
Desethylterbutylazine	62 (±12)	0.12	2.23	1.44
Diazinon	47 (±14)	0.02	3.81	4.50
Difenoconazole	75 (±13)	0.01	4.40	4.57
Diflufenican	47 (±9)	0.39	4.90	5.08
Dimethoate	62 (±6)	0.62	0.78	0.56
Diuron	70 (±9)	0.38	2.68	2.63
Epoxiconazole	62 (±10)	0.55	3.58	3.26
Fenuron	73 (±9)	0.06	0.96	1.59
Flufenacet	67 (±11)	0.63	3.20	2.86
Flurtamone	86 (±7)	0.15	2.87	5.00
Flusilazole	62 (±8)	0.13	3.81	4.68
Imidacloprid	70 (±10)	0.61	0.57	-2.89
Irgarol	48 (±8)	0.46	4.07	2.64
Isoproturon	71 (± 6)	0.05	2.87	2.79
Lenacil	59 (±9)	0.81	3.09	1.61
Linuron	61 (±7)	0.97	3.20	2.90
Metamitron	62 (±12)	0.75	0.83	0.21

Chemicals	**Recovery**	**MQL**	**log K_{OW}**	**log D**
Metazachlor	80 (±10)	0.78	2.49	3.18
Metolachlor	59 (±13)	0.03	3.13	3.31
n-Acetyl-4-aminoantipyrine	63 (±11)	0.39	-0.13	0.42
Pendimethaline	48 (±14)	0.48	5.20	4.80
Pethoxamid	64 (±13)	0.51	3.39	2.32
Picoxystrobin	48 (±12)	1.60	3.67	4.90
Pirimicarb	59 (±10)	0.02	1.70	1.91
Prochloraz	74 (±12)	0.20	4.38	3.48
Prometryn	58 (±12)	0.03	3.51	2.92
Propiconazole	62 (±13)	0.02	3.72	4.01
Prothioconazole-desthio	66 (±7)	0.80	3.05	1.96
Pyraclostrobin	50 (±8)	0.02	3.99	4.73
Simazine	58 (±11)	0.05	2.18	1.65
Spiroxamine	22 (±10)	0.05	5.51	2.07
Sulfamethazine	55 (±11)	0.93	0.14	0.44
Tebuconazole	57 (±11)	0.55	3.70	3.24
Terbutryn	61 (±8)	0.65	3.74	2.58
Terbutylazine	55 (±10)	0.59	3.40	2.14
Thiabendazole	59 (±9)	0.03	2.47	2.19
Thiacloprid	54 (±10)	0.01	1.26	2.51
Thiamethoxam	76 (±6)	0.55	-0.13	3.05
Triethyl-citrate	80 (±16)	0.13	0.71	-0.40
Trifloxystrobin	48 (±9)	0.01	4.50	5.36

Table 2.2: Detected organic micropollutants in *Dikerogammarus* spp. tissues from JDS3 (concentrations in ng g^{-1} wet weight).

	MDL	**17**	**19**	**20**	**24**	**25**	**31**	**34**	**36**	**39**	**43**	**44**	**45**	**50**	**52**	**53**	**59**	**60**	**61**
Insecticides																			
Diazinon	0.02				0.53														
Thiacloprid	0.01	0.10	0.39	0.29	0.32	0.10	+		0.27	0.10	+	+		0.10					
Fungicides																			
Difenoconazole	0.01								0.19										
Epoxiconazole	0.55										+	+							
Flusilazole	0.13	0.63	+	+	+	+	+	0.57	+	+	+	+	+	+	+	+	+	+	+
Spiroxamine	0.05				+														
Tebuconazole	0.55															+			
Propiconazole	0.02	1.25									1.35	4.17					1.05		
Herbicicdes																			
Desethylterbutylazine	0.12	+	+	+	+														
Fenuron	0.06	0.77	0.34	0.22	0.32	0.62	0.96	6.52	0.38	0.21	0.25		+	0.96	+		0.39		
Metolachlor	0.03	+	+	+	+	+	+	0.29	+				+	+		+	+		
Terbutryn	0.65	+																	
Terbutylazine	0.59	+	+	+						+	+	+	+	+	+	+	+	+	+
Wastewater chemicals																			
Carbamazepine	0.15								+	+	+			+	+	+	+	+	+
DEET	0.03	0.81		0.33	0.66		1.04		+			1.55		2.83		0.61	0.20	0.50	
1H-Benzotriazole	2.13								+			+							
5MBT	0.01	0.27	0.18	0.10	0.34	0.15	+		0.54	0.12	0.10	0.58	0.13	0.45	0.28		0.42	0.40	

+ Compound detected, but below the method quantification limit.

Body burden were compared with water concentrations from the JDS3 survey in order to obtain a better understanding of the dynamic of pollutants among biota and water compartment under real environmental conditions. Nonetheless, few compounds (e.g., metolachlor, DEET and 5MBT) were quantified both in biota and water samples. Most of the pollutants were not detected or below the quantification limit in water while detectable in biota. The present study shows that chemicals with log K_{OW} from 1 to 6 can be detected in aquatic invertebrates and it was not observe a higher frequency of hydrophobic chemicals in gammarids tissues.

2.3.3 LC-HRMS nontarget screening

In the gammarid samples from JDS3 analysed in positive ESI mode, on average 16,061 peaks (range 14,506-17,558) above a threshold of 50,000 a.u. intensity could be detected using the MZmine software (Table 2.3). Based on adduct and isotope search of the "nontarget" package, these peaks could be grouped into 12,310 components on average (range 11,091-13,888), which likely represent individual compounds showing one or more ions (i.e., monoisotopic, isotope, and adduct ions).

Table 2.3: Mean values and ranges of total peaks and component numbers, and components containing likely Cl or Br based on the isotope assignment by the "nontarget" package for the JDS3 samples. For ^{37}Cl and ^{81}Br isotopes, either unequivocal assignments or those to more than one possible isotopologue (potential assignment) are given. For values of individual samples see Table 2.5.

	Mean	**Range**
Total number of peaks	16,061	14,506-17,558
Number of components		
Total	12,310	11,091-13,888
potential ^{37}Cl isotopes	168	62-286
unequivocal ^{37}Cl isotope	106	47-168
potential ^{81}Br isotopes	41	15-73
unequivocal ^{81}Br isotopes	7	2-10
Number of components with z=1 and m/z < 450		
Total	14	5-35
^{37}Cl	7	2-14
^{81}Br	2	0-4
^{37}Cl or ^{81}Br	5	1-23
^{37}Cl or ^{34}S	0	0-1

As it was laid the focus of non-target screening on the detection of chlorinated or brominated compounds in this study, the rule-based isotope assignment of the "nontarget" package was used. It has to be noted that the non-target package does only consider relative intensities of the M+1 and M+2 isotopologues, which means that the type of isotope cannot

Table 2.4: Total peaks and component numbers, and components containing likely Cl or Br based on the isotope assignment by the "nontarget" package for the JDS3 samples. For ^{37}Cl and ^{81}Br isotopes, either unequivocal assignments or those to more than one possible isotopologue (potential assignment) are given.

	JDS17	**JDS19**	**JDS20**	**JDS24**	**JDS25**	**JDS34**	**JDS36**	**JDS39**	**JDS43**	**JDS44**	**JDS45**	**JDS50**	**JDS52**	**JDS53**	**JDS59**	**JDS61**
Total number of peaks	15668	15457	15559	15008	16720	17002	16033	15984	15690	17262	15520	17558	14506	15964	16161	16882
Number of components																
Total	11837	11488	11794	11091	12669	12810	12149	11803	12213	13528	11841	13888	11269	12454	12839	13286
potential ^{37}Cl isotopes	286	227	178	270	184	67	132	208	62	198	210	72	118	180	92	204
unequivocal ^{37}Cl isotope	166	143	107	168	121	47	84	126	47	132	131	47	79	108	47	135
potential ^{81}Br isotopes	73	43	50	65	40	15	29	49	15	41	43	26	21	49	46	48
unequivocal ^{81}Br isotopes	10	5	4	4	9	3	8	6	2	7	8	8	4	10	8	8
Number of components with z=1 and m/z < 450																
Total	26	14	11	15	6	13	10	7	9	16	18	11	11	14	35	5
^{37}Cl	14	8	5	9	5	9	7	5	4	4	8	8	6	6	7	2
^{81}Br	2	2	3	2	0	2	1	0	0	2	2	0	1	1	4	2
^{37}Cl or ^{81}Br	9	4	3	4	1	2	2	2	5	10	8	3	4	6	23	1
^{37}Cl or ^{34}S	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0

Table 2.5: List of non-target peaks of interest (Cl/Br containing, charge 1, m/z 450) in sample JDS34. Isotope assignments were taken from detection by the nontarget package.

m/z	RT (min)	Intensity (a.u.)	Isotopes assigned"	Molecular formula	Mass deviation (ppm)	Chemspider # of hits	Identity
356.1578	0.8	2.28×10^7	^{13}C and ^{37}Cl	nd[a,b]	-	nd	unknown
256.1095	10.6	9.23×10^6	^{37}Cl	$C_{13}H_{18}O_2NCl+H^+$	-1.4	1598	Dimethachlor (confirmed)
276.0816	11.2	3.55×10^6	^{37}Cl	$C_{12}H_{18}O_2NClS+H^+$	-1.0	281	Dimethenamid (confirmed)
212.0835	8.9	2.30×10^6	^{37}Cl	$C_{11}H_{14}ONCl+H^+$	-1.1	909	Unknown; candidate compound propachlor rejected (RT 10.2 min)
408.0951	9.8	8.77×10^5	^{37}Cl	$C_{16}H_{20}O_3N_3ClF_2S+H^+$	-1.1	1	unknown; Chemspider hit not likely[c]
233.0503	1.1	6.63×10^5	^{81}Br	nd, no Br	-	nd	unknown
302.1292	8.3	5.18×10^5	^{81}Br	nd, no Br	-	nd	unknown
284.0276	8.8	4.77×10^5	^{37}Cl	$C_{12}H_{14}O_2NBr+H^+$	-1.3	716	unknown
306.9426	9.2	4.23×10^5	^{37}Cl or ^{81}Br	$C_6H_{12}O_4Cl_3P+Na^+$	-1.3	5	Tris(2-chloroethyl)-phosphate (confirmed)
216.0714	1.1	3.97×10^5	$^{37}Clor$ ^{81}Br	nd, no Cl/Br	-	nd	unknown
401.1464	8.7	3.60×10^5	^{37}Cl	nd, no Cl	-	nd	unknown
440.1609	18.4	2.14×10^5	^{37}Cl	nd, no Cl	-	nd	unknown
433.2861	23.7	1.66×10^5	^{37}Cl	nd, no Cl	-	nd	unknown

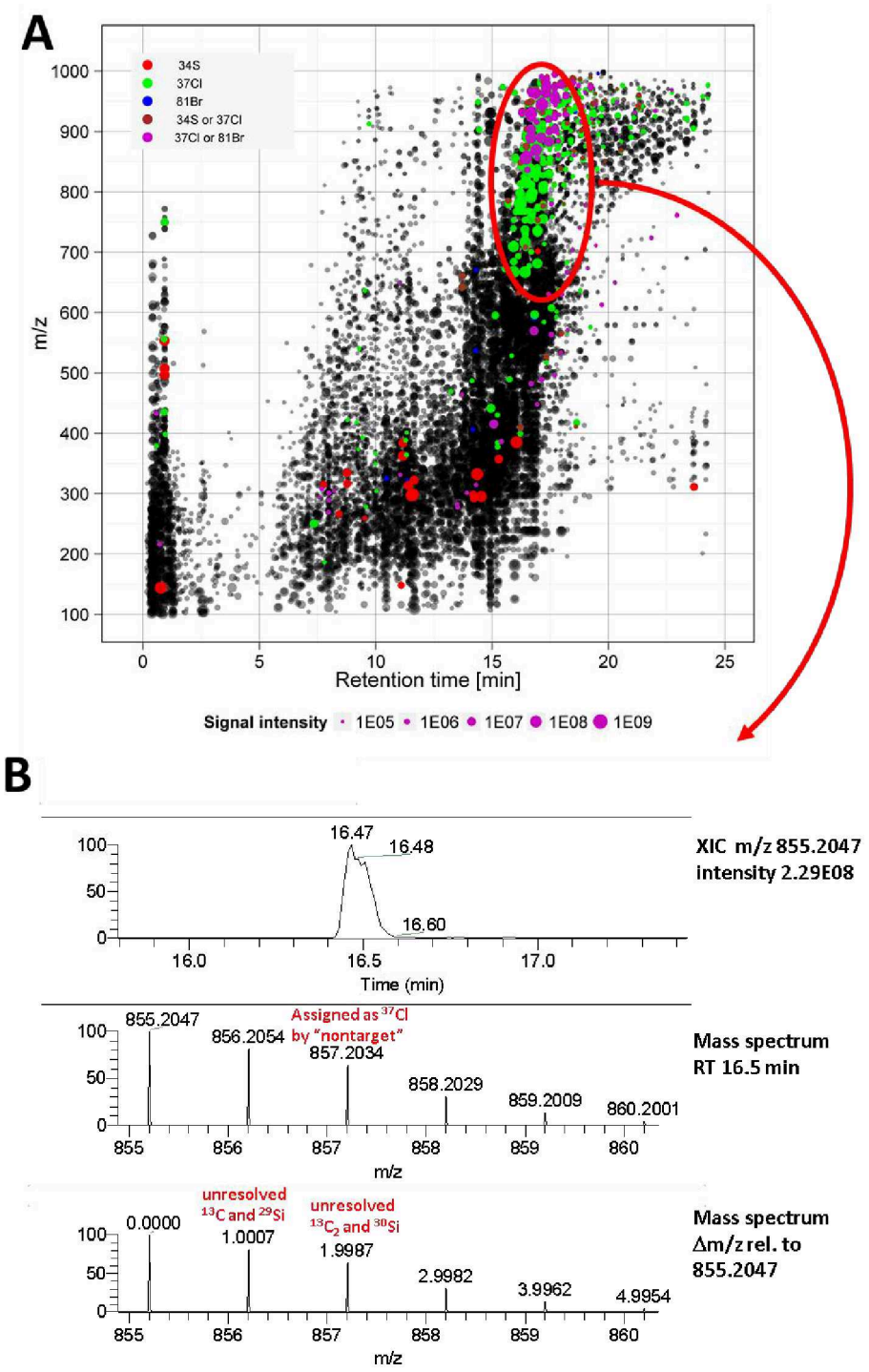

Figure 2.4: (A) m/z vs. retention time plot for sample JDS17 (positive mode) showing signal intensities as dot sizes and highlighting particular isotope signals. (B) One example for a detected compound from the m/z range 500-100 and retention time range 15-20 minutes for which a 37Cl isotope peak was assigned by the "nontarget" package, but which is likely stemming from an unresolved mixture of 13C and Si isotopes. The actual resolving power of the MS at m/z 850 is about 75,000, which does not allow distinguishing the different isotopologues.

be unequivocally assigned if isotopologues cannot be resolved by the MS and relative intensities can be explained by different isotopologues (e.g., a $^{32}S_6{}^{34}S_1$ isotopologue at 31.4%

relative intensity and a Δm/z of 1.9958 to the monoisotopic ion cannot be distinguished from a $^{37}Cl_1$ isotopologue at 31.9% relative intensity and a Δm/z of 1.9970 at a resolving power of 140,000 at the given spectral accuracy). Thus, many peaks were assigned to contain either S, Cl or Br (or two of these) based on the M+2 isotope peak intensity, and a smaller number could be unequivocally assigned (Table 2.4).

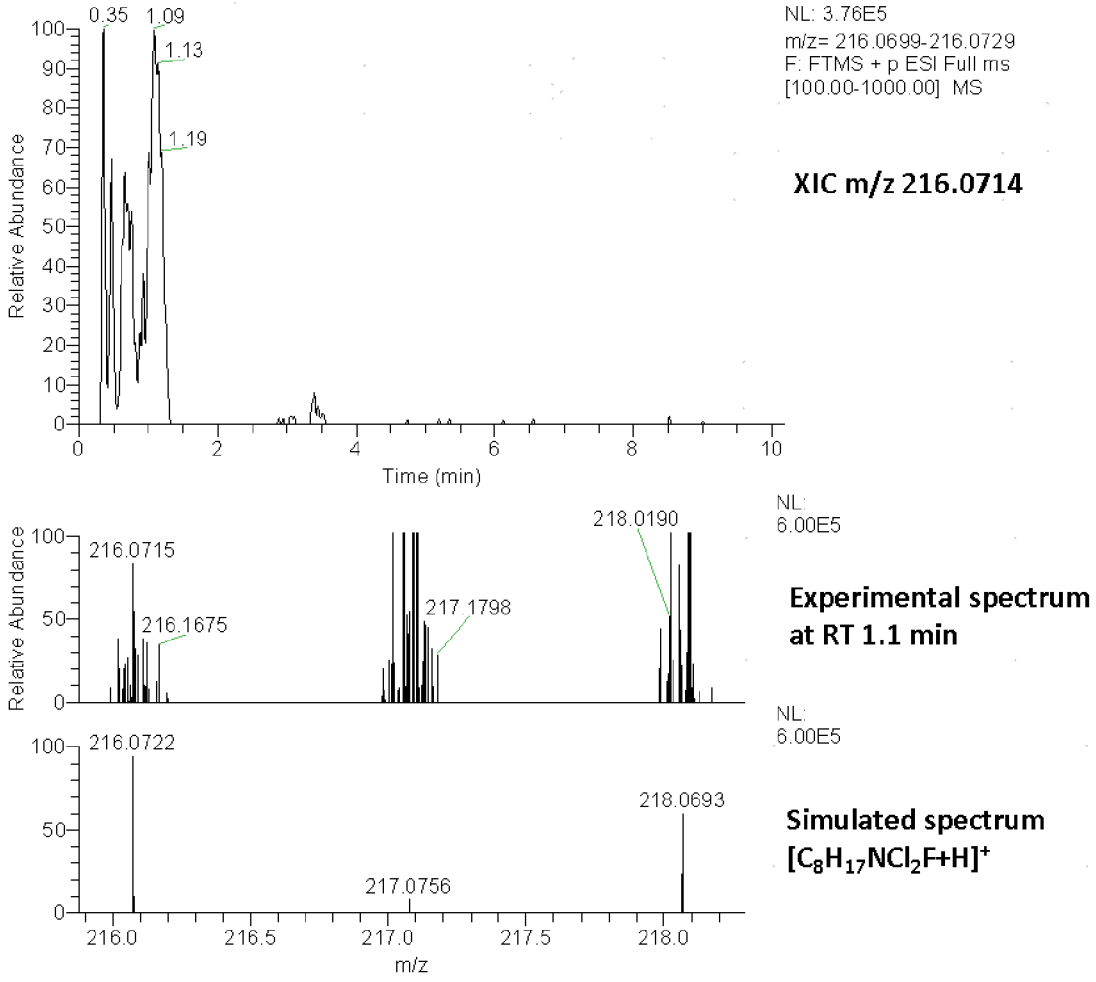

Figure 2.5: Extracted ion chromatogram of the peak of interest for compound m/z 216.0714 (retention time 1.1 minute) and the experimental full scan mass spectrum at that retention time. The theoretical mass spectrum for the plausible compound $[C_8H_{17}NCl_2F+H]^+$ was used to illustrate the position of the mass peaks, but the isotope peaks assigned by the "nontarget" package were in from other compounds.

Many high-intensity ions above m/z 500 (and doubly charged ions below that value) and retention times between 15 and 20 minutes were assigned to contain a ^{37}Cl and/or ^{34}S and/or ^{81}Br isotope, but a visual inspection of the raw mas spectra revealed that these were likely polysiloxane-containing ions, whose isotope pattern caused by several Si atoms could not be distinguished from that caused by S, Cl, or Br isotopes. This is exemplified in Figure 2.4. Due to these findings, it was focused the identification of chlorinated/brominated compounds on singly charged ions with m/z <450, which covers many small contaminant

molecules such as pesticides, pharmaceuticals and industrial chemicals. Table 2.3 shows that this resulted in relatively small numbers of 5 to 35 peaks of interest per sample. The number of Cl-/Br-containing compounds showed no significant trend along the course of the River Danube (see Table 2.4), thus the decreasing level of wastewater treatment technology along its course is not reflected in an increased accumulation of such compounds in gammarids.

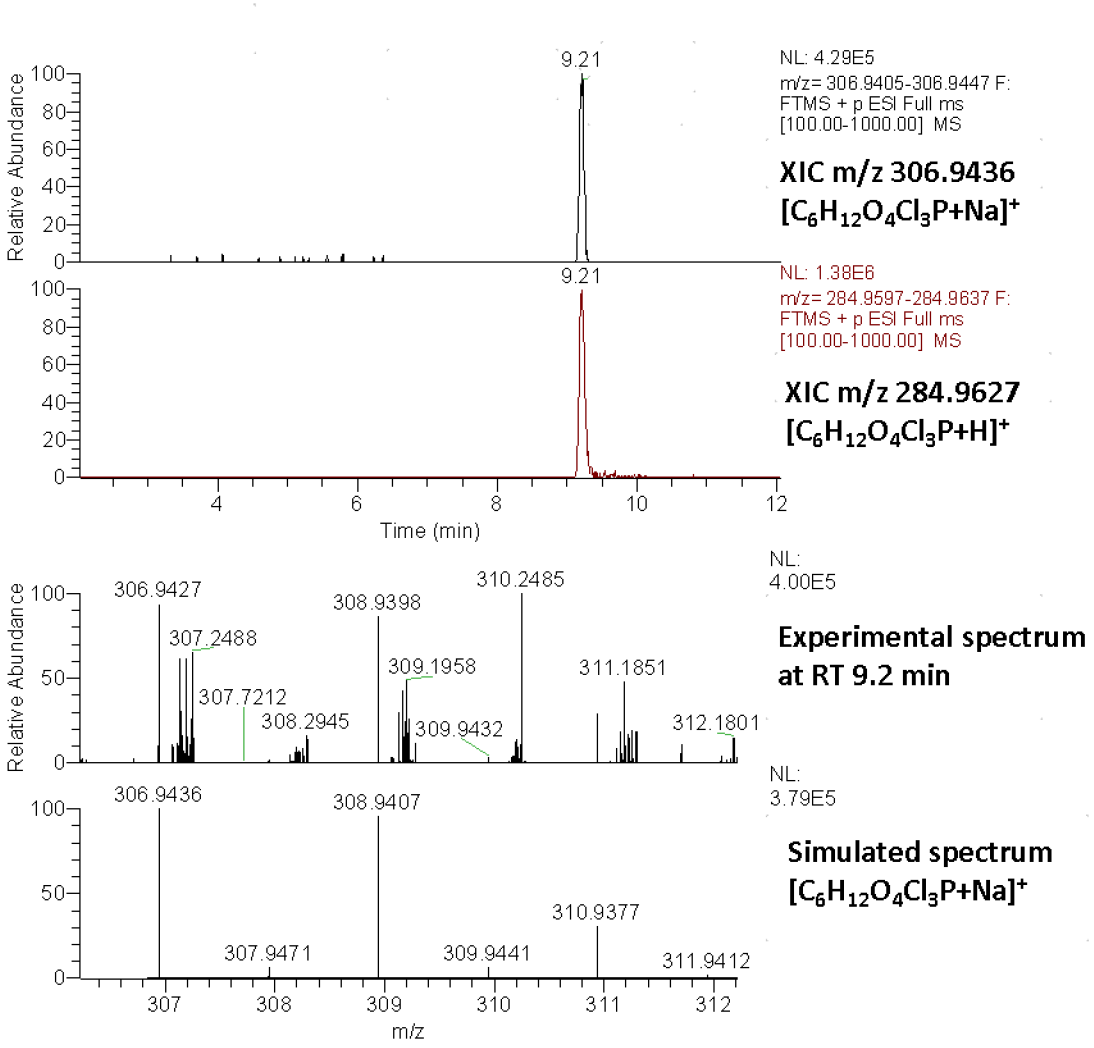

Figure 2.6: Extracted ion chromatogram of the peak of interest for compound m/z 306.9426 (retention time 9.2 minutes), the experimental full scan mass spectrum at that retention time and the theoretical mass spectrum for this compound's molecular formula. The Extracted ion chromatogram of the corresponding protonated molecule is also shown. Peak of interest for compound m/z 306.9426.

For the peaks of interest, ion chromatograms of the monoisotopic masses were extracted from the raw data files using the QualBrowser of Xcalibur and the mass spectra were checked visually. The peak intensities of these compounds were mostly below 10^6 a.u., about 30% was above 10^6, and only a few above 10^7 a.u. A visual inspection of isotope patterns revealed that in most cases for peaks $<10^6$ a.u. intensity no Cl or Br was present, but ions from co-eluting compounds were assigned as Cl or Br isotopologues by the nontarget package at the given intensity and m/z tolerances. This is illustrated for sample JDS34 as an

example in Table 2.5 and Figures 2.5-2.6. For the compound at m/z 216.0714 at a retention time of 1.1 minutes, the isotope pattern was obscured by the presence of many ions in the respective mass defect range (Figure 2.5). This was observed not only for compounds eluting close to the dead time with distorted peak shapes, but also for later eluting ones. Nevertheless, in some cases also low intensity ions were correctly assigned, as shown for the compound m/z 306.9426 at RT 9.2 (Figure 2.6). The assigned Cl isotope pattern could be confirmed as the ions were clearly separated from the large number of matrix ions at mass defects of +0.1 to +0.3 Th despite the low intensity of 4×10^5. This compound was actually a sodium adduct, but the corresponding protonated molecule was not assigned by the "nontarget" package.

For sample JDS34, three compounds could be finally identified (Table 2.5) based on a search of the determined molecular formulas in the Chemspider database. For these three compounds, among the candidate structures in Chemspider well-known environmental contaminants were found, which could in turn be confirmed by a comparison of retention times and MS/MS spectra with reference standards. Two of these compounds, dimethenamid and dimethachlor, are herbicides, a fact which coincides with the relatively high concentrations of other target herbicides in this sample. For three other compounds, a molecular formula could be determined. For the compound m/z 212.0835 at a retention time of 8.9 minutes, the subsequent Chemspider search suggested the herbicide propachlor as a plausible candidate, which could however not be confirmed by a reference standard. For the two remaining molecular formulas, no "promising" hits were found in Chemspider. These compounds would require thus more elaborated candidate selection procedure, but this was beyond the scope of this study.

2.4 DISCUSSION

2.4.1 Extraction and clean-up

Absolute recoveries of 1H-benzotriazole, carbamazepine, diuron and sulfamethazine were in the same range as reported in previous studies using sonification+96 well plate Ostro™ (Huerta et al., 2015), PuLE+SPE (Miller et al., 2015) and grinding balls+QuEChERS (Berlioz-Barbier et al., 2014). These studies were focused on individual compound groups whereas the method presented here encompasses organic micropollutants of diverse classes and a wide range of physicochemical properties making this approach more applicable in investigation facing a larger number of compounds.

Matrix effects on the analysis of environmental samples are commonly reported. The residual components of the matrix may promote either ion suppression or (less commonly) signal enhancement of the analytes in the electrospray source and cause errors that lead to inaccurate results (Gago-Ferrero et al., 2013). Moreover, matrix effects depend on the nature of the matrix and the efficiency of sample preparation (Bonfiglio et al., 1999). The procedure without any clean-up step presented the highest matrix effects, which is consistent with previous outcomes when direct injection without dilution has been used (Dams et al., 2003). Lipid removal thanks to hexane clean-up was reported. Although the lipid content is low in

gammarids ranging from 1.3% to 4.4% (Ashauer et al., 2006; Tlili et al., 2012) it still may drive signal suppression like it has been proposed in a former study for fish tissues (Huerta et al., 2013).

Regarding MQLs, a study carried out by Berlioz-Barbier et al. (2014) investigates pharmaceuticals and pesticides in *Gammarus fossarum* whose MQLs were 0.5 and 4.2 ng g^{-1} wet weight for carbamazepine and diuron respectively. In addition, Miller et al. (2015) reported MQLs of 6 and 15 ng g^{-1} wet weight in *G. pulex* for carbamazepine and sulfamethazine respectively. In this study, were achieved lower MQLs compared to those previous studies, allowing to this procedure to be tested in the biological compartment at trace concentrations.

2.4.2 Chemicals in gammarids

Benzotriazoles are widely used as dishwashing additives and are persistent in the environment, 1H-benzotriazole has been reported to be present in surface waters from the River Danube in concentrations of 213 ng L^{-1} and for 5MBT at 67 ng L^{-1} (Loos et al., 2015, 2010).

CBZ has been detected in an average concentration of 37 ng L^{-1} in the River Danube surface water samples (Loos et al., 2010). In a previous study Miller *et al*., (2015) detected CBZ at a concentration of <6 ng g^{-1} in gammarid tissues from tributaries of the River Thames, UK, which was the limit of quantification of their method. However, no concentrations in the water phase were given. CBZ is hardly sorbed to sediment and resistant to microbial degradation and therefore low concentration in surface water may be due to dilution processes (Allen et al., 2012; Tixier et al., 2003). Following the latter assumption, it can infer that observed lower concentrations in gammarids from the River Danube compared to gammarids from tributaries of the River Thames reflect low water concentrations.

DEET is one of the most commonly used active ingredients in insect repellents and recently has attracted attention with respect to environmental fate and potential hazards to aquatic organisms (Weeks et al., 2012). Thus far, DEET has not been reported in invertebrates tissues from field samples and few studies about its toxicity are available (Xue et al., 2006, 2000). The presence of DEET is consistent with former measurements performed in surface water samples in the River Danube (Loos et al., 2015).

In a previous study, metolachlor has been characterised to present genotoxic potential (DNA breakdown) using micronucleus test in fish exposed to river water samples after rainfall events in France (Polard et al., 2011). However, investigations about potential adverse effects upon gammarids or even others benthic invertebrates are still scarce.

The organophosphate diazinon and the neocicotinoid thiacloprid act via different molecular interactions, diazinon inhibiting the enzyme activity of the acetylcholinesterase (AChE), and thiacloprid disrupting the insect's nervous system by the stimulation of nicotinic acetylcholine receptors (nAChRs). Thiacloprid as a neurotoxic insecticide has been shown to

be capable to initiate drift in microcosm studies (Beketov and Liess, 2008). After diazinon exposure, a lack of mobility due to decreasing activity of AChE in *Daphnia magna* was found (Kretschmann et al., 2011a, 2011b). No data is available about the presence of these compounds in Danube surface waters, but diazinon has been measured in tributaries waters of the River Danube in Central Romania at 20 ng L^{-1} concentrations (Ferencz and Balog, 2010).

Triazole fungicides have been largely used as systemic fungicide because of their inhibition potential over enzymes involved in the biosynthesis of steroid hormones. However, this mechanism is generally active in wildlife, including mammals; according to the elevated persistence of these compounds in the environment, most of them are an environmental and human threat due to their inherent endocrine disruption features in mammals (Taxvig et al., 2007), fish (Yu et al., 2013) and invertebrates (Oetken et al., 2004).

Grab sample water concentrations may be considered as not very representative although short term changes in thus large water bodies should be limited. However, the results demonstrate that non-detectable low concentrations of a compound in water do not exclude the accumulation in biota tissue and thus risks to aquatic organisms. Body burden analysis is a complementary approach to water monitoring and helps to improve environmental risk assessment.

2.5 CONCLUDING REMARKS AND OUTLOOK

In this study, a multi-target method for the analysis of organic micropollutants of diverse chemical classes and physicochemical properties has been developed and optimised based on PuLE and QuEChERS with an additional hexane phase as clean-up step and subsequent analysis by LC-MS/MS. Due to its sensitivity, reproducibility and fast sample processing it allows to gain a picture on internal concentrations of micropollutants in aquatic organisms. Overall, 58 compounds were determined with quantification limits ranging from 0.01 to 2.13 ng g^{-1} wet weight and with an average reproducibility of 5.8% (RSD) in macroinvertebrate samples.

The optimised method was applied to gammarid samples from JDS3 project. In total 13 pesticides and four wastewater-derived pollutants were detected in the freshwater invertebrate *Dikerogammarus* spp. With regard to non-target screening, the sample preparation method was as well suitable and we could demonstrate the successful identification of several compounds. However, the automated detection of isotope patterns was hampered by the complex matrix particularly for peaks of lower intensity. To overcome this drawback, a further clean-up of the extracts could be a solution, but this would reduce the compound domain covered by this method.

This study suggests that invertebrate tissues represent a good biological proxy of organic micropollutants burdens in order to assess water quality providing an integrative history of chemical deposition.

CHAPTER 3

Occurrence, freely dissolved concentration, chemical activities and baseline toxicity of organic contaminants in freshwater ecosystems: multi-compartment analysis

ABSTRACT

Many chemicals are persistent to biodegradation and depending of their physicochemical features they can enter aquatic systems in freely dissolved form or bound to organic matter and thus might undergo partitioning processes. Both the quantification and prediction of freely dissolved concentrations is a primordial step in the understanding of the bioavailable fraction of the chemicals and thus their final fate in the environment. Additionally, chemical activity, an analogous concept to freely dissolved concentration, may help in order to get a better comprehension of the potential fate and distribution of chemical in the water-sediment phases. In this study, applying equilibrium partitioning theory was investigated whether equilibrium was hold between the water-sediment and biota compartment in a freshwater system. In addition, hazard assessment was estimate using baseline toxicity approach for chemicals with a wide range of hydrophobicity. Overall, sediment compartment exhibited both the highest freely dissolved concentrations and chemical activities. Furthermore, significant differences were observed between water and biota based on chemical activity. In fact, the results suggest that sediment might act as a source of contamination towards the water phase in the River Holtemme. In this study is extended this finding, sediments source of contamination towards the surrounding waters, to organic chemicals with a log K_{OW} values between -1 to 5. Additionally, calculated hazard strongly depended on which compartment is analysed, for instance, highest hazard can be observed if chemical activities of contaminated sediments are considered for assessment.

In preparation in a slightly modified form as:

Pedro A. Inostroza, Riccardo Massei, Romy Wild, Martin Krauss and Werner Brack. Freely dissolved concentration, chemical activities and baseline toxicity: insights of a multi-compartment analysis in a freshwater system.

3.1 INTRODUCTION

The exposure of aquatic organisms to organic contaminants in surface waters may have adverse effects on survival, fitness and reproduction of individuals but also on populations and ecosystems (Brown et al., 2009; Vörösmarty et al., 2010). Many chemicals are persistent to biodegradation in wastewater treatment plants (WWTPs) and thus present in surface waters in varying concentrations (Reemtsma et al., 2006). Compounds such as pesticides may show distinct concentrations peaks in surface water due to input events resulting from spray-drift during application, surface run-off during rain events and/or field drainage (Schulz, 2004). Depending on their physicochemical properties, contaminants enter aquatic systems in freely dissolved form or bound to dissolved or suspended particulate organic matter (DOM or POM), and may undergo further redistribution processes. These processes ultimately drive their fate and bioavailability to organisms, as has been demonstrated mainly for hydrophobic contaminants (Lohmann et al., 2004; Neff, 1984; Tlili et al., 2012).

The chemical activity concept has been shown to be a useful approach for understanding the environmental fate and distribution of chemicals, but can be also useful for relating exposure to toxicity (Di Toro et al., 1991; Mackay et al., 2014; Mayer and Holmstrup, 2008; Reichenberg and Mayer, 2006; Trapp et al., 2010). According to equilibrium partitioning theory (EqP), an organic chemical is assumed to partition between water, sediments (predominantly organic matter) and biota (predominantly lipids and proteins) until equilibrium is reached between these compartments (Di Toro et al., 1991; Reichenberg and Mayer, 2006). The bioavailability of a compound mainly depends on the freely dissolved concentration (C^{fd}) (Kraaij et al., 2003; Urrestarazu Ramos et al., 1998), which refers to chemicals in an aquatic solution that are not bound to particles or to dissolved organic carbon (Mayer et al., 2000). The concept of C^{fd} has been successfully applied in several studies where bioconcentration and toxicity were observed to be regulated by C^{fd} rather than by total concentration of the organic contaminants (Lang et al., 2015; Mayer and Reichenberg, 2006; Reichenberg and Mayer, 2006; Witt et al., 2009). In fact, freely dissolved concentrations in equilibrium with sediment and biota and chemical activity are based on the same concept. They quantify the potential for spontaneous physicochemical processes, such as diffusion and partitioning and each of them can be derived from the other (Reichenberg and Mayer, 2006).

Differences in chemical activity drive diffusion and partitioning processes and determine whether a specific environmental compartment acts as source or sink of organic contaminants in a multi-compartment system (Reichenberg and Mayer, 2006). Therefore, chemical activity is a relevant parameter for the assessment and management of the risk of organic contaminants in the environment. Chemical activity also plays an important role for partitioning processes into biological membranes and thus baseline toxicity. It provides a direct approach for relating external concentrations (i.e. in water, sediment, and biota) to baseline effect concentrations by normalising effect concentrations to partitioning to partition coefficients. This approach allows for comparison of effect concentrations across compounds,

species and environmental media (Reichenberg and Mayer, 2006; Smith et al., 2010). The occurrence of baseline toxicity requires a chemical activity of approximately 0.01 to 0.1 for non-polar neutral organic chemicals under long-term exposure in aquatic toxicity tests (Bobra et al., 1983; Mayer and Reichenberg, 2006; Reichenberg and Mayer, 2006). Thus, chemical activity may be used to differentiate baseline from excess toxicity using empirical data.

The determination of chemical activities of organic contaminants in different compartments of aquatic ecosystems may help to translate water to sediment and biota concentrations and vice versa assuming equilibrium, respective estimate the degree of equilibrium if measured concentrations in different compartments are available (ECETOC, 2016). The assessment of equilibrium strongly depends on the partition coefficients between water, sediments and biota used for estimating chemical activity. Two major approaches that will be used also in this study are log K_{OW}-based estimations (Di Toro et al., 2000) and polyparameter linear free energy relationships (ppLFERs) considering different types of lipids and proteins. The latter became broadly available by the establishment of a generally LSER database allowing for the calculation of partition coefficients between an extensive set of biological and non-biological matrices including storage and membrane lipids and different types of proteins (Endo et al., 2015).

Due to its remarkable gradient of anthropogenic influences with clearly defined sources of pollution (CHAPTER 4), the River Holtemme (Saxony-Anhalt, Germany) was chosen as a case of study for a multi-compartment analysis of organic micropollutants in water sediments and the model invertebrate *Gammarus pulex* that was selected based on its ubiquitous occurrence in European freshwater systems and its important ecological function breaking down coarse organic matter (Friberg et al., 1994; Jażdżewski, 1980). This species has been previously used as a model organism for assessing both adverse effects (Cold and Forbes, 2004) and uptake of organic micropollutants under laboratory conditions (Ashauer et al., 2012). The overarching goal of this chapter was to investigate for which compounds equilibrium partitioning based on log K_{OW} or LSER is able to explain the concentrations in the different environmental compartments for the compounds in a freshwater system. It should be investigated whether there are systematic deviations from equilibrium partitioning dependent on calculated partition coefficients suggesting limits of the domain of hydrophobicity-based equilibrium partitioning approaches or compounds specific deviations suggesting actual disequilibrium and helping to reveal the function of different compartments as sinks or sources of these chemicals. Additionally, biological implications of the chemicals in each environmental compartment were assessed based on their chemical activities throughout the hazard assessment approach.

3.2 METHODOLOGY

3.2.1. Reagents, chemicals and consumables

A list of 86 analytes with a wide range of hydrophobicity (log K_{OW} from -1.61 to 5.51) was selected based on their occurrence in water samples and sediments (see Appendix

Table B.1). These compounds belonged to different classes of pollutants such as pesticides, pharmaceuticals, and industrial chemicals and some of their main transformations products.

Methanol (gradient grade), acetonitrile (HPLC grade), acetone (HPLC grade), ethyl acetate (HPLC grade), sodium hydroxide (analytical grade), formic acid (analytical reagent grade, 98%), and sodium chloride were supplied by Sigma-Aldrich and primary secondary amine (PSA) by Agilent. For LC-MS/MS and LC-HRMS analyses, methanol, water and formic acid of LC-MS grade (ChromaSolv, Sigma-Aldrich) were used. Analytical standards were obtained from different sources. Stock solutions of these standards (1 mg mL^{-1}) were prepared in methanol (MeOH) and stored in amber vials (20 mL) at -20°C in the dark. Mixed solutions of 10 μg mL^{-1} were prepared in methanol and used for method development and calibration.

3.2.2 Study area and sampling

The multi-compartment analysis was performed in the River Holtemme as an example for a typical small central European stream, which stretches over 47 kilometres in the Bode catchment, Saxony-Anhalt, Germany. The Holtemme catchment is characterised in its upper stretch mainly by forest while its lower stretch is dominated by intensively used agricultural areas and impacted by the medium-sized towns of Wernigerode and Halberstadt (Figure 3.1). Discharges of two municipal wastewater treatment plants (WWTPs) serving approximately 150,000 inhabitants and together with agricultural activities represent the main pollution sources in its catchment (Reuter et al., 2003).

Water, sediment and macroinvertebrate samples were collected in October 2014 in an integrated sampling campaign along the course of the river and principal tributaries in order to obtain a longitudinal profile in the freshwater system integrating water-sediment and biota compartments. Water samples were always collected at the same sites where sediments or biota were sampled (Figure 3.1).

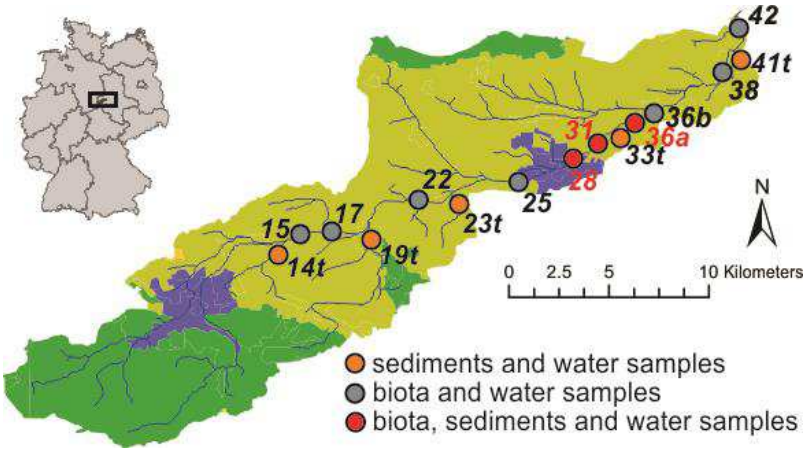

Figure 3.1: Map showing location of sampling sites in the River Holtemme and its tributaries (marked by "t" after the site number). In orange sampling sites where sediments and water samples were collected: in grey sampling sites for *G. pulex* and water samples and red where *G. pulex*, water and sediments samples were collected. Green colours represent forest; olive colours represent agricultural landscapes and blue main cities.

3.2.3 Sample preparation and extraction

3.2.3.1 Grab water samples

Grab water samples were collected at 15 sites (Figure 3.1) directly into 2 mL autosampler vials using a 1-mL pipette, transported to the laboratory in a cooling box at 0°C and stored in the laboratory at -20°C until analysis. Prior to analysis, water samples were thawed, 25 μL of methanol and 25 μL of an internal standard mixture containing 31 isotope-labelled compounds were added to yield a final concentration of 100 ng/L in vial.

Contents of dissolved organic carbon (DOC) and particulate organic carbon (POC) were determined according to Kamjunke et al. (2013). Briefly, all water samples were filtered through glass fibre filters (Whatman GF/F). Subsequently, the filter was analysed for POC (and PN) (using an Elementar Vario EL cube) and the filtrate was analysed for DOC (using a DIMATOC 2000), both measurements were based on high-temperature combustion. Deionised water was used as a blank for all samples. The DOC and POC data were used in order to calculate freely dissolved concentrations in each environmental compartment in the River Holtemme.

3.2.3.2 Sediments samples

Surface sediment samples were collected at eight sites using a pre-cleaned stainless steel scoop (Figure 3.1). Aliquots of the top 5 cm from 5-10 spots within an area of 5-25 m^2, depending on the size of the river/stream, were pooled in pre-washed stainless steel bowls and thoroughly mixed. Only three samples could be taken from the River Holtemme itself, due to a lack of sedimentary areas along the river, while five were taken from tributaries. All sediment samples were collected under oxic conditions. Samples were transported in a cooling box at 0°C. Samples were homogenised overnight, freeze-dried, sieved to <63 μm and stored at -20°C for further analysis. Total organic carbon content (TOC) was measured according to DIN 19539 on a LECO C-230 Carbon Analyser by solid combustion. The method is based on combustion and the subsequent detection of produced CO_2. A total of 0.2 g dry weight sediment was used for the analysis. The instrument was first heated to 400°C in oxygen atmosphere to measure total organic carbon. Then the temperature was increased to 900°C in nitrogen atmosphere to determine the total inorganic carbon. The potential accumulation of organic micropollutants depends on the sediment's TOC. Thus, only the TOC data was of interest for this study and was used to normalise sediment concentrations.

Pressurised liquid extraction (Dionex ASE 200) and subsequent clean-up steps were performed according to Massei *et al.* (in preparation) with minor modifications. Briefly, freeze dried sediments were transferred to stainless steel ASE cells prepared with a 27 mm glass fibre filter (Dionex, Olten, Switzerland). In total 5 g of sediments were delivered to the cell. In addition, in order to increase the solvent channelling, 1.25 g of diatomaceous earth were added to each extraction cell. The cells were extracted at 100°C using a mixture of ethyl acetate, acetone (50:50, EtAc) for a total of two extraction cycles. Besides, two blanks (diatomaceous earth) were run in parallel to check for possible contamination.

The clean-up procedure was based on normal-phase chromatography using alumina/silica gel columns (6% and 3%, respectively). The ethyl acetate/acetone (EtAc) extract was mixed with deactivated silica gel and the solvent was evaporated with a rotary evaporator until dryness. The silica was then loaded onto the alumina/silica gel column. To protect the packed column against turbulences during the filling of eluents a layer of diatomaceous earth was added on top. Three different solvents (hexane, dichloromethane and methanol) were used in succession and collected in two different fractions in order to separate compounds according to their polarity. The dichloromethane/methanol fraction was evaporated close to dryness and re-dissolved in 1 mL MeOH and filtered with cellulose acetate syringe filter (pore size 0.45 μm). Internal standard (100 ng/mL) was added before injection to take matrix effect into account.

3.2.3.3 Gammarids samples

Gammarus pulex specimens were sampled at ten sampling sites (Figure 3.1) following a standardized sampling protocol described by Hering et al. (2004). Briefly, 20 habitat-weighted samples (total sampled area 1 m²) were taken from each site with a Surber sampler (500 μm mesh). From each sample, 24 individuals were stored at -20°C for body burden analysis of organic micropollutants. Samples of different size classes were collected and pooled in order to avoid bias produced by different life stages.

Gammarus pulex samples were extracted using a multi- and non-target screening method based on pulverised liquid extraction (PuLE) and a modified QuEChERS with an additional hexane phase (CHAPTER 2). Briefly, 900 mg thawed gammarids were homogenised in 4 mL acetonitrile/water (1:1 *v/v*) and 1 mL of hexane using an Ultra-Turrax® T-25 (IKA) for 1 minute and vortexed for another minute. A total of 4 mL of homogenate were thoroughly mixed with 800 mg of anhydrous $MgSO_4$ and 200 mg of NaCl. The mixture was immediately shaken for 1 minute using a vortex mixer and centrifuged at 4,000×*g* for 5 minutes. Aliquots of 3.5 mL of the acetonitrile phase were transferred to glass centrifugation tubes containing 50 mg of PSA and 400 mg of anhydrous $MgSO_4$. The tubes were vortexed for 60 seconds, centrifuged at 4,000×*g* for 5 minutes and the supernatant was concentrated under a nitrogen stream at room temperature to dryness. Finally, the residues were reconstituted in 500 μL of MeOH and filtered with a PTFE syringe filter (pore size 0.45 μm, Chromafil).

3.2.4 LC-MS/MS analysis

All analyses were performed using a 1260 Infinity LC system (Agilent) coupled to a QTrap 6500 MS (ABSciex) with IonDrive™ Turbo V ion source. Concisely, a Poroshell 120 C18 column (50×4.6 mm, 2.7 μm particle size, Agilent) was installed between the auto sampler and the pump in order to trap background contaminants from the eluents, degasser or pump. Aliquots of 10 μL of extract were injected onto a Kinetex C18 column (50 × 3.0 mm, 2.6 μm particle size, Phenomenex). Analytes were separated by gradient elution at a flow rate of 0.4 mL/min using LC-MS grade water (A) and LC-MS grade MeOH (B), both containing 0.1% of formic acid. The initial content of 5% B was held for 1 minute and increased to 95%

B over 5.2 minutes. After 11.4 minutes of elution with 95% B, the column was re-equilibrated for 5 minutes to the initial composition. Following electrospray ionization (ESI), the QTrap instrument was operated in scheduled multiple reactions monitoring (sMRM) mode switching between positive and negative ionization.

Water samples were analysed with LC-MS/MS by direct injection of 100 μL of water. For separation, a 1260 Infinity LC system (Agilent) equipped with a reversed-phase LC column (Kinetex C18 50×2.1 mm; 2.6 μm particle size; Phenomenex) and a gradient elution with water and methanol (both containing 0.1% formic acid) with a flow rate of 0.35 mL min^{-1} were used. The LC was coupled via an electrospray ionisation source to QTrap 6500 MS (ABSciex), which was operated in sMRM mode.

The method quantification limits (MQLs) were determined as described previously by Wells et al. (2011). MQLs were determined as the lowest concentration of a compound that can be reliably quantified (99% confidence interval) in the matrix in question. Results were not corrected for recovery, because method-matched calibrations were carried out. So, the losses during sample preparation are covered and the internal standard calibration accounts for matrix effect.

3.2.5 Calculation of C^{fd} and chemical activity

Freely dissolved concentrations were derived from total concentrations for each of the environmental compartments (water, sediment and biota) and their subsequent chemical activities were calculated using the following equations:

For water, freely dissolved water concentration (C_W^{fd}) was predicted with the two-carbon equilibrium partitioning model (Eqn 3.1) as proposed by Schwarzenbach et al., (2005) assuming equilibrium conditions:

$$C_W^{fd} = \frac{C_W^t}{1 + (DOC\ K_{DOC}) + (POC\ K_{POC})} \tag{3.1}$$

where C_W^t, it is the total concentration in the water sample, DOC is the dissolved organic carbon concentration (Kg/L) and POC is particulate organic carbon concentration (Kg/L), K_{DOC} is the equilibrium partitioning coefficient (L/Kg) of the chemical between DOC phase and the freely dissolved phase of water, K_{POC} is the equilibrium partitioning coefficient (L/Kg) of the chemical between POC phase and the freely dissolved concentration of water.

The predicted relationship between K_{DOC}=0.08K_{OW} with 95% confidence limits was used when experimental K_{DOC} data was missing (Burkhard, 2000). Experimental investigations have shown that K_{POC} is approximately equal to K_{OW} for many chemicals (Dean et al., 1993; Eadie et al., 1992, 1990). Thus, C_W^{fd} was calculated applying Eqn 3.2 using K_{OW} values estimated by the software KowWin v1.68 submodel in EPISuite v4.11 (US Environmental Protection Agency (USEPA), 2012).

$$C_{\mathrm{W}}^{\mathrm{fd}} = \frac{C_{\mathrm{W}}^{\mathrm{t}}}{1 + \mathrm{POC} \times K_{\mathrm{OW}} + \mathrm{DOC} \times 0.08 K_{\mathrm{OW}}} \tag{3.2}$$

Assuming organic carbon as only relevant phase for the absorption of organic micropollutants, equilibrium freely dissolved concentration in the pore-water ($C_{\mathrm{S}}^{\mathrm{fd}}$) was calculated from organic carbon fraction normalised sediment concentrations ($C_{\mathrm{S}}^{\mathrm{t}}$) according to Eqn 3.3, with K_{OC} being the partitioning coefficient between sediment organic carbon and pore-water and f_{OC} the organic carbon fraction in sediment. The K_{OC} was predicted using K_{OW}-based values in KOCWIN v2.00 submodel in EPISuite v4.11.

$$C_{\mathrm{S}}^{\mathrm{fd}} = \frac{C_{\mathrm{s}}^{t}}{f_{\mathrm{OC}} \quad K_{\mathrm{OC}}} \tag{3.3}$$

Assuming lipids and proteins as the only relevant phases for absorption of micropollutants, the internal freely dissolved concentrations in gammarids ($C_{\mathrm{B}}^{\mathrm{fd}}$) were calculated as follows:

$$C_{\mathrm{B}}^{\mathrm{fd}} = \frac{C_{\mathrm{B}}^{t}}{(f_{\mathrm{LIPID}} K_{\mathrm{OW}} + f_{\mathrm{PROTEIN}} K_{\mathrm{PW}})} \tag{3.4}$$

where ($C_{\mathrm{B}}^{\mathrm{t}}$) is related to the concentration in *G. pulex*'s tissues, f_{LIPID} the lipid fraction and K_{OW} being a reasonable parameter in lack of an experimental K_{LIPID}. Both lipid and protein content were not measured in this study. Instead, lipid content values reported by Ashauer et al. (2010, 2006) and protein content by Fredrickson & Reid (1988) were used. K_{PW} was estimated using K_{PW}=0.7K_{OW} according Schwarzenbach et al (2005).

The C^{fd} of an organic chemical was converted to chemical activity by normalising with the sub-cooled water solubility (Reichenberg and Mayer, 2006). Chemical activity for each environmental compartment was calculated as follow:

$$a = \frac{C_{\mathrm{W,S,B}}^{\mathrm{fd}}}{S_{\mathrm{L}}} \tag{3.5}$$

where $C_{\mathrm{W,S,B}}^{\mathrm{fd}}$ is related to the freely dissolved concentration of each environmental compartment (W=water, S=sediment and B=biota), and S_{L} is the sub-cooled liquid solubility which was calculated by the SPARC on-line calculator (http://archemcalc.com/sparc-web/calc/#/multiproperty, 2016-06-12).

LSER fits

Linear solvation energy relationships (Eqn 3.6) were used as an alternative to log K_{OW}-based approaches to estimate sediment-water and biota-water partition coefficients. LSER calculations were based on organic carbon-water, protein-water, membrane lipid-water and storage lipid-water partitioning applying the system parameters *v,e,s,a,b* and *c* given in the open UFZ-LSER database (Endo et al., 2015). The molecular descriptors *V* [(cm^3 mol^{-1})100] as McGowan's characteristic volume, *E* (cm^3/10) as the excess molar refraction, *S* as

the polarity/polirisability parameter and A and B as the hydrogen acidity and basicity, respectively, were calculated with the ACD/Percepta platform (ACD labs, v2016).

$$\log K_{\mathrm{L,P}} = vV + eE + sS + aA + bV + c \tag{3.6}$$

The overall sediment-water and biota-water freely dissolved concentrations were calculated according to Eqn 3.3 and an extended version of Eqn 3.4 considering the two different types of lipids (storage and membrane lipid). For the calculation of biota-water partition coefficients, the following composition of *G. pulex* was assumed: f_{PROTEIN}=0.47 (Frederickson & Reid 1988), $f_{\mathrm{STORAGE\ LIPID}}$=0.045 and $f_{\mathrm{MEMBRANE\ LIPID}}$=0.015 (Ashauer et al., 2010).

3.2.6 Interphase disequilibrium

The distribution of a chemical between compartments in an ecosystem is most effectively described as the sediment-water concentration quotient (Burkhard et al., 2008), which is further defined as:

$$\Pi_{\mathrm{SOC}} = \frac{C_{\mathrm{SOC}}}{C_{\mathrm{W}}^{\mathrm{fd}}} \tag{3.7}$$

where C_{SOC} is the concentration of chemical in sediment, normalised by sediment organic carbon, and $C_{\mathrm{W}}^{\mathrm{fd}}$ is the concentration of chemical that is freely dissolved in water. By expressing the concentration of chemical in sediment as an organic carbon normalised basis and the concentration of chemical in water as a freely dissolved basis, this quotient is a metric of the degree to which the chemical's distribution between the surface sediment and the water column approaches or derives from a condition of thermodynamic equilibrium for the water body. Same criteria may be used to describe the distribution between biota-water and biota-sediment:

$$\Pi_{\mathrm{B,Lip}} = \frac{C_{\mathrm{B,Lip}}}{C_{\mathrm{W,S}}^{\mathrm{fd}}} \tag{3.8}$$

where $C_{\mathrm{B,Lip}}$ is the concentration of a chemical in biota, normalised to lipid content, and $C_{\mathrm{W,S}}^{\mathrm{fd}}$ is the concentration of chemical that is freely dissolved in water (W) or sediment (S).

According to Burkhard *et al.* (2008) when all the organic carbon is assumed to be biogenic, the relationship between C_{SOC} and $C_{\mathrm{W}}^{\mathrm{fd}}$ are described by the TOC equilibrium partitioning coefficient (K_{SOC} or K_{POC}) which have been approximated by the octanol-water partition coefficient K_{OW} (Di Toro et al., 1991; US Environmental Protection Agency (USEPA), 2000) or fraction ($f_{\mathrm{OC,DOC}}$) thereof (Seth et al., 1999). Therefore, using field measured concentration quotients, Π_{SOC} and $\Pi_{\mathrm{B,Lip}}$, apparent disequilibria metric may be calculated on basis of chemical activity as follows:

$$disequilibrium_{\mathrm{SW}} = \frac{C_{\mathrm{S}}^{\mathrm{fd}}}{S_{\mathrm{L}}} \Big/ \frac{C_{\mathrm{W}}^{\mathrm{fd}}}{S_{\mathrm{L}}} = a_{\mathrm{S}}/a_{\mathrm{W}} \quad (3.9)$$

for biota-water as follows:

$$disequilibrium_{\mathrm{BW}} = \frac{C_{\mathrm{B}}^{\mathrm{fd}}}{S_{\mathrm{L}}} \Big/ \frac{C_{\mathrm{W}}^{\mathrm{fd}}}{S_{\mathrm{L}}} = a_{\mathrm{B}}/a_{\mathrm{W}} \quad (3.10)$$

for biota-sediment as follows:

$$disequilibrium_{\mathrm{BS}} = \frac{C_{\mathrm{S}}^{\mathrm{fd}}}{S_{\mathrm{L}}} \Big/ \frac{C_{\mathrm{S}}^{\mathrm{fd}}}{S_{\mathrm{L}}} = a_{\mathrm{B}}/a_{\mathrm{S}} \quad (3.11)$$

Note that these relationships are basically the ratio of the chemical activities in the defined environmental compartment.

3.2.7 Hazard assessment

In order to assess the environmental hazard of the chemicals in each compartment, the baseline toxicity approach was used. Toxicity data (e.g., LC_{50} or EC_{50s}) can be expressed in terms of chemical activity as follows:

$$Ea_{50} = EC_{50}/S_{\mathrm{W}}^{\mathrm{L}} \quad (3.12)$$

Median effective activity (Ea_{50}) values were calculated in this study according to Eqn. 3.12 defining baseline toxicity for polar microcontaminants. The predicted baseline toxicity was derived following the same criteria used by Reichenberg and Mayer (2006) where median effective concentrations (EC_{50}) were translated to median effective activity by normalising by the sub-cooled liquid solubility. Furthermore, the activities of individuals compounds contained in the mixture were added. The sum of baseline toxicity is an indicator of the baseline toxic potential of the mixture, because baseline toxicity of mixtures follows the concept of concentration addition (Di Toro et al., 2000; Escher and Hermens, 2002).

Because in general toxicity data is not reported in terms of internal concentrations (i.e., body burden), predicted baseline toxicity from water was used in order to assess the hazard of chemicals in gammarid samples. This assumption was applied based on that all total concentrations in gammarids were previously converted to their freely dissolved form and then to chemical activity as explained above. Alternatively, the internal EC_{50} can also be estimated from external EC_{50} using toxicokinetic (TK) models. Nevertheless, the latter was far of the scope of this study.

3.2.8 Statistical analysis

Freely dissolved concentrations and chemical activity were analysed by a one-way ANOVA (Tukey test). But, when data were not normally distributed non-parametric method were used (Kruskal-Wallis test). In cases of significant differences between any data sets, a *post hoc* test according to Dunn was used to identify groups that differed significantly using the R-package dunn.test. Differences were considered statistically significant at $p<0.05$. When pattern differences were identified, the similarity percentage (SIMPER) subroutine was

used to identify which pollutants contributed the most to the observed differences. Non-metric multi-dimensional scaling plots (NMDS) were constructed to display clustering patterns among pollutants and their environmental compartments. Multivariate analysis was performed with nonparametric statistical software PRIMER V.6.1.11. In the present study, median rather than average was chosen as the statistical parameter to present a single value for the sampling sites in order to avoid the dominance of relatively large values.

3.3 RESULTS

3.3.1 Total concentrations in the River Holtemme

From the 86 organic contaminants investigated, 63, 52 and 17 compounds were detected in water, sediment and gammarid samples, respectively (Figure 3.2; for details see Appendix Table B.2, B.3 and B.4). The detected compounds exhibited log K_{OW} values ranging from -1.4 to 4.9 in water, from -0.2 to 5.5 in sediments, and from -0.2 to 5.5 in gammarids (Appendix Table B.5).

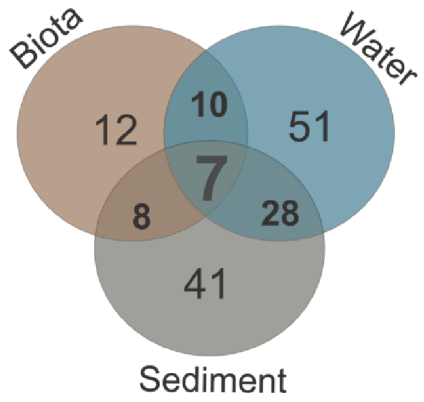

Figure 3.2: Summary of chemicals quantified in each environmental compartment in the River Holtemme.

The general patterns of the total concentrations are plotted in Figure 3.3. A slight increase was observed in the median concentrations in the biota compartment with a peak at the sampling site in kilometre 17 (*st*17) located downstream of the first wastewater treatment plant. In addition, for water compartments a peak at the kilometre 31 (*st*31) was observed right after the second wastewater treatment plant in the River Holtemme. Overall, tributaries and river samples for both sediment and water were in the same range of median concentrations (Figure 3.3).

For gammarids, only one compound, the insecticide thiacloprid, was quantified in all the sampling sites at the River Holtemme with concentrations ranging from 0.47 to 2.42 ng/g wet weight (Appendix Table B.2). Additionally, three compounds were quantified at all sites downstream of the first wastewater treatment plant (*st*17) until the last sampling site nearby the confluence with the River Bode. These compounds were the fungicide propiconazole, the pharmaceutical carbamazepine and the industrial chemical 4-/5-methyl-1H-benzotriazole (both isomers could not be separated with the applied method) with concentrations ranging

from 1.74 to 3.85, from 1.53 to 2.83 and from 0.48 to 1.78 ng/g wet weights, respectively (Appendix Table B.2). The highest concentration peak (13.08 ng/g wet weights) was found for the herbicide prosulfocarb at *st*36a. The latter sampling site also exhibited the highest frequency of quantified compounds in gammarid tissues and for most compounds the highest concentration (n=11, for details see Appendix Table B.2).

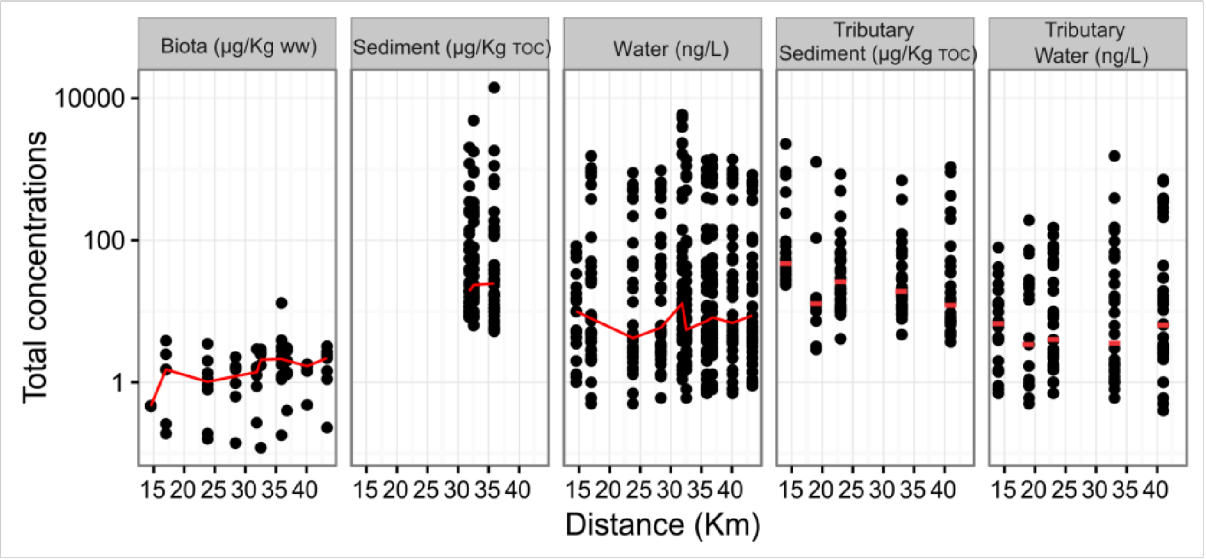

Figure 3.3: Total concentrations per environmental compartment along the River Holtemme and its tributaries (right two columns). The red line and red dots represent the median concentration at each site.

In sediment samples forty one compounds were quantified and twelve of them were quantified in all sampling sites, including both tributaries and the main course the River Holtemme (Appendix Table B.3). In general, concentrations in tributaries and the river were in the same range for most compounds with only two remarkable exceptions, the herbicide diflufenican and the industrial chemical triethyl citrate. Both compounds showed maximum concentrations in a tributaries up to 5-fold and 6-fold higher than in the River Holtemme, respectively (tributary *st*23t and *st*14t, respectively; Appendix Table B.3). Pharmaceuticals and industrial chemicals typically exhibited higher concentrations than pesticides. The only exception was 2-aminobenzimidazole (transformation product of the fungicide carbendazim) which reached a concentration 14.06 µg/g TOC at the sampling site *st*36a (Appendix Table B.3).

Overall, fifty one compounds were quantified in water samples at the River Holtemme. However, fourteen compounds were quantified along all sampling sites in water samples (Appendix Table B.4). Eight of them were either pharmaceutical or industrial chemicals with remarkable differences in concentrations. While pesticides concentrations ranged from 0.5 to 44.2 ng/L, pharmaceuticals and industrial chemicals escalated up to 5.8 µg/L. Most of the compounds showed the highest concentration peaks at the site *st*28 followed by the sampling site *st*36a (Appendix Table B.4) where a rainwater drainage outfall and a small water reservoir are located, respectively.

The detected chemicals exhibited log K_{OW} values ranging from -1.4 to 4.9 in water, from -0.2 to 5.5 in sediments, and from -0.2 to 5.5 in gammarids (Appendix Table B.5). The spatial distribution of chemicals (normalised by frequency and categorised according to log K_{OW}) in each environmental compartment for each site are given in Figure 3.4. A cut-off value of log K_{OW} equal 3 was established to distinguish amongst hydrophilic and hydrophobic chemicals (represented by a black solid line in Figure 3.4). Overall, about 75% of the detected compounds in water exhibited log K_{OW} values below 3, whereas it were about 50% in sediments and about 60% in gammarids (Figure 3.4). No remarkable differences in the log K_{OW} distribution were observed between the main course of the River Holtemme and its tributaries. There was an exceptional gammarid sample (*st*15) that showed the highest fraction of hydrophilic chemicals (75%) with a remarkable presence of the compounds, imidacloprid and fenuron, with log K_{OW}≤-1 (Figure 3.4C).

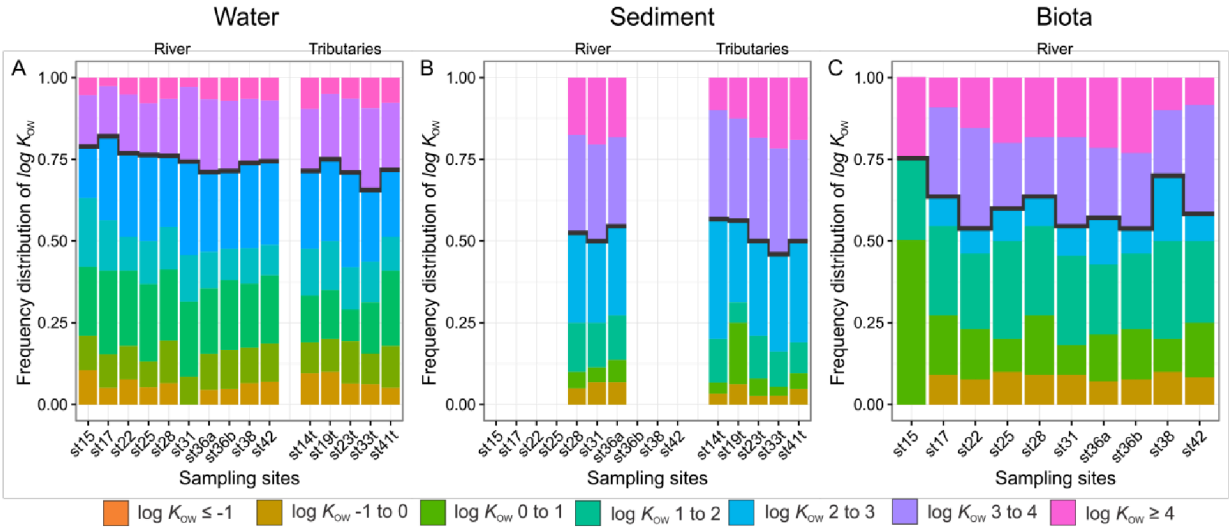

Figure 3.4: Distribution of organic contaminants according to log K_{OW} values. The y-axis represents chemicals normalised by the weight of their frequency distribution. Distribution according to (A) water, (B) sediment, and (C) biota (gammarid tissues) and sites located in the river or its tributaries. Tributaries only for water and sediment and are labelled by "t" at the end of each site. Each colour represents a log K_{OW} category arbitrarily defined and solid back line represents the cut-off value of log K_{OW}=3.

3.3.2 Total and freely dissolved concentrations

Freely dissolved concentrations were calculated for biota, sediment and water samples along the River Holtemme. No significant differences were determined for C^{fd} between tributaries and the main course of the River Holtemme for sediment and water samples (*post hoc* Dunn's-test after FDR corrections, p>0.05). However, significant differences amongst the analysed environmental compartment were calculated (*post doc* Dunn's-test after FDR corrections, p<0.05, Appendix Figure B.1). Sediments showed the highest C^{fd}, followed up by biota samples and finally water samples (1.83×10^{-7} and 2.51×10^{-7} and 6.72×10^{-9} g/L respectively, Figure 3.5). Most of the C^{fd} concentrations were rather constant with the exception of calculated C^{fd} in biota samples, which showed a slight drop at the sampling site *st*28 (kilometre 28, Figure 3.5). However, not significant differences were observed in C^{fd} between sampling sites along the River Holtemme. These are the first measurements of freely

dissolved concentrations of organic micropollutants characterised with a broader hydrophobicity range (log K_{OW} ranged from -2.2 to 5.51) in a freshwater system influenced by different anthropogenic pressures.

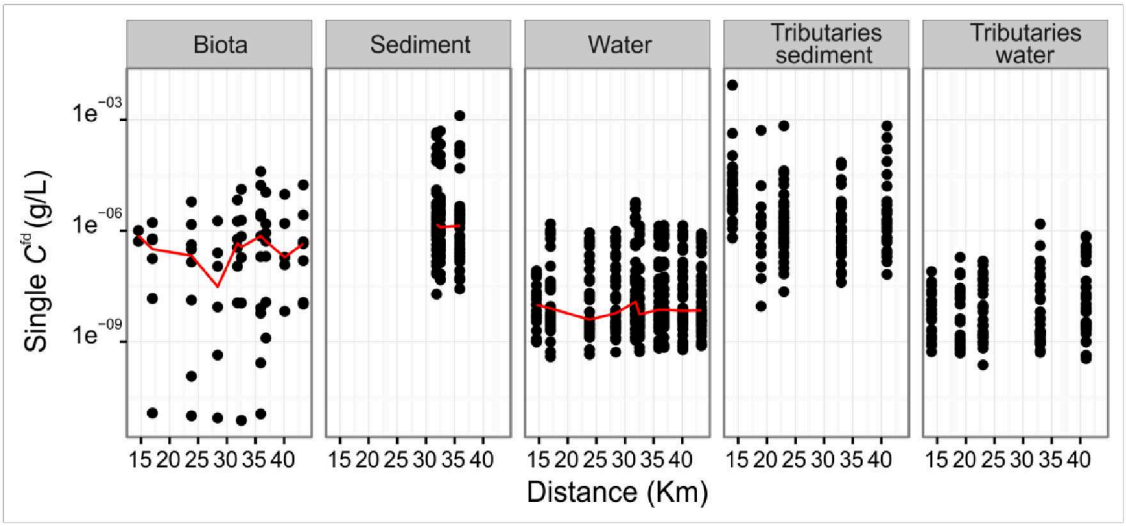

Figure 3.5: Freely dissolved concentrations in the River Holtemme and its tributaries in biota, sediment and water (g/L). The red line represents the median C^{fd} at each site along the river.

3.3.3 Partitioning coefficients

LSER approach was compared to the traditional or simplified octanol-water partitioning coefficient for both lipids (K_{LIPID}) and proteins ($K_{PROTEIN}$) in order to investigate the biota compartment. High and significant correlations were determined for both K_{LIPID} and $K_{PROTEIN}$ (Figure 3.6). Therefore, chemical activities and disequilibrium analysis were calculated using K_{LIPID} and $K_{PROTEIN}$ based on the octanol-water partitioning coefficient (K_{OW}) and LSER.

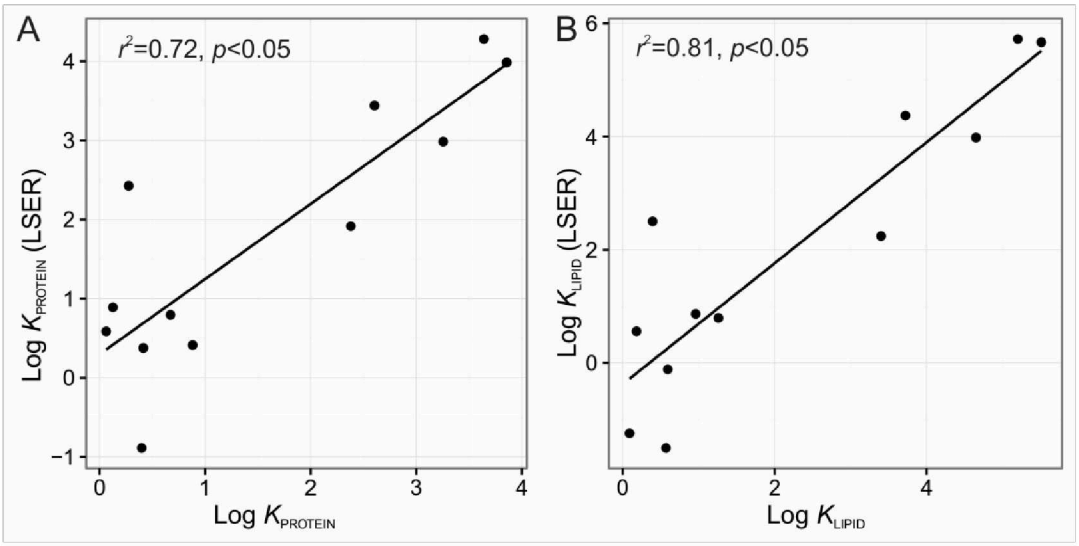

Figure 3.6: Relationship between (A) $K_{PROTEIN}$ and (B) K_{LIPID} based on K_{OW}-approach and LSER approach. Each point represents the compound determined in biota tissues at the River Holtemme.

3.3.4 Chemical activity and disequilibrium

Chemical activities were determined for all compounds in each environmental compartment in order to quantify their chemical potential. Polar organic micropollutants in the sediment compartment showed the highest chemical activities followed up by biota and finally the water compartment (1.86×10^{-5}, 6.3×10^{-8} and 2.24×10^{-8} respectively, Figure 3.7), with significant differences amongst environmental compartments (Kruskal-Wallis *post hoc* Dunn's test $p<0.05$, Appendix Figure B.2). Detail information about individual chemical activities by compound per environmental compartment is presented in the appendix section (Appendix Figure B.3-B.12).

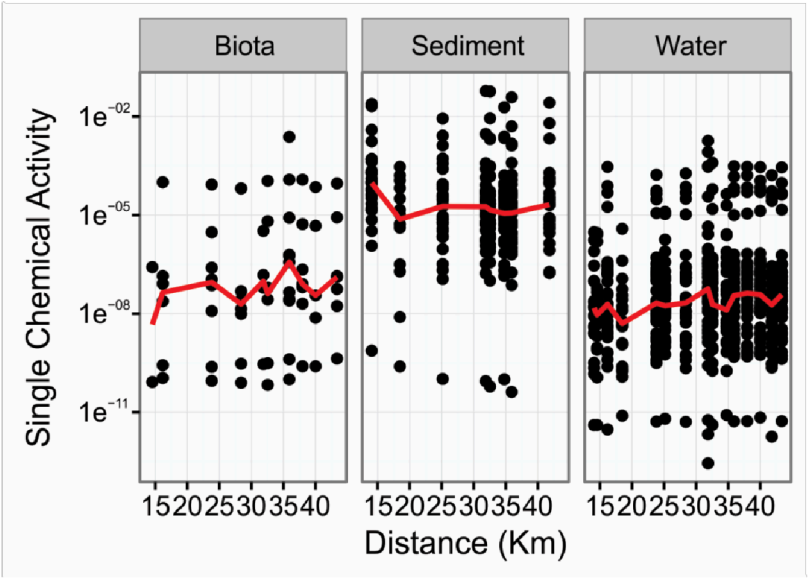

Figure 3.7: Chemical activity for all compounds in each environmental compartment along the River Holtemme. The red line represents median chemical activity.

The 10,11-dihydroxy-10,11-dyhidrocarbamazepine (CBZ-diol), transformation product of carbamazepine, showed the highest chemical activity in biota followed up by carbamazepine (Appendix Figure B.4). Overall, chemical activities were rather constant in biota samples. In the sediment compartment, the insecticide diazinon, carbamazepine, and CBZ-diol reached the highest chemical activities (Appendix Figure B.5 and B.8). Chemical activities were rather constant as in the biota phase. However, few compounds showed peaks activity, the herbicides diflufenican, prometryn, simazine and terbutryn (Appendix Figure B.6), and the industrial chemical triethyl citrate (Appendix Figure B.8). Most of these deviations occurred at the sampling site *st*25t (tributary) and downstream of the second wastewater treatment plant (*st*31) for terbutryn. Finally in the water phase, diazinon showed the highest chemical activity (Appendix Figure B.9). Insecticides together with pharmaceutical and industrial chemicals exhibited constant chemical activities. Nevertheless, a high variation was observed for herbicides and few fungicides mainly due to lower activities in tributaries. A slight pattern was observed for pharmaceutical and industrial chemicals with a notably increase of the chemical activity along the river (Appendix Figure B.12).

Based on these individual changes on chemical activity, disequilibrium (*Π)* was assessed along the River Holtemme between the environmental compartments. A mode of comparison disequilibrium between the environmental phases was calculated using the simplified K_{OW} model and LSER approach. In general, similar disequilibria patterns were observed for the biota compartments (Figure 3.8). Therefore, based on the simplicity and of the K_{OW} model, all further results are based on K_{OW} and derived K_{LIPID} and $K_{PROTEIN}$.

Disequilibrium values were determined using the chemical activities data in each environmental compartment and their coefficient of variation were 49%, 14% and 17% for Biota-Water, Biota-Sediment and Sediment-Water respectively. The higher variation was observed in the Biota-Water compartment and may be due to the inherent feature of biological tissues of storage compounds compared to water samples. Grab sampling was the sampling strategy used to collect surface water in this study, which represent a snapshot of the chemical pollution in the water phase.

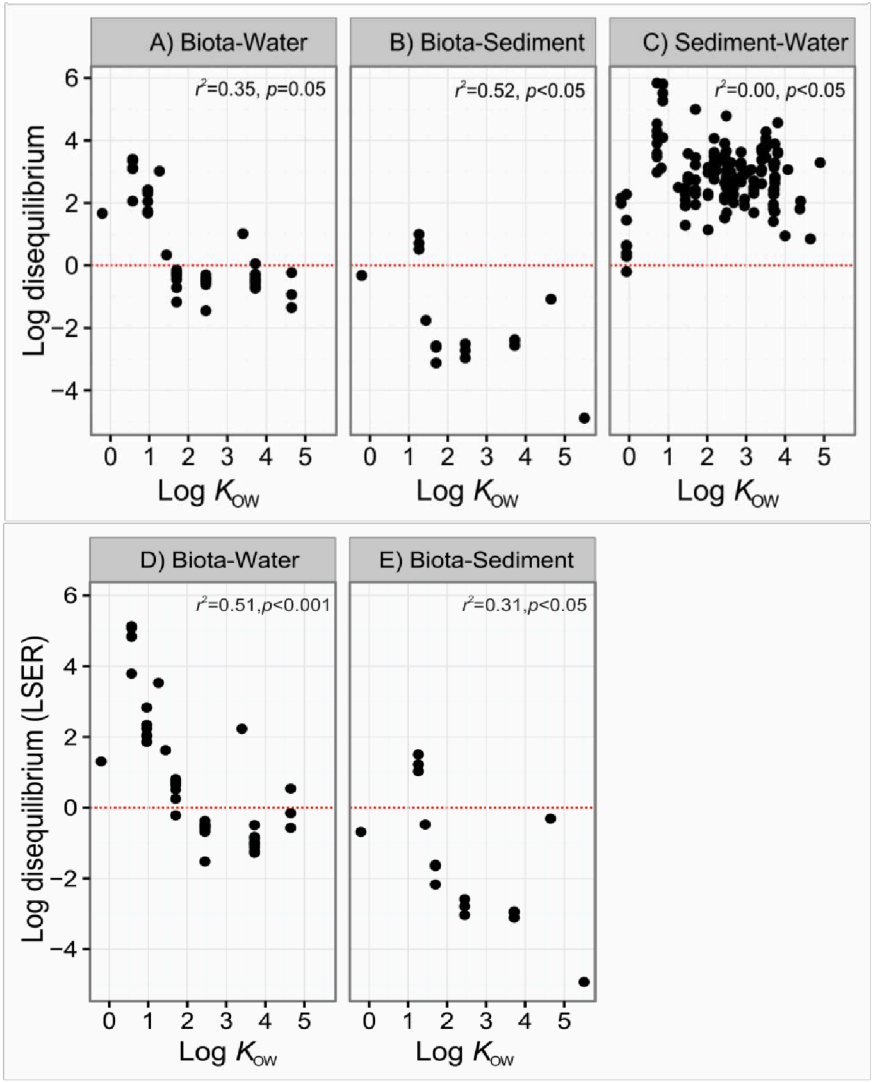

Figure 3.8: Measured disequilibria values in the River Holtemme. Each *Π* was normalised by its respective partitioning coefficient (K_{OC} for sediment, K_{DOC} for water and K_{OW} for biota) and S_L (Upper plot). Disequilibria values using LSER approach (Bottom plot). Some compounds were measured in more than sampling site therefore more than one black dot is plotted. Dotted red line represents equilibrium (Log disequilibria=0).

Accumulation of chemicals in biota compared to water is characterised by behaviour of chemicals with log K_{OW} of 1.5 to 5 very close to equilibrium (1H-benzotriazole, 4-/5-methyl-1H-benzotriazole, terbutylazine, carbamazepine and propiconazole). However, disequilibrium towards biota was observed for the most hydrophilic compounds (Figure 3.8A) (CBZ-diol, imidacloprid, fenuron and thiacloprid). While for chemicals in biota compared to sediment, most of the disequilibrium was observed toward the sediment compartment. The only exceptions were the carbamazepine transformation product CBZ-diol (log K_{OW}=-0.21) and the insecticide thiacloprid (log K_{OW}=1.26) with activity ratios close to zero indicating equilibrium between biota and sediment (Figure 3.8B). Furthermore, the Π values exhibited a trend increasing disequilibrium towards water and sediment with increasing hydrophobicity (Figure 3.8, p<0.05). Finally for the sediment-water phases, the distribution of all quantified compounds showed clear disequilibrium towards the sediment phase without a clear trend based on log K_{OW} (Figure 3.8C). Furthermore, disequilibria between sediment and water showed the most pronounced distribution towards the sediment phase of 1 to 4 orders of magnitude, and for very hydrophilic compounds up to 6 orders of magnitude (Figure 3.8C).

Overall, chemicals quantified in more than one sampling site exhibited a narrow variance in their disequilibria per compartment (below factor of ten). For instance disequilibria for propiconazole ranged from -2.84 to -2.39 in Biota-Sediment and from -0.56 to 0.18 in Biota-Water and finally from 1.73 to 2.44 in Sediment-Water phases.

3.3.4 Hazard assessment

Potential toxicity was calculated based on toxicity data (EC_{50}) and S_L values, henceforth median effective activity (Figure 3.9). The median effective activity calculated in this study was compared to the baseline toxicity range (0.1-0.01) defined for non-polar organic chemicals (in gray in Figure 3.9). Most of the compounds analysed in this study were distributed in the range of 0.01 and 10 for baseline potential or above of it. However, 13 compounds from different compound classes reached harmful median effective activity. These compounds exhibited a wide range of log K_{OW} values from -0.21 to 4 (Figure 3.9). Only one compound, the insecticides diazinon reached chemical activities in the environment capable to exert adverse effects in biota. Diazinon exhibited high median effective activities for both water and sediment phases but only sediments reached the harmful values.

Furthermore, given the additive nature associated with baseline toxicity, it is also possible to sum the chemical activities associated with mixtures organic chemicals to assess the potential risk from the mixture. Once the baseline toxicity was calculated only for compounds presented in all compartments along the river, both the median and minimum Ea_{50} were plotted together with the summed chemical activities determined in each sampling site in order to assess the toxic potential (Figure 3.10). Overall, most of the sites were in the range of the minimum and medium *Ea*50 and few sites upstream of the first WWTP did not reach the minimum threshold set up for organic micropollutants in this study.

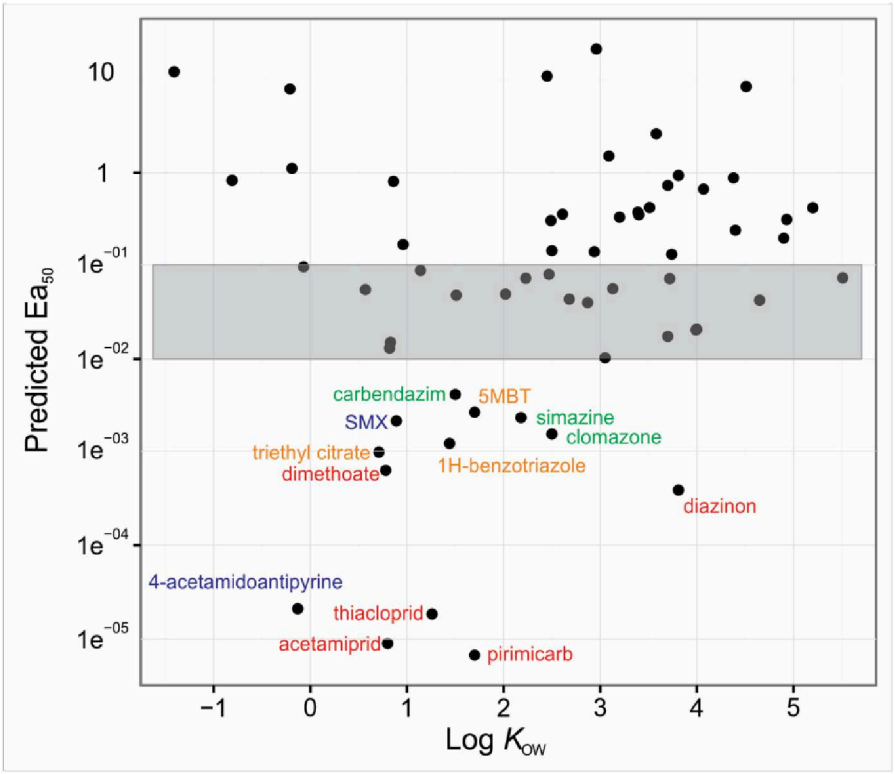

Figure 3.9: Median effective activity for organic micropollutants with log K_{OW} ranging from -1.5 to 5.5. In red insecticides, green herbicides, blue pharmaceuticals and orange industrial chemicals. In gray is highlighted 0.1-0.01 threshold effective toxicity.

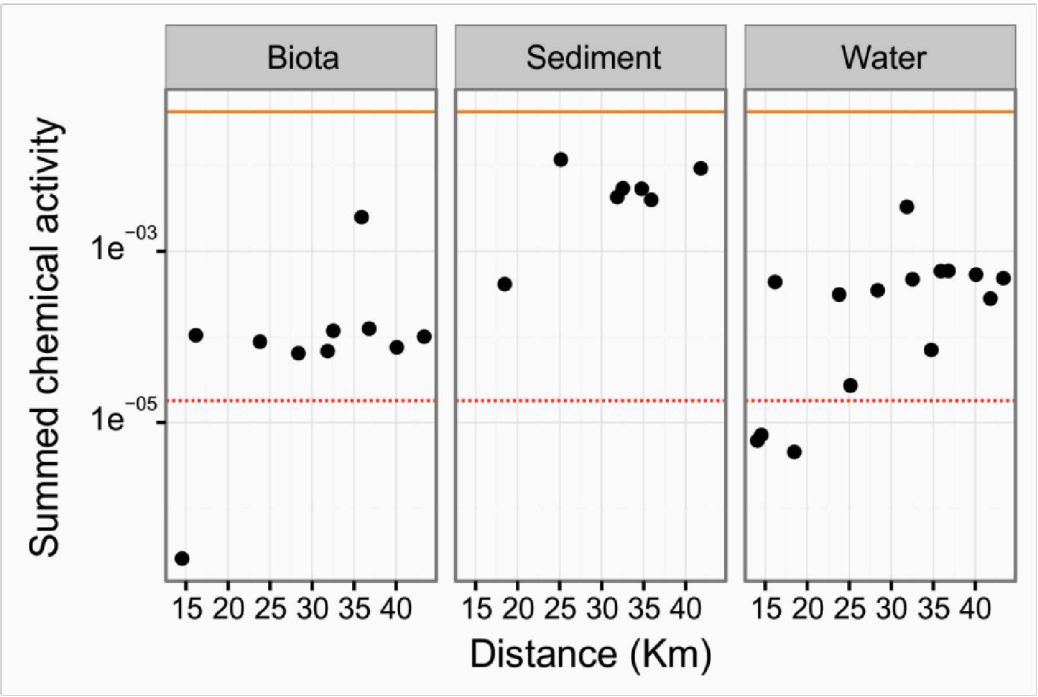

Figure 3.10: Summed chemical activities and their potential risk associated with mixture effect. The red dashed line represents the minimum effective activity and in orange the median effective activity calculated for the River Holtemme considering all the environmental compartments.

3.4 DISCUSSION

3.4.1 Chemical activity and equilibrium

Multi-compartment assessment indicated that chemical activities are an excellent predictor for body burdens of many chemicals in *Gammarus pulex*. This sound reasonable when water content range up to 85% in *G. pulex* (Maazouzi et al., 2011). However, this holds only for compounds with a log K_{OW} above about 1.5 (1H-benzotriazole, 4-/5-methyl-1H-benzotriazole, terbutylazine, carbamazepine, propiconazole and prosulfocarb). Very hydrophilic may accumulate to a higher amount in biota than expected from equilibrium partitioning into lipids or proteins (CBZ-diol, imidacloprid, fenuron and thiacloprid). This is in line with expectations since for these compounds, lipids may be no longer the predominant phase for accumulation and nonlipid material such as protein may play an important role (deBruyn and Gobas, 2007; Endo et al., 2012). Furthermore, hydrophobicity may not be longer the primary driving force but other more specific interactions become important (Burkhard et al., 2008; Escher and Hermens, 2002; Kukkonen and Oikari, 1991). Thus, the results are no indication for disequilibrium but for strong deviation of actual K_{OW} values from those defined on the basis of hydrophobicity. Interestingly, for some chemicals such as the insecticide thiacloprid strong non-hydrophobicity driven accumulation in biota can be observed and may lead to enhanced hazards. Additionally, increased chemical activities for wastewater-derived chemicals occurred downstream of wastewater treatment plant in biota samples. This is in line with higher chemical activities determined for personal care product in wastewater treatment plants effluents (Gobas et al., 2015).

Chemical concentrations but also estimated chemical activities in sediments using predicted partition coefficients are much less reliable predictors for concentrations in biota. For most compounds accumulation in biota was 2 to 3 orders of magnitude below of what has been expected from sediment activity under equilibrium conditions. This is very much in line with apparent disequilibrium between sediment and water with activities in sediments, which are above those in water two orders of magnitude. This may be partly caused by an underestimation of partition coefficients between sediments and water. This may be particularly true for the very hydrophilic chemicals (Burkhard, 2000; Burkhard et al., 2008). Similarly to biota, it is expected that for these compounds hydrophobicity-driven partitioning is less important than more specific interactions with polar groups in sediment organic matter or even with the mineral components. For many moderately hydrophobic compounds real disequilibrium might play a role. That means that these compounds might be emitted bound to particles and only slowly equilibrate with the water phase and biota.

Apparent disequilibrium between biota and sediment have been reported in marine environment (Jahnke et al., 2012) and freshwater systems (Jahnke et al., 2014a, 2014b). An organism may be in disequilibrium with its environment for several reasons, including slow uptake kinetics of hydrophobic organic chemicals, biomagnification, or biotransformation. Another plausible explanation of the observed biota-sediment disequilibrium may be the far simplistic model used in the study. Previous studies have shown that hydrophilic compounds can partition into proteins as function of the compounds hydrophobicity (Schwarzenbach et

al., 2005). Because many benthic organisms contain more protein than fat, an accurate physicochemical prediction might have to include consideration of uptake by proteinaceous materials (Lohmann et al., 2004).

Furthermore, the higher chemical activities in sediments relative to water can also be driven by ongoing sediment OC diagenesis that can reduce the sorptive capacity of the sediments and thereby increase the chemical activity of persistent chemicals in the sediment (Gobas and MacLean, 2003). But in this study was hypothesised that sediment disequilibrium might be due to the transport from agricultural areas of sediment already burden with pesticides. Thereby, contaminated soil that might exhibited higher load of pesticides are transported by rain and/or spray-drift processes to the river. Therefore, this foreign source of sediment does not get into equilibrium during transport and ended up acting as a source of pollution. Another hypothesis might be related with the role of primary producers and the subsequent sorption of chemicals to phytoplankton (Nizzetto et al., 2012). Unfortunately it was far of the scope of this study to explore primary production, but it cannot be discarded the contribution of these organisms in the freshwater system.

The multi-compartment analysis was based on seven compounds quantified at all environmental phases. Overall, these seven compounds were in line with the general disequilibrium pattern described above for all quantified compounds. However, three compounds such as CBZ-diol, thiacloprid and prosulfocarb showed up deviation from the general pattern. CBZ-diol, transformation product of the drug carbamazepine, and the insecticide thiacloprid deviated significantly towards the biota interphase compared to water and additionally showed up equilibrium behaviour in the biota-sediment interphase. Conversely prosulfocarb exhibited general equilibria behaviour amongst all environmental compartments. A plausible explanation for the biota-water deviation for CBZ-diol may be the own metabolism of the parent compound, carbamazepine, once it is uptake from the water. *Gammarus pulex*, used as proxy for the biota compartment, has the biochemical mechanism to cope with breakdown of carbamazepine as it has been empirically demonstrated (Meredith-Williams et al., 2012) and thus considerable accumulation may occur in their tissues. Thiacloprid deviation may be more related to inherent features of the compound. This compound belongs to the neonicotinoid class of insecticides and thus it exhibits a high selectivity and specificity. Neonicotonoids are not protonated but instead have an electronegative tip consisting of a nitro or cyano phamacophore that imparts potency and selective for the insect nicotinic acetylcholine receptor (nAChR) (Tomizawa and Casida, 2004). The equilibrium of CBZ-diol and thiacloprid against sediments is in concordance with direct uptake through diet. This is consistent with higher body burden of 4-nonylphenol achieved by dietary uptake in *G. pulex* under experimental conditions (Gross-Sorokin et al., 2003).

Thus, the results suggest that contaminated suspended matter and sediments in the River Holtemme might act as a source for contamination of the water phase. This idea is widely accepted that sediment could act as source of pollution in freshwater (Aouadene et al., 2008; Burkhard et al., 2008; Warren et al., 2003) and marine systems (Neff, 1984; Wiklund et

al., 2003). However, the later has been well established for hydrophobic organic chemicals with log K_{OW}>5 (Burkhard et al., 2008; Wiklund et al., 2003). Here, it was extended this finding to organic chemicals with a wide range of log K_{OW} values (-1 to 5). However, due to the small amounts of sediments found in the river, the role of contaminated sediments for water quality and exposure of *G. pulex* is expected to be limited. *Gammarus pulex* typically feeds on litter but lives only partially in contact with fine sediments. Thus, exposure via the water phase is probably the major pathway. This is in line with the excellent agreement of chemical activities in water and biota with equilibrium conditions.

3.4.2 Hazard assessment

Hazard assessment was based on hydrophobicity driven baseline toxicity as a minimum toxicity approach. Baseline toxicity is the minimal toxicity a single compound can cause when entering membranes (Escher et al., 2002). Baseline toxicity is particularly relevant for complex environmental mixtures, since all chemicals can contribute to baseline mixture toxicity. Even if all of them are below the threshold level for individual toxicity, the underlying cumulative baseline toxicity might determine the overall toxic effect (Escher et al., 2002). Estimated hazards strongly depend on which compartment is considered. Highest hazards can be observed if chemical activities of sediment contaminants are used for assessment. However, as discussed above, sediments seem to be a poor predictor for accumulating contamination.

Chemical activity in water could be shown to reasonably predict body burdens and thus are expected to be a good basis for hazard assessment when internal effect concentrations are not measured. In most of the sites, summed chemical activities are below the median baseline toxicity predicted in this study. However, results showed that sites under the influence of point-source of pollutants (e.g., sites at kilometre 32 and 36) are close to the median baseline toxicity. Thus, due to these relatively narrow differences between summed chemical activity and the predicted baseline toxicity adverse effects cannot be excluded in those specific sites.

3.5 CONCLUDING REMARKS AND OUTLOOK

Society is facing a variety of challenges in environmental risk assessment (ERA): growing concerns about the effects of multiple stressors (both chemical and non-chemical); risk associated with exposure to complex mixture; and demands to quantify local site-specific risks. Here, a detail analysis of organic contaminants affecting an important component of the macroinvertebrate community, *G. pulex*, a representative species of the Biological Quality Elements (BQEs) according to the European Union Water Framework Directive (EU WFD) for a typical Central European Rivers is provided. Besides, surrounding sediments and overlying water phases were analysed in order to have a more integrative picture of the distribution of organic micropollutants in a freshwater river-system.

The multi-compartment approach used in this study may be improved in several ways. With respect to sediment, it may be taken in consideration to include black carbon

measurement as well as black carbon partitioning coefficient for more precise outcomes. Likewise, the idea that organic micropollutants bioaccumulation occurs only in the lipid phase of the organisms might be quite simplistic and erroneous. This may be especially problematic for organisms with relatively low lipid contents as compared to other abundant tissues such as protein or lignin. Furthermore, the use of stationary sampling devices such as membranes and/or large volume water sampler may contribute to have a better understanding of the chemical in the water phase and have a more holistic picture for further comparison based on long-term exposure. Another improvement would be to measure both K_{DOC} and K_{POC} and even more measure directly concentrations associated to POM or DOM in order to verify the modelling approach. Additionally, the findings in this study reinforce more integrative assessments of the aquatic system based on multi-compartments analysis.

CHAPTER 4

Evidence of disrupted genetic variability, mutation rate and gene flow under multiple anthropogenic threats: insight of a model freshwater population in the Holtemme River

ABSTRACT

Environmental pollution including mutagens from wastewater effluents and discontinuity by man-made barriers are considered typical anthropogenic pressures on microevolutionary processes that are responsible for the loss of biodiversity in aquatic ecosystems. Here, the effects of wastewater treatment plants, weirs and other stressors on the invertebrate species *Gammarus pulex* were tested at the population genetic level combining evolutionary ecotoxicology, body burden analysis and testing for exposure to mutagens. Exposure to chemical pollution alone and in combination with the presence of weirs resulted in a depression of allelic richness in native *G. pulex* populations. The results suggest that the input of a mutagenic effluent from a wastewater treatment plant resulted in a strong increase in private alleles over the affected populations. In addition, the presence of weirs along the river disrupted the migration across the river and thus the gene flow between *G. pulex* upstream and downstream. This study provides strong evidence that the assessment of genetic variation including private alleles together with the contamination mutagenic and nonmutagenic chemical pollution offers new insights into the regulation of genetic population structure and highlights the relevance of emerging anthropogenic pressures at the genetic level.

Submitted in a slightly modified form as:

Pedro A. Inostroza, Iván Vera-Escalona, Anna-Jorina Wicht, Martin Krauss, Werner Brack and Helge Norf. Anthropogenic stressors shape genetic structure: insights from a model freshwater population along a land use gradient. Environmental Science & Technology 2016, 50(20), 11346-11356.

4.1 INTRODUCTION

Organic micropollutants such as pesticides, biocides, pharmaceuticals, personal care products, and industrial chemicals are ubiquitous in the aquatic environment (Schwarzenbach et al., 2006). Even if their ecological effects in the environment are still poorly studied, they are considered to pose emerging anthropogenic pressure on microevolutionary processes responsible for the current loss in biodiversity (Brown et al., 2009; Medina et al., 2007). Most of these chemicals occur at low concentrations. Nevertheless, many of them raise significant environmental health concerns, especially when occurring as mixtures in the environment (Altenburger et al., 2004). Organic micropollutants enter surface water bodies from a variety of sources such as effluents of wastewater treatment plants (WWTPs), untreated wastewaters, urban runoffs, and by leaching from agricultural lands (Reemtsma et al., 2006).

There is particular concern that anthropogenic pressures such as land use change, input of chemicals into the aquatic ecosystems may affect the genetic structure of natural populations and impair ecological functions in freshwater ecosystems (Brown et al., 2009). Recent studies reporting alterations in the genetic structure are closely related to bottlenecking due to pollution (Fratini et al., 2008; Gardeström et al., 2008; Matson et al., 2006), pollution-induced natural selection (Bridges and Semlitsch, 2001; Theodorakis and Shugart, 1997), ecological sinks (Baker et al., 2001; Theodorakis, 2001) and increased mutations rates (Rinner et al., 2011; Theodorakis et al., 2006). From a population genetic perspective, the exposure to chemical pollution may result in loss of genetic variation and a decrease in fitness, a process referred to as genetic erosion as proposed by van Straalen and Timmermans (van Straalen and Timmermans, 2002). Although not in all cases exposure and adaptation to pollution result in a loss of genetic variability (Bach and Dahllöf, 2012; McMillan et al., 2006; Whitehead et al., 2003), the authors suggest that the reduction of genetic variation is one of the most common effects of long-term exposure to anthropogenic toxicants. Genetic erosion in the sense of a loss in allelic richness and diversity may impair population fitness and consequently decrease the adaptive potential of biota toward future stressors (Bijlsma and Loeschcke, 2012). In concordance with population genetics theory, a population with low genetic diversity appears to be less adaptable to environmental changes (Brown et al., 2009).

Hence, the effects of pollutants on genetic population structure may be more disruptive for ecosystem functioning than individual-level effects: When water quality improves, metabolic and molecular processes of organisms may return to nonimpacted status within days to weeks, whereas the genetic population structure may be irreversibly disrupted (Bickham et al., 2000; Theodorakis, 2001). Moreover, both genotoxic (i.e., mutagenic) and non-genotoxic pollutants can cause direct and indirect heritable effects as DNA base substitutions, deletions or duplications, and reproduction impairments, and alterations of dispersal patterns, respectively (Bickham, 2011; Rose and Anderson, 2005).

However, chemical pollution is typically not the only factor influencing genetic variation in multiple-stressed aquatic environments. For example, man-made barriers in

streams and rivers can cause severe effects on both ecosystem structure and functioning by inducing serial discontinuity (Kindlmann and Burel, 2008; Mueller et al., 2011; Ward and Stanford, 1983), higher sedimentation in upstream waters, and altered nutrients fluxes (Allan and Castillo, 2007). Accordingly, many studies have reported adverse effects of dams and weirs on freshwater fish populations including alterations in gene flow and reproduction impairments (Hansen et al., 2014; Junker et al., 2012). Conversely, Weiss and Leese (2016) found that colonization history influenced the genetic population structure of *Gammarus fossarum* in highly human-impacted landscapes, whereas in-stream barriers such as weirs and/or barrages had rather limited effects. The past decade has delivered a number of studies in landscape genetics and evolutionary toxicology dedicated to the effects of global pressures (e.g., pollution, or fragmentation and destruction of habitats) on genetic patterns (Bickham, 2011; Manel and Holderegger, 2013). Although considerable efforts were spent to reveal such evolutionary impacts, most of the available studies on genetic variation only explored the effects of single stressors, which does not reflect reality in most aquatic ecosystems (Nõges et al., 2016). Despite an increasing number of investigations on genetic variation in wildlife, our understanding about potential ecological effects of reduced genetic variation is still limited (Hughes et al., 2008). Recently, it was suggested to extend environmental risk assessment to novel approaches including ecological and evolutionary functional genomics (van Straalen and Feder, 2012).

While risk assessment is typically based on external toxicant concentrations in waters and sediments, there is increasing awareness that body burden is a key to exposure of and effects on organisms (Rappaport and Smith, 2010). At the same time the body burden may provide time integrated patterns of bioavailable pollutants whereas key date samplings only provide a snapshot of often highly dynamic concentrations in waters. Since the enormous complexity of chemical mixtures in the environment often prevents a comprehensive analysis of chemical contamination, bioanalytical tests can be involved for a more holistic effect-based characterisation of contamination (Altenburger et al., 2015). For example, mutagenicity testing of tissue extracts and water samples may provide information on contaminants impacting the genetic structure of aquatic organisms directly, and genetic population analysis has the potential to provide new insights into such pressures.

Due to its remarkable gradient of anthropogenic influences with clear defined sources of pollution, the River Holtemme (Saxony-Anhalt, Germany) was chosen as a test case for anthropogenic-derived stressor effects as it combines typical features of many central European rivers in close proximity. *Gammarus pulex*, a benthic shredder amphipod, is ubiquitous in European running waters (Jażdżewski, 1980). It plays a key function in freshwater ecosystems, particularly by breaking down coarse particulate organic matter (MacNeil et al., 1997) and by linking organic material to higher-level consumers such as fishes (Friberg et al., 1994). *G. pulex* has previously been used as model organism for assessing both adverse effects (Cold and Forbes, 2004) and uptake of organic micropollutants under laboratory conditions (Ashauer et al., 2012).

In order to characterise the impact of chemical pollution and other stressors on population genetic structure in a typical Central European small river, we examined the change of genetic variation of a freshwater invertebrate population depending on the occurrence of particular pollution sources and other stressors along the River Holtemme. Evolutionary ecotoxicology and body burden analysis of the invertebrate *G. pulex* were combined and supplemented with mutagenicity testing with the Ames fluctuation test (AFT) at selected sites. Preliminary tests indicated that mutagenic contamination might occur at distinct river stretches. Thus, three major hypotheses were addressed: (1) exposure to chemical pollution (e.g., wastewater-derived pollutants and pesticides) leads to a depression of genetic variation in native *G. pulex* populations, (2) mutagenic water contamination results in observable genetic effects in these populations, and (3) the presence of man-made barriers is prone to affect the migration of biota, and therefore a certain level of differentiation is expected along an asymmetric freshwater system.

4.2 METHODOLOGY

4.2.1 Study area and sampling strategy

The River Holtemme is located in the Bode catchment (Saxony-Anhanlt, Germany; Figure 4.1) and is 47 kilometre long. Its course starts in a mountain brook of high water quality before becoming an increasingly polluted and channelized lowland river. The River Holtemme catchment is characterised by semi natural forest in the upstream sections, and agricultural areas and medium-sized towns in the central and lower sections.

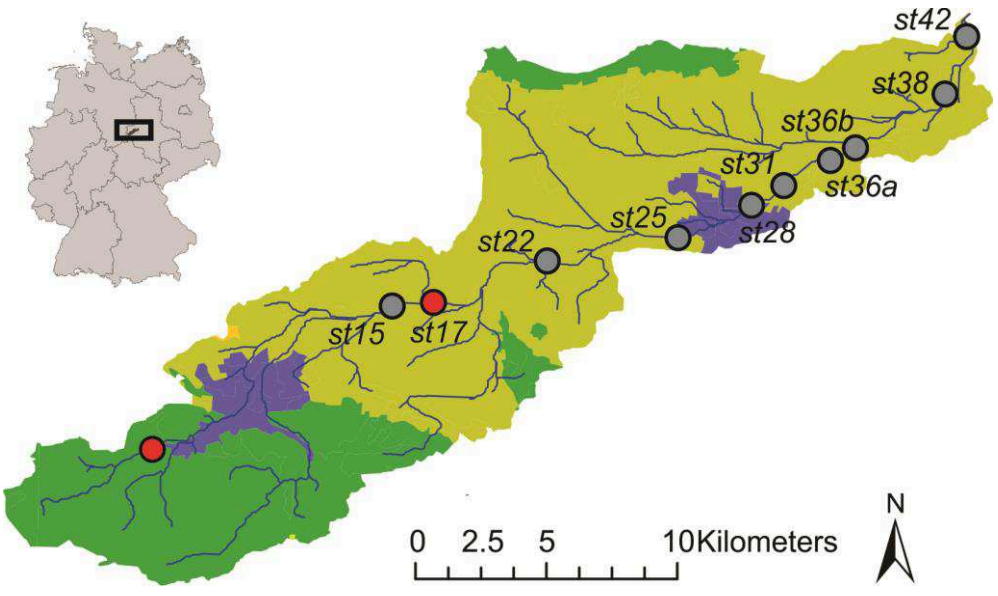

Figure 4.1: Location of the study area; in grey sampling sites where *G. pulex* were collected both to genetics and chemicals analysis along the River Holtemme. Red indicate points where water samples were collected using a LVSPE sampler for mutagenicity assays (AFT). Green colour represents forest; olive colour represents agricultural landscapes and blue main cities.

Effluents of two WWTPs serving approximately 150,000 inhabitants, together with agriculture, represent the main source of pollution (Reuter et al., 2003). Stressors were categorised based on their presence/absence and the degree of influence according to the results of a key date sampling campaign in October 2014 including measurements of a variety of environmental variables. Briefly, in order to assess the correlation between population genetics responses and environmental stressors two matrices were used. The environmental pressures matrix was defined as explained in Table 4.1.

Table 4.1: Definition and categorisation of multiple stressors in the River Holtemme.

Category	definition	weight	description
I	high/direct influence	1	occurrence of stressors on-site
II	moderate/indirect influence	0.66	stressors <1.5 km upstream or distant from sites
III	low/tailed influence	0.33	stressors <2.5 km upstream or distant from sites
IV	no influence	0	stressors >2.5 km from sites

Macroinvertebrates were sampled from ten sites along the River Holtemme (Figure 4.1) in October 2014 following a sampling protocol by Hering et al. (2004). Briefly, 20 habitat-weighted samples were taken from a total area of 1 m^2 at each site with a Surber sampler (500 μm mesh size). A subset of 24 specimens of *G. pulex* per sample was taken for genetic analyses. The remaining sample was preserved in 96% ethanol for further abundance analysis or frozen for chemical analysis. For genetic and body burden analyses specimens from different size classes were chosen in order to avoid biases produced by different ages of specimens.

4.2.2 Body burden and chemical analysis

A list of 74 analytes with a wide range of hydrophobicity (log K_{OW} from -0.21 to 5.51) was selected for body burden analysis based on their occurrence in water and sediments. These compounds belonged to different classes of pollutants such as pesticides, pharmaceuticals, industrial chemicals and some of their main transformation products. Organic micropollutants were extracted from *G. pulex* using multi- and non-target screening methods based on pulverised liquid extraction (PuLE) and a modified QuEChERS with an additional hexane phase (CHAPTER 2). Briefly, 900 mg thawed gammarids were homogenised in 4 mL acetonitrile: water (1:1 *v/v*) and 1 mL of hexane using an Ultra-Turrax® T-25 (IKA) per 1 minute and subsequently vortexed for 1 minute. Aliquots of 4 mL of homogenised samples were thoroughly mixed with 800 mg of anhydrous $MgSO_4$ and 200 mg of NaCl, vortexed again and centrifuged at 4,000×*g* for 5 minutes. Aliquots of 3.5 mL of supernatant were transferred to glass centrifugation tubes containing 50 mg of PSA and 400 mg of anhydrous $MgSO_4$. After vortexing and centrifugation the supernatant was concentrated and dried under nitrogen stream at room temperature. Finally, the residues were reconstituted in 500 μL of MeOH and filtered with a PTFE syringe filter (pore size 0.45 μm, Chromafil®) for further analysis using a 1260 Infinity LC system (Agilent) coupled to a QTrap 6500 MS (ABSciex) with IonDrive™ Turbo V ion source.

4.2.3 Organic micropollutants as chemical stressors

Based on the equilibrium partitioning theory (Di Toro et al., 1991; Reichenberg and Mayer, 2006), freely dissolved water concentrations of organic micropollutants can be predicted using appropriate partitioning coefficients and the total measured concentration in *G. pulex*. Freely dissolved concentrations (C^{fd}) (μg/L) of organic micropollutant can be estimated as follows:

$$C^{\mathrm{fd}} = \frac{C^{\mathrm{t,G}}}{f_{\mathrm{LIPID}} K_{\mathrm{OW}}} \tag{4.1}$$

where $C^{\mathrm{t,G}}$ is the total measured concentration (μg/Kg) in *G. pulex*, f_{LIPID} the lipid fraction and K_{OW} being a reasonable parameter in lack of an experimental K_{LIPID}. Lipid content was not measured in this study. Instead, values reported by Ashauer et al., (2010, 2006) were used (1.34% wet weight). The K_{OW} values were obtained from the software KowWin v1.68 submodel in EPISuite v4.11 (US Environmental Protection Agency (USEPA), 2012).

Freely dissolved concentrations (C^{fd}) were converted to chemical stress using the toxic units (TUs) approach (Sprague, 1970). To derive respective TUs, measured pollutant concentrations were scaled to inherent toxicity of each pollutant towards the model organism *G. pulex*. In cases where *G. pulex* data was missing, toxicity data from the model organism *D. magna* were used:

$$\mathrm{sTU} = \log \Sigma \left(\frac{C_{\mathrm{i}}^{\mathrm{fd}}}{EC_{50,\mathrm{i}}} \right) \tag{4.2}$$

where C^{fd} is the freely dissolved concentration of the compound *i*, and $EC_{50\mathrm{i}}$ is the respective median acute effect concentration in a standard toxicity test (48h). The summed TU (*s*TU) was calculated, including all the compounds detected in each tissue sample (TU Gam). For *s*TUs, the suggested threshold value for observed acute effects in the field is ≥ -3.0 (Liess et al., 2008). The summation of all TUs is based on the principle of toxic additivity; as the number of components in a toxic mixture increases, the range of deviation from toxic additivity is suggested to decrease (Warne and Hawker, 1995). Concentrations below the limit of quantification were excluded from the calculation of TUs in order to avoid overestimations.

4.2.4 Microsatellite analysis

The DNA of *G. pulex* was extracted using the NucleoSpin® 96 Tissue Kit (Macherey-Nagel) following the manufacturer's recommendations. Concentration and quality of DNA was measured using a UV-Vis nanophotometer (NanoDrop Technologies Inc.). Nine microsatellite markers were amplified by polymerase chain reactions (PCR) (Table 4.2). Five loci were amplified and genotyped using primers previously developed for *G. pulex* (Gergs et al., 2010). Four additional markers were originally designed for the sibling species *G. fossarum* and validated for *G. pulex* (Danancher et al., 2009; Westram et al., 2010). Primers

were optimised and combined in two multiplex PCR (Table 4.2). Reverse primers were pigtailed (Brownstein et al., 1996) and forward primers were fluorescently labelled (6-FAM, HEX or TET) for genotyping. PCR were performed in a final volume of 11 μL containing 5.5 μL 2× Phire Hot Star II PCR Master Mix (with 1 U Taq polymerase, dNTPs and $MgCl_2$), 0.55 μL DMSO, 0.44 μL Q-Solution (Qiagen), 1 μL of DNA, 0.30-0.60 μM primer (Table 4.2), and PCR-grade water. PCR conditions were as follows: 30 seconds at 98°C followed by 30 cycles with 5 seconds at 98°C, 15 seconds at the annealing temperature of 58°C, 10 seconds at 72°C and finally 1 minute at 72°C. PCR products were purified by ethanol/EDTA (ethylenediaminetetraacetic acid) precipitation protocol (PRISM, 2010) and resuspended in 10 μL HiDi formamide (Thermo Scientific). Each sample was mixed with 0.1 μL ROX size standard 500 (MCLAB) for genotyping, denatured at 90°C for 2 minutes, immediately chilled on ice, and separated on an ABI Prism 3130XL Genetic Analyzer (Applied Biosystems). Visualisation and genotyping of microsatellite markers was performed in GeneMapper 4.0 (Applied Biosystems).

4.2.5 Genetic variation and differentiation

Genotyped microsatellite data were analysed with MICRO-CHECKER 2.2.3 (van Oosterhout et al., 2004) for detection and correction of null alleles and stutter peaks. The presence of outliers was evaluated using an F_{ST} outlier detection method in LOSITAN (Antao et al., 2008) by running 5×10^5 simulations with a confidence interval of 0.95. Allelic frequency, and richness, and the observed and expected heterozygosity were calculated. The Exact tests of Hardy-Weinberg equilibrium (HWE; 10^6 steps in the Markov Chain Monte Carlo (MCMC) and 10^5 dememorization steps), and Linkage Disequilibrium (LD; 10^4 permutations) were performed with Arlequin 3.5 (Excoffier and Lischer, 2010). GENALEX 6.5 (Peakall and Smouse, 2012) was used for calculating unbiased expected heterozygosity (uH_E), which is a better metric for genetic diversity when sample sizes are low (Pruett and Winker, 2008). The False Discovery Rate (FDR) was calculated to correct the results for multiple tests in both HWE and LD. In addition, differentiation indices between sites by calculating pairwise Jost's D (Jost, 2008) and Wright´s F_{ST} (Weir and Cockerham, 1984) were compared using R (R Development Core Team, 2008) and the diveRsity package (Keenan et al., 2013). Population bottlenecks were identified using BOTTLENECK 1.2.02 (Cornuet and Luikart, 1996). Two phase models (TPMs), default settings and combinations of 95% single-step mutations and 5% multistep mutations were used, with a variance of 30 among multiple-step mutations (10^4 replications), and the significance was tested with the Wilcoxon test. Finally, a point-estimator method based on linkage disequilibrium (Do et al., 2014) (NeEstimator, version 2.0.2) restricted to alleles with frequencies >0.02 was used to compare rough estimates of effective population sizes (N_e).

Table 4.2**:** Primers sequences and concentrations used for nine microsatellite loci in *G. pulex* species.

Name	**Primer Sequence**	**Motif**	**Multiplex**	**Primer Concentration**	**Reference**
gapu-8	F: GAGCGTCATCATTTCCATCC	$(AT)_8$	A	0.45 μM	(Gergs et al., 2010)
	R:*GCCAATCAGGGAACTGAGAA			0.45 μM	
gapu-9	F: CTATGCCCAAGCTGGTTGTT	$(ATT)_9$	B	0.30 μM	(Gergs et al., 2010)
	R:*TTCGCGTCATTCACTCGTAG			0.30 μM	
gapu-23	F: CAGCAAGTGGTGCAGCTAAA	$(GCA)_{11}$	A	0.45 μM	(Gergs et al., 2010)
	R:*CAGCCACATCGAAGCTGTAA			0.45 μM	
gapu-29	F: CCTGCTCAGTAACAGCCTCA	$(TTAA)_4/(AT)_4$	B	0.50 μM	(Gergs et al., 2010)
	R:*TCAAATCGAGAAGGCTACAACA			0.50 μM	
gapu-30	F: AAGTCGTTGCCATTGCTCTC	$(GT)_5/(ACA)_5/(CAA)_{4+5}$	A	0.45 μM	(Gergs et al., 2010)
	R:*TCTTGGAGAGGGTGAGGTTG			0.45 μM	
gam2	F:	$(CATA)_{13}$	single	0.45 μM	(Danancher et al.,
	R:*ATCGCAGTGGCTCTCTGAC			0.45 μM	
gam4	F: TCTGCTGACAACAACTACTTCAAC	$(TAC)_{26}$	single	0.45 μM	(Danancher et al.,
	R:*CATGGCGCAACTAACCAGC			0.45 μM	
gamfos10	F: GGCTGGGCTAGTTGTATTGC	$(CTA)_{10}$	A	0.60 μM	(Westram et al., 2010)
	R:*AAGACGACTAAGGGGTCTGC			0.60 μM	
gamfos28	F: ACCTCTCCATCCCTGATGC	$(AC)_{13}$	single	0.45 μM	(Westram et al., 2010)
	R:*CATCGACCCGTCAGTATGTG			0.45 μM	

* indicates “pigtail” (see Materials and methods).

4.2.6 Population Structure

The presence of distinct population clusters was assessed using STRUCTURE 2.3.4 (Pritchard et al., 2000), assuming an admixture model and correlated allele frequencies with LOCPRIOR turned off. STRUCTURE was run for K=1 to K=n+1, where n was the maximum number of sites sampled in the River Holtemme. Ten independent runs were conducted for each K, with 2×10^5 burn-in periods, followed by 2×10^6 MCMC steps for each site. The likelihood results were collected and assessed in STRUCTURE HARVEST (Earl and von Holdt, 2012). The Evanno method (Evanno et al., 2005) was used to detect the number of clusters. The Greedy algorithm in CLUMPP 1.1.2 (Jakobsson and Rosenberg, 2007) was used to create a single plot based on ten independent runs, and the final graphic results were generated in DISTRUCT 1.1 (Rosenberg, 2004). In addition, assignment probability values (Q-values) were derived in order to obtain differentiation measures based on Bayesian analysis.

4.2.7 Directional relative migration

A recent approach was used to calculate directional relative migration, henceforth gene flow. The method is explained in detail by Sundqvist *et al.*(2016). Briefly, this approach calculates a directional component of genetic differentiation using any classical measures of differentiation such as Nei's G_{ST} (Nei, 1973) or Jost's D (Jost, 2008). Directional D-values (D_d) were then calculated the same way as regular D-values, with the exception that the samples were compared to the pool of migrants instead of to each other (Jost, 2008). Only upstream-downstream measures, consistent with our unidirectional asymmetric case of study, were used to calculate gene flow. To test whether gene flow was significantly higher in one direction than the other (i.e., asymmetric migration), 95% confidence intervals were calculated from 10^3 bootstrap iterations. Gene flow between the 10 different sites was normalised and varied between zero and one, yielding a relative measure of direction of migration between the different sample sites. Directional relative migration rates were calculated using the "divmigrate" function from the R-package diveRsity. Values below 0.20 were discarded from the data in order to identify major gene flow barriers (Sjöqvist et al., 2015).

4.2.8. Statistical analyses

Spatial patterns of genetic variation were investigated using allelic richness (A_R) and private alleles (N_{PA}, i.e. alleles occurring in only one cluster) in a rarefaction analyses with HP-RARE 1.1 (Kalinowski, 2005); a higher frequency of private or rare alleles in polluted sites can be used as a proxy for differences in mutation rates between sites (Theodorakis et al., 2006; Whitehead et al., 2003). Generalized linear models were calculated to identify which of the variables (i.e., distance, abundance, and several physical-chemical parameters of the water samples) were relevant to explain variation in the response variable. Sample independence was tested using Fisher's exact tests across loci and per locus (10^5 MCMC replicates). Inbreeding (F_{IS}) with 95% confidence interval (10^4 bootstrap replicates), was calculated using the R-package diveRsity. Measures of genetic differentiation was evaluated

using the multivariate Mantel test, which was based on calculation of genetic and environmental distance measures between every pair of populations, with 10^4 random permutations using the R-package ade4 (Dray and Dufour, 2007). The genotype accumulative curve was constructed by randomly sampling *x* loci and counting the number of observed multi locus genotypes using the R-package *poppr* (Kamvar et al., 2014). These curves are useful for determining the minimum number of loci necessary to discriminate population structure with 95% of confidence.

Canonical ordinations were conducted to assess population genetics responses to anthropogenic pressures. Each stressor was normalised by $\log_{[X+1]}$ transformation before detrended correspondence analysis on population genetics data, which revealed a linear gradient requiring a redundancy analysis (RDA). RDA was performed on four population genetic responses against the environmental variables explained in Table 4.1. For the variable WWTP, the compound carbamazepine was used as proxy for wastewater (Bahlmann et al., 2014; Nakada et al., 2008). Concentrations were normalised according the highest concentration determined in the River Holtemme (Table 4.3). Statistical significance of RDA axes and environmental parameters were assessed using a permutation test with 10^4 random permutations. Multivariate analysis was performed using the R-package vegan (Oksanen et al., 2015). Significant differences between groups were identified using one-way ANOVA and Duncan's *post hoc* test. Differences were considered significant when $p<0.05$.

4.2.9 Mutagenicity analysis by the Ames fluctuation test (AFT)

AFT was performed in order to assess the on-site mutagenic potential of gammarid tissues and water samples collected at two sampling sites (Figure 4.1) before and after the presence of emergence of private alleles as indicators of mutagenicity in *G. pulex*. To obtain a sufficient water volume we used an on situ large volume solid phase extraction device (LVSPE). At the sampling sites *st*2 and *st*17 (Figure 4.1), subsamples of water from the River Holtemme were continuously collected over 28 days resulting in final sample volumes of 130 to 210 litres. Water samples were filtered through a glass fiber filter cartridge (Sartorius GF+ Midicap, 0.63 μm) to remove suspended particulate matter and passed through a tailor-made SPE column filled with 10 grams of Chromabond® HR-X (Macherey-Nagel). The mixture was eluted with three different solvents: ethyl acetate: methanol, methanol(2% ammonia) and methanol (1.2% formic acid) in order to obtain acidic, basic, and neutral compounds from the resin. All extracts were combined, neutralised, filtered (Whatman GF/F) and reduced in volume to a final concentration factor of 1000.

For AFT aliquots of gammarid and water extracts were dried under nitrogen stream and residues were reconstituted in 80 μL in dimethyl sulfoxide (DMSO). The AFT was carried out with *Salmonella typhimurium* tester strains TA98 as described by Hug et al. (2015). The mutagenic activity was determined from the exponential fit of the dose-response curves using the slope of the curve (*b*) expressed as revertants per L sample in L methanolic extract (Gallampois et al., 2013).

4.3 RESULTS

4.3.1 Body burden as proxy for chemical stressor

The selected organic micropollutants are typically detected in sediment and water samples in European freshwater systems due to the influences of WWTP and agriculture. It was detected and quantified a total of 17 out of 74 organic micropollutants in *G. pulex*'s tissues (Table 4.3). Concentrations ranged between 0.47–3.22 ng g^{-1} wet weight for insecticides, 0.12–3.85 ng g^{-1} wet weight for fungicides, 0.19–13.08 ng g^{-1} wet weight for herbicides, and 0.48–3.92 ng g^{-1} wet weight for wastewater-derived chemicals. Notably, wastewater-derived pollutants such as carbamazepine (CBZ) and its transformation product 10,11-dihydroxycarbamazepine (CBZ-diol), and the corrosion inhibitors 1H-benzotriazole and 4- and 5-methyl-1H-benzotriazole (5MBT), which could not be separated, were detected. It was found a general increase in both numbers and the concentrations of wastewater chemicals in *G. pulex* in the course of the River Holtemme with strong peaks after WWTPs, while the number of detected compounds and their concentrations were low at site *st*15 before the first WWTP (Figure 4.2). Sampling site *st*36a exhibited the highest internal concentrations of organic micropollutants in gammarids along the river course. This sampling site was mainly characterised by elevated concentrations of wastewater-derived chemicals and a peak in the herbicide prosulfocarb.

Table 4.3: Detected organic micropollutants in *G. pulex* (concentrations in ng g^{-1} wet weight). Symbol + means pollutant was detected but under the method quantification limit (MQL).

	MQL	**st15**	**st17**	**st22**	**st25**	**st28**	**st31**	**st36a**	**st36b**	**st38**	**st42**
Insecticides											
Imidacloprid	1.11	+	+	1.13	+	1.26	2.46	3.14	2.02	1.79	3.22
Thiacloprid	0.03	0.47	1.51	1.35	1.67	1.64	1.75	2.30	1.39	1.44	2.42
Fungicides											
Flusilazole	0.24			+							+
Spiroxamine	0.09	+	0.19	0.16	0.14	+	0.12	0.18	+		
Tebuconazole	1.00		+	+	+	+	+	+	+	+	+
Propiconazole	0.05		3.85	3.49	2.27	2.94	2.92	2.13	3.06	1.74	2.69
Herbicides											
Atrazine	1.22							+		+	
Diflufenican	0.71								+		
Fenuron	0.11	0.46	0.26	0.19		0.27		1.11	0.40		0.23
Pendimethalin	0.88			0.92				2.10			
Prosulfocarb	0.82				0.97	+	+	13.08	2.80	+	+
Terbutryn	1.18		+	+			+	+	+		
Terbuthylazine	1.07										1.45
Wastewater chemicals											
Carbamazepine	0.29		2.48	2.01	1.53	1.54	2.65	2.79	2.83	1.69	2.19
CBZ-diol	1.14		+	+	+	+	+	1.23	+	+	+
1H-Benzotriazole	3.85		+	+	+	+	+	3.92	+	+	+
5MBT	0.03		1.52	0.79	0.63	0.88	1.75	1.78	1.30	0.48	1.11

A wide range of toxic units (TUs) was calculated for the different organic micropollutants based on freely dissolved concentrations (Appendix Table C.1). The insecticides imidacloprid and thiacloprid reached concentrations supporting individual TU higher than -3.0 for *G. pulex*, which is a value above which chronic effects can be expected (Liess et al., 2008). All sampling sites showed *s*TU values higher than -3.0. The highest value was calculated for the chemicals detected at the mouth of the river (*s*TU = -0.07).

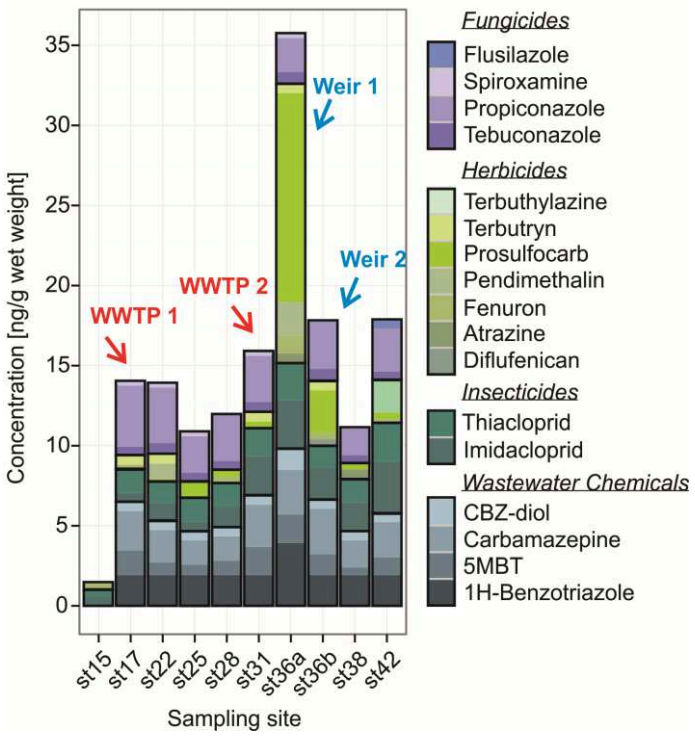

Figure 4.2**:** Organic micropollutants in *G. pulex*. Chemicals are clustered by class of pollutant. The presence of wastewater treatment plants (WWTP) and weirs is highlighted by coloured arrows. Detailed chemicals concentrations are listed in Table 4.3.

4.3.2 Genetic diversity and population structure

The genotype accumulative analysis determined an asymptote and a decrease in variance in eight microsatellite loci (Appendix Figure C.1). No outliers were detected in any of the nine loci (Appendix Figure C.2). Wright's *F*-statistics, especially the inbreeding coefficients (F_{IS}) ranged from -0.421 to -0.144 (Table 4.4) indicating outbreeding (i.e. individuals are less related to each other than expected under a random mating model, suggesting an increase in heterozygosity). The lowest F_{IS} was detected near the mouth of the River Holtemme (*st*41; F_{IS}=-0.421), and highest F_{IS} (F_{IS}=-0.144) was observed in the agriculturally impacted midstream of the river (*st*22). Although specific locus departure from Hardy-Weinberg equilibrium (HWE) occurred, no global deviation of populations from HWE was detected. No evidence of Linkage Disequilibrium (LD) was observed after applying FDR approach.

General population genetic metrics for each sampling site were calculated: the number of alleles (*N*), allelic richness (A_R), private alleles (N_{PA}), and unbiased expected (uH_E) and

observed heterozygosity (H_O). The obtained results are listed in Table 4.4. Smallest values occurred at *st*17 for *N*, A_R, uH_E, and differentiation values (*Q*). Moreover, highest N_e was predicted at site *st*17 characterised by a permanent discharge of treated wastewater from the first wastewater treatment plant effluent to the River Holtemme.

Table 4.4: Genetic variability calculated using nine microsatellite loci per sampling site. *N*, number of alleles; A_R, allelic richness; H_O, observed heterozygosity; uH_E, unbiased expected heterozygosity; F_{IS}, inbreeding coefficient, assignment probability values represent differentiation (*Q*), N_{PA}, average number of private alleles and effective population size (N_e). Delta distance represents the distance from the spring of the river to the sampling site.

Pop ID	Δ distance	*N*	A_R	H_O	uH_E	F_{IS}	*Q*	N_{PA}	N_e
st15	14.54 kms	30	3,09	0.398	0.330	-0.210	0.09	0.20	∞ (85.7-∞)
st17	17 km	27	2.80	0.407	0.347	-0.209	0.07	0.46	∞ (193.5-∞)
st22	23.81 km	35	3.28	0.562	0.496	-0.144	0.26	0.36	41.2 (15.8-∞)
st25	28.39 km	31	3.30	0.616	0.476	-0.327	0.69	0.33	57.9 (15.9-∞)
st28	31.86 km	34	3.76	0.648	0.512	-0.319	0.83	0.09	∞ (57.0-∞)
st31	32.54 km	33	3.61	0.615	0.542	-0.177	0.89	0.17	31166 (28.3-∞)
st36a	35.91 km	33	3.61	0.648	0.538	-0.209	0.87	0.17	28.6 (12.2-∞)
st36b	36.81 km	30	3.25	0.574	0.460	-0.259	0.35	0.31	13.6 (6.1-38.1)
st38	40.1 km	30	3.25	0.458	0.389	-0.224	0.16	0.31	∞ (138.0-∞)
st42	43.35 km	34	3.58	0.627	0.461	-0.421	0.48	0.33	∞ (31.1-∞)

Genetic diversity overall increased (A_R; R^2=0.340; p<0.05) from upstream to downstream sites, but was lower after wastewater treatment plant effluents and weirs (Figure 4.3A). A remarkable increase in private alleles as an indicator for mutagenicity effects in *G. pulex* downstream the first wastewater treatment plant was detected (*st*17; Figure 4.3B), followed by a significant reduction after the rainwater drainage (*st*28), and a subsequent increase downstream of the second wastewater treatment plant (*st*31, Figure 4.3B). Genetic differentiation based on distance was assessed using Mantel tests between Wright's fixation indexes (F_{ST}) or the assignment probabilities (*Q*-values), and the geographic distances between sampling sites. Mantel tests did not show a pattern of isolation by distance (IBD) (Mantel test; p>0.05; Figure 4.3C). Conversely, a significant divergence (p<0.001) in the differentiation pattern was observed shortly after the presence of physicals barriers (i.e. weirs) in the River Holtemme (sites *st*36a-*st*36b and *st*38; Figure 4.3C). Bottleneck analysis revealed that two sites along the river underwent population bottlenecks (*st*31 and *st*36a; Appendix Table C.2): The first site (*st*31) is located downstream of two main point-source of chemicals, i.e. a rainwater drainage (*st*28) and the effluent of the second wastewater treatment plant (*st*31). The second site (*st*36a) is located in small reservoir water created by the influence of the weir.

Estimates of N_e were generally variable with very wide confidence intervals (Table 4.4). For five sampling sites, N_e was estimated as infinite, and the upper limit of the 95% confidence interval (CI) in most cases reached infinite. Hence, the lower bound of the CI might be the most informative parameter estimated providing reasonable limits of N_e (Waples and Do, 2010). In particular, sampling site *st*17 exhibited highest N_e values along the River

Holtemme. It was also analysed if the observed declines in genetic diversity (see above) correlated with changes in abundances or N_e. However, only a negative correlation was found between N_e and allelic richness (Appendix Figure C.3), where the site with the lowest genetic diversity (*st*17) exhibited the highest N_e (Appendix Figure C.3).

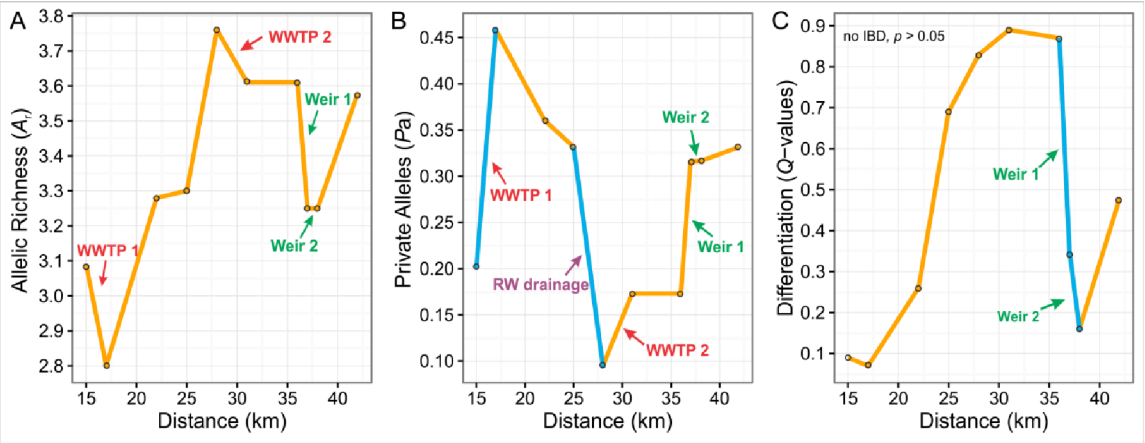

Figure 4.3: (A) Positive and significant trend of the genetic diversity along the river (R^2=0.340; p<0.05). (B) Private alleles as proxy of mutation rates along the river. (C) Differentiation using assignment probability values (Q-values) derived from Bayesian analysis in STRUCTURE along the River Holtemme, Mantel tests revealed no IBD. For all figures, green arrows represent the influence of the weirs, red arrows the influence of wastewater treatment plants (WWTPs), and pink arrow the weight of the rainwater drainage (RW drainage). Black circles represent sampling sites and light blue lines represent significance (p<0.05).

For the River Holtemme, the Evanno method revealed two delta *K* peaks; the first and highest with a *K*=2 and a second with a *K*=4 (Appendix Figure C.4 details on Evanno values). The general pattern, with an intermediate cluster, supports the lack of IBD (Figure 4.4A). Specimens from sampling sites *st*15, *st*17, *st*36b and *st*38 formed the first cluster; whereas individuals from *st*25, *st*28, *st*31 and *st*36a formed a second cluster (Figure 4.4A). STRUCTURE analysis revealed two sites with an admixture pattern: *st*22, after the first wastewater treatment plant, and *st*42 located at the confluence of the River Holtemme and the River Bode. STRUCTURE revealed two remarkable changes in population structure. The first change is gradual and started at site *st*17 to *st*25 (separated by about 10 km from each other) (Figure 4.4B) and second and most striking occurred downstream of the first weir (between sites *st*36a and *st*36b), despite their close spatial proximity of few hundred meters (Figure 4.4C).

Regarding gene flow, the relative migration network (Figure 4.4D) illustrates significant migration rates in the River Holtemme (95% CI, 10^3 bootstrap iterations). Upstream populations (i.e. *st*15 and *st*17) showed higher gene flow rates than populations within the central reach (*st*25, *st*28, *st*31, *st*36a) and downstream sites (*st*42). No migration, and thus gene flow, was detectable across the weirs (between sites *st*36a and *st*36b, as well as *st*36b and *st*38, respectively; Figure 4.4D).

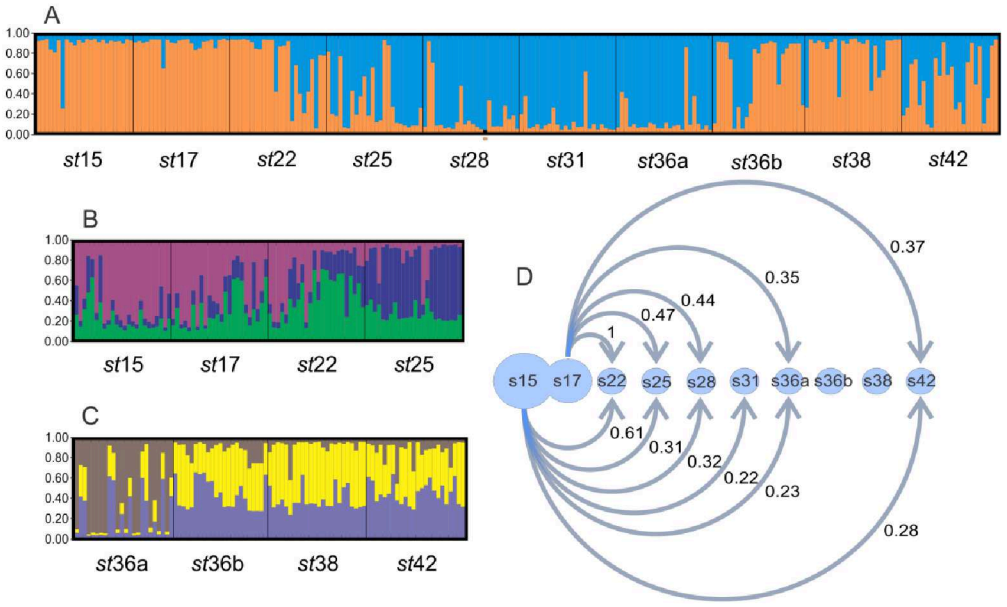

Figure 4.4: Estimated population structure in *G. pulex*. (A) Results shown are for *K*=2 clusters using full data set. Each individual's genotype is represented by a thin vertical line which is partitioned into coloured sections in proportion to the estimated membership. (B) Results shown are for *K*=3 clusters using subdivided dataset corresponding to upper part of the river. (C) Results shown are for *K*=3 clusters using subdivided dataset corresponding to lower part of the river. (D) Unidirectional relative migration network. Arrows represent significant upstream-downstream relative migrations along the Holtemme (CI 95%; 10^3 bootstrap iterations). Size of sites represents contribution to the gene flow. All sampling sites are labelled below the figures and they correspond to those shown in Figure 4.1.

4.3.3 Mutagenicity in gammarid and water extracts

Both gammarid and water samples extracts upstream and downstream of the first wastewater treatment plant were analysed for mutagenicity with AFT in order to confirm mutagenicity as indicated by the occurrence of private alleles in *G. pulex*. While gammarid extracts did not show any significant mutagenicity activity, water extracts sampled downstream of the first wastewater treatment plant displayed significant mutagenicity after metabolic activation with S9 (Table 4.5).

Table 4.5**:** Results of AFT. Test performed using tester strain TA98 without (-S9) and with metabolic activation (+S9). Tests performed in triplicated. * Significant mutagenic activity

Samples	**TA 98 -S9**		**TA98 +S9**	
	Average number of revertants	Standard deviation	Average number of revertants	Standard deviation
Sildstedt August 2014	4.57*	2.38	20.95*	10.90
Sildstedt September 2014	3.43*	1.78	19.05*	9.91
Sildstedt October 2014	1.90	0.99	8.38*	4.36
Wernigerode August 2014	1.90	0.99	6.86*	3.57
Wernigerode September 2014	2.67	1.39	3.81*	1.98
Wernigerode October 2014	2.67	1.39	4.95*	2.58
K-	0.88	0.81	1.50	1.15
K+	48.0	0.00	48.0	0.00

4.3.4 Relationship between multiple stressors and genetic variability

Redundancy analysis (RDA) was performed using four categories of anthropogenic stressors and four indicators of the population genetic responses in gammarid populations (Table 4.1 and Appendix Table C.3). The first two axes of the RDA altogether explained 44.59% of variance (Figure 4.5), with RDA1 explaining 38.97% of variance. The variable weir and *s*TU Gam, representing chemical stress (C^{fd} translated to chemical stress), were environmental stressors with high correlation. RDA1 showed that *s*TU Gam explained most of the variance in allelic richness. Furthermore, *s*TU Gam correlated with differentiation (F_{ST}) and unbiased expected heterozygosity (μH_E). In RDA2 the WWTP variable correlated with private alleles (*P*a), but the whole explanatory power of this axis was only 5.62%. All multiple linear regressions (MLRs) were significant (p<0.001; Appendix Table C.4).

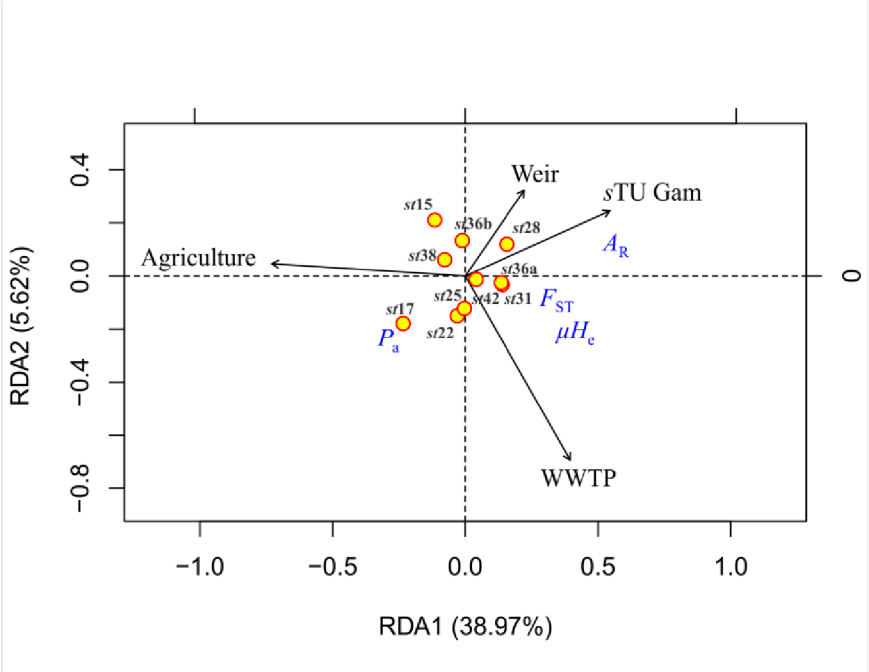

Figure 4.5: Redundancy analysis (RDA) plot showing the ordination of four main population genetics responses in green (Allelic richness: A_r; Fixation index: F_{ST}; unbiased expected heterozygosity: uH_E and Private alleles: *P*a) in the River Holtemme under the presence of multiple stressors in black. All multiple linear regressions are significant (p<0.001; Appendix Table C.4).

4.4 DISCUSSION

4.4.1 Genetic diversity patterns

In the present study, body burden analysis and evolutionary ecotoxicology were successfully combined to provide novel insights into the linkage between anthropogenic pressures and population genetic responses in a multiple-stress scenario. In general, genetic diversity in *G. pulex* at the River Holtemme was higher than *G. fossarum* inhabiting forested and agricultural landscapes in pre-alpine rivers based on allelic richness and expected heterozygosity (Alp et al., 2012). These differences in genetic diversity may be attributed either to geographic differences or to a higher pollutant load observed in the River Holtemme

compared to the pre-alpine watercourse. However, pollutant data were not reported by Alp et al. (2012).

One of the major challenges in evolutionary ecotoxicology lies in the ability to distinguish genetic variations caused by anthropogenic pressures from those naturally occurring due to natural environmental conditions (Hoffmann and Willi, 2008). It is generally assumed that genetic diversity increases with increasing distance from the source of the river due to higher downstream migration of genotypes especially when compensating strategies such as upstream migration and dispersal are lacking (Excoffier et al., 2009). Even though this general pattern trend was confirmed, remarkable deviations from this pattern were observed and successfully linked to anthropogenic pressures.

In this study, it was possible to link reductions in genetic diversity to the occurrence of chemical stress (i.e., body burden of organic micropollutants) and physical barriers in a long-term exposure scenario, thus supporting the first working hypothesis. A first decrease in genetic diversity was detected at sampling site *st*17 characterised by a direct influence of treated wastewater and a significant increase in organic micropollutants. This sampling site showed the smallest values for several genetic population metrics such as allelic richness, number of alleles, unbiased heterozygosity and assignment probabilities values as well as the highest values for metrics related with mutagenicity. The second decrease in genetic diversity could be attributed to the combined action of the second wastewater treatment plant (*st*31) and the first weir (*st*36a) in the River Holtemme. Both sampling sites suffered from recent bottleneck processes as demonstrated in this study. Although the decrease in genetic diversity started after the wastewater treatment plant, it became prominent only in that stretch at the weirs. The first weir creates a characteristic pool leading to higher residence time of water and increased sedimentation of fine particulate matter, which may together enhance the retention and bioconcentration of organic micropollutants. In fact, the highest loads of micropollutants were quantified here in both the sediments (unpublished data) and the gammarid tissues. Therefore, it is suggested that combined action of chemical stress and physical barriers was responsible for the observed drop in genetic diversity at this site.

It was also tested if the observed declines in genetic diversity correlated with changes in gammarid abundances or N_e, considering the lower borders of N_e as previously suggested (Waples and Do, 2010). However, no correlation was evident confirming the assumption that drops in genetic diversity was directly related to anthropogenic pressures. This in line with previous studies in which even severe reductions in Ne did not result in substantial reductions in genetic diversity (Pimm et al., 1989). Therefore, it was concluded that genetic erosion observed in this study was linked to chemical stress arising from long-term exposure to organic micropollutants. Particularly population located downstream of the wastewater treatment plant (*st*17) and upstream of the weir (*st*36a) probably experienced more intense exposure to chemicals, which may have triggered population declines and genetic drift in that stretch of the river (Bickham, 2011). In agreement with this, Coutellec and coworkers (2013) reported that the multiple exposure to toxic agrochemicals with multiple modes of action might increase stochastic genetic drift.

4.4.2 Pollutant induced genetic structure changes

Two different effects of anthropogenic pressures on the genetic structure in the River Holtemme were observed: a gradual alteration exerted by chemicals in line with literature (Bickham et al., 2000; Mussali-Galante et al., 2014) and a drastic alteration associated with the combined effect of the physical barrier and the high load of organic micropollutants at *st*36a. Furthermore, divergences in genetic differentiation (Q or F_{ST} values) can be attributed to the action of weirs that may disrupt migration and hence gene flow between gammarid populations in upstream and downstream waters. The short distance between two physical barriers (*st*36a/b and *st*38; 900 m) may have enhanced population differentiation (see Q-values in Table 4.4). This disruption of migration creates a diverged population immediately below the weir with significant levels of differentiation supporting the third hypothesis. A similar pattern was observed previously by Sjöqvist et al. (2015) leading to the conclusion that distance alone is a poor predictor at both small and regional geographic scales. Divergences in differentiation have also been reported in fish populations due to the action of weirs (Hansen et al., 2014; Koizumi et al., 2006; Vera-Escalona et al., 2015). Conversely, Weiss and Leese (2016) found no effect of in-stream barriers in *G. fossarum* inhabiting highly human-impacted landscapes. It is suggested that together with empirical data, this argues for combined action of different stressors (i.e., organic micropollutants and weirs).

4.4.3 Private alleles as mutation proxy

Private alleles are commonly used as proxies for relative mutation rates (Mengoni et al., 2000; Nadig et al., 1998; Theodorakis et al., 2006; Theodorakis and Shugart, 1997; Whitehead et al., 2003). It was found strong indications of increased mutagenicity of the first wastewater treatment plant (*st*17) reflected by an increase in private alleles, probably due to pollutant-induced *de novo* mutations or selection of rare genotypes as reported by Theodorakis et al. (2006). This assumption is supported by the significant mutagenicity in the Ames fluctuation test performed with water samples from this site. Gammarid extracts did not show significant mutagenicity activity probably due to the limited amount of biomass available for extraction and testing (900 mg wet weight) and to the metabolisation of the causative compounds (as indicated by the effect of S9). The observed mutagenicity could not be explained by the target chemicals detected in gammarids. Although weak mutagenic activities have been reported for the insecticide imidacloprid and the industrial chemical 1H-benzotriazole (Bagri et al., 2016; Dunkel and Simmon, 1980) in different *in vitro* assays and propiconazole, a nonmutagenic fungicide, has been demonstrated to exert carcinogenic effect after long-term exposure (Shane et al., 2012), these chemicals are probably contributing only a minor extent to the observed effect.

The individual compounds or mixtures causing the mutagenicity downstream of the first wastewater treatment plant and their sources are unknown. However, the absence of higher numbers of private alleles downstream of the second wastewater treatment plant suggests that the causes may be expected beyond the municipal wastewater both wastewater treatment plants are treating. Since both wastewater treatment plants follow similar conventional mechanical and biological treatment approach without advanced oxidation

methods, there is no indication that the observed mutagenicity is produced during treatment processes. Thus more specific (e.g., industrial) sources may come into consideration. Another plausible explanation is that recent bottleneck processes associated with the combined action of the second wastewater treatment plant and the first weir swept away some of the private alleles in this stretch of the river.

These results emphasize that, due the high complexity of contamination mixtures in the environment, chemical analysis needs to be supplemented with effect-based approaches in order to avoid overlooking unknown toxicants such as the mutagens in this study. The use of both tissue and water extracts is highly recommended for chemical analysis and biotesting, particularly when compounds of interest and their environmental fates are unknown. The present study provides strong indication that environmental mutagenicity as measured with AFT (Hug et al., 2015; Ohe et al., 2004) in environmental matrices is actually reflected in native organisms by the occurrence of private alleles, thus confirming the second hypothesis. In fact, two conditions, low genetic diversity and low genetic differentiation, were met at site *st*17 where the highest private alleles were detected. This has been described as "an ideal scenario" for detecting *de novo* mutations as previously observed for mosquito fish exhibiting pollution-induced genetic mutations (Rinner et al., 2011). The suggested relationship between private alleles and mutagenic compounds opens new insights into adverse effects of genotoxicants on the genetic variation of invertebrate populations in freshwater ecosystems.

4.5 CONCLUDING REMARKS AND OUTLOOK

In conclusion, it is provided evidence for combined effects of multiple anthropogenic pressures on the genetic structure of freshwater biota using *G. pulex* as a model. Furthermore, discernible signals of pollutant-induced genetic changes due to chemical stress and evidence of constraints in gene flow due to physical barriers were determined in a typical central European river. Different population genetic responses were observed downstream of both wastewater treatment plants along the river suggesting that presumably similar pressures (here: wastewater treatment plants) may cause different population genetic responses. Wastewater treatment plants should not be regarded as monotonous sources of pollutants to the aquatic environment but as complex, dynamic and diverse sources of thousands of chemicals, which effects may interact with other stressors such as in-stream barriers. This study shows that chemical pollution may be a main driver for population shifts in a multiple stressed scenario. Therefore, multidisciplinary strategies are recommended in order to bare trends induced by anthropogenic activities. This is because genetic population response may be biased and masked by natural environmental conditions.

The approach developed in this chapter, combination of evolutionary ecotoxicology and body burden of emerging organic microcontaminants, has the potential to be applied both to higher biological organisation level such as fishes and broader geographical scales.

CHAPTER 5

Synthesis and challenges

While the environmental risk assessment is traditionally built on chemical concentrations determined in water and/or sediments samples, the body burden of chemicals in biota may contribute to opening new insights in the field of risk assessment. In order to test this hypothesis, new analytical methods have to be developed or optimised using cutting-edge analytical approaches. One strategy could be the development of narrow but highly precise methods for single classes of chemicals or wider multi-target class methods. Multi-target screening methods have the advantages of having a unique extraction procedure and further a robust method performance even when the analytes are characterised with different physical-chemical properties. In CHAPTER 2, the second strategy was chosen and a robust and highly selective multi-target screening method was developed and optimised for invertebrate freshwater biota. Although the method was developed, validated and applied in different gammarids species around European water systems with reliable outcomes, the method has the potential to be applied in other biological environmental matrices for instance fishes. The method reached pretty good recoveries and lower matrix effects in tissues exhibiting an average lipid content of 4%, then in other biological matrices with higher lipid content it would be expected to have at least a similar achievements. Moreover, the developed method is utterly suitable for non-target analysis how is shown in CHAPTER 2. The latter analysis is a pivotal tool in effect-directed analysis (EDA). EDA focus in unravel the ecological risk of complex mixtures of chemicals in the environment. Therefore, promising studies can be carrying out using this method in further investigations.

Understanding the fate and ecological hazard of emerging micropollutants is a challenging task in the ecotoxicology field. Primarily due to the complexity of the environment itself with several variables ruling ecological processes and hardly described mechanistic processes in aquatic systems. Therefore, a multi-compartment analysis was performed in different environmental compartments such as water, sediments and biota based on equilibrium partitioning theory (CHAPTER 3). Based on total concentration of chemicals, hypothetical freely dissolved concentrations and corresponding chemical activities were calculated for 63, 52 and 17 compounds detected in water, sediment and gammarid samples respectively. Significant differences both for freely dissolved concentrations and chemical activities were observed in the multi-compartment analysis. Sediment compartment exhibited both highest chemical and hazard potential and additionally it was in disequilibrium regarding water and biota phases. Results suggest that contaminated suspended matter and sediments act as source of chemical contamination towards the water phase. Chemical activity and baseline toxicity is well understood for non-polar organic hydrophobic chemicals. However, in this chapter the fate and potential toxicity was extended for emerging

organic microcontaminants with a broad hydrophobicity (log K_{OW} range from -1 to 5). This approach may be improved considering partitioning coefficient for back carbon in sediments.

How has been demonstrate in the former chapters, chemical pollution is not based on the occurrence of single chemicals rather complex mixtures in the environment. Therefore, in order to bare adverse ecological consequences in the field, novel and/or integrative approaches with considerable holistic foresight must be develop. In CHAPTER 4, an integrative tool is proposed using environmental chemistry, particularly body burden, and molecular biology, especially evolutionary ecotoxicology, in order to unravel adverse effects at genetic level in the model invertebrate population *G. pulex* along land use gradient. Using the multi-target screening method developed in CHAPTER 2 and outcomes from CHAPTER 3, several genetic population responses were determined in the analysed invertebrate population in a highly anthropogenised aquatic system. Along the River Holtemme several anthropogenic pressures were identified. For instance, the river course hosts two wastewater treatment plants and two weirs. Specific shift in genetic diversity, translated to genetic erosion, were unravelled only downstream of wastewater treatment plants and the first weir. These results open new insight about the role of the wastewater treatment plants as source of mutagenic complex mixtures to the aquatic system. Moreover, the presence of physical stress, represented throughout the weirs constrains significantly the flow of genetic information along a water course. However, one of the main disadvantage of the tool develop in CHAPTER 4 is the need of monitoring strategies in order to disclose deleterious outcomes at genetic or genome level. Because only using wider spatial or temporal dataset reliable genetic/genomic population patterns can be obtained.

Hence, chemical pollution and its consequences are a global scale problem that we have to face with holistic strategies, proactivity and novel ideas in order to maintain viable the current aquatic systems. Thus, unless we, like society, change to a green or sustainability way of production of our goods and services, being aware of the effects of pollutants in the environment, chemicals in aquatic environments will remain a major environmental changer force for many centuries.

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APPENDIX A

Supplementary information for Chapter 2

Table A.1: Acid dissociation constant (pKa), octanol-water partitioning coefficient (log K_{OW}), average retention times in minutes (t_R), molecular weight (MW in g mol^{-1}), multiple reaction monitoring (MRM) transitions, for each transition for each analyte, transformation product (TP).

	CAS number	pKa[1]	log K_{ow}	MW	t_R	Q1 > Q2	Compound group
1H-benzotriazole	95-14-7	8.79	1.44	119.1	6.37	120.0 > 65.0	corrosion inhibitor
2-aminobenzimidazole	934-32-7	8.11	0.86	133.2	0.55	134.0 > 91.9	TP of carbendazim
2,6-dichlorobenzamide	2008-58-4	-	0.77	190.0	5.05	190.0 > 173.0	TP of dichlobenil
4-acetamidoantipyrine	83-15-8	-	-0.13	245.3	6.36	246.0 > 228.1	pharmaceutical
5-methyl-1H-benzotriazole	136-85-6	8.87	1.70	133.2	7.50	134.0 > 76.9	corrosion inhibitor
10,11-dihydroxy-10,11-dihydrocarbamazepine	58955-93-4	8.24	-0.21	270.2	7.61	270.9 > 179.9	TP of carbamazepine
Acetamiprid	160430-64-8	4.16	0.80	222.7	7.28	223.1 > 126.1	insecticide
N-Acetyl-sulfamethoxazole	21312-10-7	5.88	1.21	295.3	7.44	195.9 > 134.0	conjugate of sulfamethoxazole
Atrazine	1912-24-9	3.20	2.61	215.7	8.63	216.0 > 174.0	herbicide
Azoxystrobin	131860-33-8	-	2.50	403.4	8.97	404.1 > 372.0	fungicide
Bentazone	25057-89-0	2.03	2.80	240.3	8.27	238.9 > 131.9	herbicide
Boscalid	188425-85-6	-	2.96	343.2	9.05	343.1 > 306.9	fungicide
Bromoxynil	1689-84-5	5.11	5.46	276.9	8.51	273.8 > 78.9	herbicide
Caffeine	58-08-2	10.4	-0.07	194.2	6.6	195.1 > 138.0	pharmaceutical
Carbamazepine	298-46-4	-	2.45	236.3	8.37	237.2 > 194.9	pharmaceutical
Carbendazim	10605-21-7	4.28	1.50	191.2	5.82	192.0 > 159.9	fungicide
Chloridazone	1698-60-8	-	1.14	221.6	7.23	222.1 > 103.9	herbicide
Chlorotoluron	15545-48-9	-	2.41	212.7	8.53	213.1 > 71.9	herbicide
Chloroxuron	1982-47-4	-	3.70	290.7	9.27	291.1 > 72.0	herbicide
Clomazone	81777-89-1	-	2.50	239.7	8.90	240.2 > 124.9	herbicide
Clothianidin	210880-92-5	-	0.70	249.7	6.97	250.0 > 175.1	insecticide
DEET	134-62-3	-	2.02	191.3	8.64	192.1 > 118.9	insect repellent
Desethylatrazine	6190-65-4	3.38	1.51	187.6	7.38	188.0 > 146.0	TP of atrazine
Desethylterbutylazine	30125-63-4	3.35	2.23	201.7	8.30	202.1 > 145.9	TP of terbutylazine
Desisopropyl-atrazine	1007-28-9	3.41	1.36	173.6	6.49	174.1 > 103.9	TP of atrazine
Diazinon	333-41-5	4.18	3.81	304.3	9.67	305.0 > 169.0	insecticide
Diclofenac	15307-86-5	4.00	4.51	296.1	9.55	293.9 > 249.8	pharmaceutical

	CAS number	pKa[1]	log K_{ow}	MW	t_R	Q1 > Q2	Compound group
Difenoconazole	119446-68-3	2.24	4.40	406.3	9.86	406.0 > 251.1	fungicide
Diflufenican	83164-33-4	10.27	4.90	394.3	9.95	395.1 > 265.9	herbicide
Dimethoate	60-51-5	-	0.78	229.3	7.08	230.1 > 198.8	insecticide
Diuron	330-54-1	-	2.68	233.1	8.73	232.9 > 71.9	herbicide
Ethofumesate	26225-79-6	-	2.70	286.3	8.98	287.1 > 121.2	herbicide
Epoxiconazole	133855-98-8	2.26	3.58	329.8	9.38	330.0 > 121.0	fungicide
Fenuron	101-42-8	-	0.96	164.2	6.89	165.1 > 72.0	herbicide
Fenpropimorph	67564-91-4	8.49	4.93	303.5	8.56	304.4 > 147.1	fungicide
Fipronil	120068-37-3	-	4.00	437.2	9.45	434.9 > 330.0	biocide (insecticide)
Flufenacet	142459-58-3	-	3.20	363.3	9.32	364.0 > 194.3	herbicide
Flurtamone	96525-23-4	3.64	2.87	333.3	9.02	334.1 > 247.0	herbicide
Flusilazole	85509-19-9	2.32	3.81	315.4	9.45	316.1 > 246.9	fungicide
Imidacloprid	105827-78-9	-	0.57	255.7	6.98	256.1 > 209.0	insecticide
Irgarol	28159-98-0	5.68	4.07	253.1	8.84	253.9 > 198.0	biocide (herbicide)
Isoproturon	34123-59-6	-	2.87	206.1	8.67	207.1 > 72.0	herbicide
Lenacil	2164-08-1	6.60	3.09	234.3	8.67	235.2 > 153.0	herbicide
Linuron	330-55-2	11.94	3.20	249.1	8.99	249.0 > 160.0	herbicide
Metamitron	41394-05-2	2.78	0.83	202.2	6.99	203.0 > 175.1	herbicide
Metazachlor	67129-08-2	2.34	2.49	277.1	8.61	278.0 > 134.0	herbicide
Metolachlor	51218-45-2	-	3.13	283.8	9.40	284.0 > 251.9	herbicide
Myclobutanil	88671-89-0	2.27	2.94	288.8	9.19	230.0 > 173.9	fungicide
p-toluenesulfonamide	70-55-3	10.46	0.82	171.2	6.76	172.0 > 90.9	industrial chemical
Pendimethalin	40487-42-1	10.52	5.20	281.3	10.43	282.1 > 211.9	herbicide
Pethoxamid	106700-29-2	-	3.39	295.8	9.34	296.2 > 131.1	herbicide
Picolinafen	137641-05-5	11.87	5.37	376.3	10.23	377.1 > 238.0	herbicide
Picoxystrobin	117428-22-5	-	3.67	367.3	9.50	368.0 > 205.1	fungicide
Pirimicarb	23103-98-2	4.99	1.70	238.3	7.05	239.1 > 182.1	insecticide
Prochloraz	67747-09-5	2.75	4.38	376.7	9.49	376.0 > 307.9	fungicide
Prometryn	7287-19-6	5.71	3.51	241.4	8.61	242.2 > 158.0	herbicide

	CAS number	pKa[1]	log K_{ow}	MW	t_R	Q1 > Q2	Compound group
Propiconazole	60207-90-1	2.24	3.72	342.2	9.66	342.1 > 158.9	fungicide
Propoxycarbazone	145026-81-9	3.39	2.66	398.4	8.39	399.1 > 199.0	herbicide
Prosulfocarb	52888-80-9	-	4.65	251.4	10.02	252.2 > 91.0	herbicide
Prothioconazole-desthio	120983-64-4	2.26	3.05	312.2	9.40	312.1 > 70.1	TP of Prothioconazole
Pyraclostrobin	175013-18-0	-	3.99	387.8	9.73	388.1 > 194.0	fungicide
Simazine	122-34-9	3.23	2.18	201.7	8.17	202.1 > 124.0	herbicide
Spiroxamine	118134-30-8	9.34	5.51	297.5	8.71	298.3 > 144.0	fungicide
Sulfamethazine	57-68-1	6.99	0.14	278.3	6.51	279.0 > 186.0	pharmaceutical
Sulfamethoxazole	723-46-6	6.16	0.89	253.3	6.85	253.9 > 155.9	pharmaceutical
Tebuconazole	107534-96-3	2.27	3.70	307.8	9.58	308.2 > 70.0	fungicide
Terbutryn	886-50-0	5.72	3.74	241.4	8.64	242.1 > 186.0	biocide (herbicide)
Terbuthylazine	5915-41-3	3.18	3.40	229.7	9.09	230.0 >173.9	herbicide
Terbuthylazine-2-hydroxy	66753-07-9	-		211.3	7.10	212.2 > 156.0	TP of terbuthylazine
Thiabendazole	148-79-8	4.08	2.47	201.2	6.53	202.0 > 175.0	fungicide/preservative
Thiacloprid	111988-49-9	1.62	1.26	252.7	7.59	253.0 > 126.0	insecticide
Thiamethoxam	153719-23-4	-	-0.13	291.7	6.36	292.0 > 211.1	insecticide
Triethyl citrate	77-93-0	11.82	0.71	276.3	8.14	276.9 > 157.0	plasticiser
Trifloxystrobin	141517-21-7	2.37	4.50	408.4	9.92	409.1 > 186.1	Fungicide

[1] calculated using Calculator Plugins, Instant JChem 2012, ChemAxon (www.chemaxon.com), only given for $1 < pK_a < 12$; - when no ionisable atoms found.

Table A.2: Processing steps and settings used for MZmine 2.17

MZmine step	**Settings**
Peak detection	Noise cut-off 200; mass resolution 100,000
Chromatogram building	Min. time span 0.1 min, min. height 50,000 a.u, mass tolerance 4
Smoothing	Filter width of 7
Peak deconvolution	Local minimum search; chromatographic threshold 89%; search
Peak list alignment	Join aligner, m/z tolerance 0.001; weight for m/z 70, retention time tolerance 0.2 min; weight for RT 30
Filter for duplicates	m/z tolerance 0.001; retention time tolerance 0.2 min

Table A.3: Settings used for the R "nontarget" package.

Step	**Settings**
Pattern search (rule based)	Cut off intensity = 5000; Isotopes: ^{13}C, ^{15}N, ^{34}S, ^{37}Cl, ^{81}Br; RT tolerance = 0.04 min; m/z tolerance 2 ppm, intensity tolerance = 0.2; small m/z tolerance 0.5 ppm; rules 1-9, 11 true, rule 10 false (see details in package documentation)
Adduct search	Adducts: ESI+ =M+H, M+Na, M+K, M+NH4, M+CH3OH+H, M+2H, M+H+Na, M+2 Na, M+H+NH4 ESI- = M-H, M+Cl, M+FA-H, M-2H RT tolerance = 0.04 min; m/z tolerance 2 ppm
Homologue series search	Elements: C, H, O, Si; charge 1, 2 (ESI+) or -1,-2 (ESI-); Δ m/z; min. 12 Da, max 80 Da; Δ RT min ESI+ 0 min/ ESI- 0.05 min, max. 3 min; m/z tolerance 3 ppm, retention time tolerance 0.3; minimum length of series =3

Table A.4: The *P*-values for multiple-test comparisons for tested extraction procedures. The *P*-values for absolute recoveries are below the diagonal and for matrix effects are above the diagonal.

	FP	FP+ QuEChERS	FP+ SPE	PuLE+ QuEChERS	PuLE+ QuEChERS+ Hexane	PuLE+ SPE
FP	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
FP+ QuEChERS	< 0.001	-	0.001	0.07	< 0.001	0.03
FP+SPE	< 0.001	0.012	-	0.12	0.04	< 0.001
PuLE+ QuEChERS	< 0.001	0.101	0.367	-	< 0.001	< 0.001
PuLE+ QuEChERS+ Hexane	< 0.001	< 0.001	< 0.001	< 0.001	-	< 0.001
PuLE+ SPE	0.07	< 0.001	< 0.001	< 0.001	< 0.001	-

Table A.5: Recoveries and matrix effect (ME) for each extraction procedure tested. FastPrep (FP), FP+QuEChERS (FP+Q), FP+SPE (FP+S), Pulverised liquid extraction+SPE (PuLE+S), PuLE+QuEChERS (PuLE+Q), PuLE+QuEChERS +Hexane (PuLE+Q+H).

	FP		**FP+Q**		**FP+S**		**PuLE+S**		**PuLE+Q**		**PuLE+Q+H**	
Analyte	Recovery	ME	Recovery	ME	Recovery	ME	Recovery	ME	Recovery	ME	Recovery	ME
1H-Benzotriazole	48	-30	33	-53	71	24	58	45	73	43	56	40
n-Acetyl-4-aminoantipyrine	33	-47	25	-41	17	-19	11	-9	57	-2	63	5
5-Methyl-1H-benzotriazole	42	-33	39	-28	30	-26	14	-29	55	-12	50	-15
10,11-Dihydroxydihydrocarbamazepine	37	-49	48	-36	52	-37	32	-46	53	-29	71	-28
Acetamiprid	46	-46	45	-47	75	-14	42	-38	52	-40	56	-8
Atrazine	45	-45	70	-6	77	-12	37	-43	51	-25	72	-2
Azoxystrobin	1	-99	39	-10	42	-7	0	-98	7	-60	127	6
Bentazone	20	-75	30	-53	43	-44	19	-68	30	-57	93	-32
Boscalid	61	-24	69	-12	13	-24	10	-14	67	-4	55	2
Carbamazepine	30	-59	53	-26	72	-15	24	-60	48	-24	69	-16
Caffeine	29	-60	42	-44	40	-50	23	-61	41	-47	69	-38
Chloridazone	27	-63	32	-24	71	-14	25	-55	31	-49	48	-17
Chlorotoluron	25	-62	35	-51	60	-19	23	-58	36	-48	69	-15
Chloroxuron	27	-62	54	-31	52	-33	31	-52	49	-29	88	-20
Clomazone	43	-41	61	-23	58	-27	39	-38	62	-22	78	-19
Clothianidin	38	-48	46	-29	60	-11	30	-24	54	-35	62	-9
DEET	62	-18	73	-7	26	-19	20	-13	33	-51	77	-9
Deisopropylatrazin	52	-29	52	-28	53	-21	38	-28	55	-24	54	-22
Desethylatrazine	18	-73	53	-10	66	-14	19	-68	34	-30	57	-14
Desethylterbutylazine	45	-38	49	-27	56	-17	43	-15	57	-12	62	-17
Diazinon	48	-39	72	-3	74	6	51	-9	62	-8	47	-20
Difenoconazole	23	-75	37	-48	39	-45	21	-62	43	-44	75	-39
Diflufenican	51	-32	56	-27	45	-41	28	-47	65	-21	47	-22
Dimethoate	26	-66	39	-52	59	-27	29	-51	44	-43	62	-11
Diuron	31	-55	37	-51	69	-15	35	-40	40	-47	70	-27
Epoxiconazole	58	-29	58	-24	37	-34	25	-33	66	-13	62	-13

	FP		FP+Q		FP+S		PuLE+S		PuLE+Q		PuLE+Q+H	
Analyte	Recovery	ME	Recovery	ME	Recovery	ME	Recovery	ME	Recovery	ME	Recovery	ME
Fenuron	36	-46	50	-31	58	-26	29	-50	46	-40	73	-19
Flufenacet	28	-63	44	-33	59	-29	26	-56	38	-49	67	-24
Flurtamone	28	-58	34	-55	62	-22	33	-44	43	-41	86	-30
Flusilazole	53	-33	69	-19	63	-23	43	-31	69	-11	62	-11
Imidacloprid	32	-60	35	-52	53	-32	38	-41	49	-36	70	-34
Irgarol	37	-54	55	-36	73	-22	36	-38	55	-39	48	-17
Isoproturon	29	-65	44	-48	60	-27	31	-45	49	-42	71	-22
Lenacil	18	-77	33	-45	45	-46	18	-72	25	-63	59	-40
Linuron	66	-11	71	-13	52	-22	33	-24	66	-12	61	-14
Metamitron	37	-54	35	-48	72	-16	29	-54	37	-51	62	-10
Metazachlor	34	-47	40	-43	55	-31	31	-49	57	-23	80	-17
Metolachlor	51	-34	59	-19	7	-33	6	-23	63	-7	59	-5
Pendimethaline	10	-90	33	-43	26	-57	13	-74	57	-18	48	-19
Pethoxamid	28	-56	42	-42	58	-21	27	-53	46	-38	64	-16
Picoxystrobin	26	-63	30	-70	50	-42	32	-58	38	-48	48	-35
Pirimicarb	0	-	62	-18	49	-38	39	-36	64	-18	59	-22
Prochloraz	49	-35	53	-28	63	-7	39	-26	56	-20	74	-16
Prometryn	31	-60	36	-58	59	-20	36	-41	52	-39	58	-14
Propiconazole	48	-33	56	-28	65	-15	45	-24	61	-16	62	-16
Prothioconazole-desthio	29	-54	36	-50	61	-21	32	-45	42	-42	66	-29
Pyraclostrobin	0	-	0	-	46	-14	37	-22	64	-10	50	-6
Simazine	43	-43	62	-19	44	-35	29	-49	60	-17	58	-35
Spiroxamine	16	-80	57	-17	63	-20	31	-59	49	-29	22	-25
Sulfamethazine	51	-33	21	-35	54	-15	40	-26	51	-34	55	-27
Tebuconazole	38	-44	42	-36	24	-35	21	-32	62	-6	57	-2
Terbutryn	35	-47	40	-45	65	-15	38	-33	54	-29	61	-17
Terbutylazine	36	-57	44	-45	67	-19	41	-41	54	-38	55	-16
Thiabendazole	22	-72	32	-51	59	-24	27	-55	35	-51	59	-18

	FP		FP+Q		FP+S		PuLE+S		PuLE+Q		PuLE+Q+H	
Analyte	Recovery	ME	Recovery	ME	Recovery	ME	Recovery	ME	Recovery	ME	Recovery	ME
Thiacloprid	12	-86	53	-35	50	-27	13	-79	63	-20	54	-18
Thiamethoxam	36	-49	48	-38	49	-37	32	-49	54	-29	76	-27
Triethyl-citrate	39	-54	66	-22	55	-34	33	-37	65	-24	80	-23
Trifloxystrobin	26	-59	65	-14	70	-1	34	-39	66	-1	48	6

Table A.6: Mean recovery and mean matrix effect per sample size. Standard deviations are in brackets.

Sample size individuals (g wet weight)	**Recovery (%)**	**Matrix effect (%)**
10 (0.3)	84 (±11)	-16 (±11)
30 (0.9)	79 (±16)	-21 (±16)
50 (1.5)	61 (±15)	-39 (±15)
75 (2.2)	63 (±17)	-37 (±17)
100 (3.0)	63 (±18)	-37 (±18)

Table A.7: Contamination found in the matrix used for method development and intra sample derivation in two measurements. Concentrations listed in ng g^{-1} wet weight.

Compounds	**mean concentration**	**standard deviation**
1H-Benzotriazole	4.53	0.88
Caffeine	3.05	0.55
Carbamazepine	0.18	0.03

Figure A. 1: Overview map produced for JDS3, by the ICPDR-International Commission for the Protection of the Danube River.

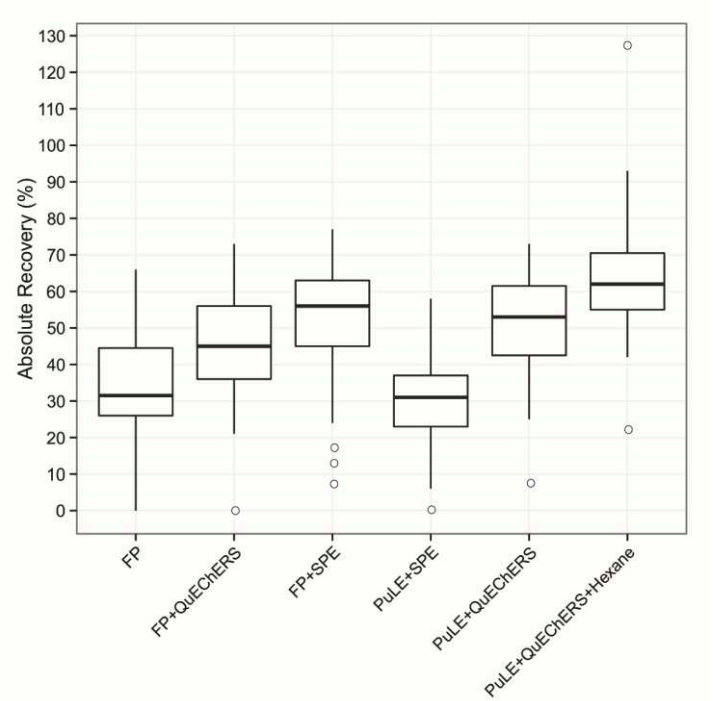

Figure A.2: Absolute recoveries of combined homogenisation and clean-up procedures tested. The selected method in this study, PuLE+QuEChERS+Hexane, exhibited the highest absolute recoveries and the lowest matrix effect (*post hoc* FDR; $p<0.001$).

APPENDIX B

Supplementary information for Chapter 3

Table B.1: Acid dissociation constants (pK_a), octanol-water partitioning coefficients (log K_{OW}), soil organic carbon-water partitioning coefficients (K_{OC}), molecular weights (MW, g mol^{-1}), water solubility (S_w; mg/mL) of the target compounds; TP = transformation product.

	CAS number	pKa[1]	log K_{OW}[1]	K_{OC}	MW	S_w	Compound group
1H-Benzotriazole	95-14-7	8.79	1.44	145	119.1	86.50	corrosion inhibitor
2-Aminobenzimidazole	934-32-7	8.11	0.86	175	133.2	3.32	TP of carbendazim
2,6-Dichlorobenzamide	2008-58-4	-	0.77	30	190.0	0.39	TP of dichlobenil
4-Acetamidoantipyrine	83-15-8	-	-0.13	240	245.3	-	pharmaceutical
5-Methyl-1H-benzotriazole	136-85-6	8.87	1.70	145*	133.2	3.1	corrosion inhibitor
10,11-Dihydroxy-10,11-dihydrocarbamazepine	58955-93-4	8.24	-0.21	29	270.2	0.01	TP of carbamazepine
Acetamiprid	160430-64-8	4.16	0.80	200	222.7	2.95	insecticide
N-Acetyl-sulfamethoxazole	21312-10-7	5.88	1.21	72	295.3	-	conjugate of sulfamethoxazole
Acesulfame	55589-62-3	3.02	-1.33	4	163.1	270	sweetener
Aspartame	22839-47-0	8.53	-2.2	25	294.3	0.65	sweetener
Atrazine	1912-24-9	3.20	2.61	225	215.7	0.03	herbicide
Azoxystrobin	131860-33-8	-	2.50	2812	403.4	0.006	fungicide
Bentazone	25057-89-0	2.03	2.80	55	240.3	0.57	herbicide
Boscalid	188425-85-6	-	2.96	809	343.2	0.004	fungicide
Bromoxynil	1689-84-5	5.11	5.46	302	276.9	0.09	herbicide
Caffeine	58-08-2	10.4	-0.07	9552	194.2	16	pharmaceutical
Carbamazepine	298-46-4	-	2.45	83	236.3	0.24	pharmaceutical
Carbendazim	10605-21-7	4.28	1.50	225	191.2	0.008	fungicide
Chloridazone	1698-60-8	-	1.14	13800	221.6	0.38	herbicide
Chlorotoluron	15545-48-9	-	2.41	196	212.7	0.074	herbicide
Chloroxuron	1982-47-4	-	3.70	2820	290.7	0.003	herbicide
Clomazone	81777-89-1	-	2.50	300	239.7	1.1	herbicide
Clothianidin	210880-92-5	-	0.70	123	249.7	0.34	insecticide
Cotinine	486-56-6	8.8	0.21	130	176.2	117	pharmaceutical
Cyclamate	100-88-9	1.71	-1.61	12	179.2	130	sweetener
DEET	134-62-3	-	2.02	300	191.3	11.2	insect repellent
Desethylatrazine	6190-65-4	3.38	1.51	105	187.6	3.2	TP of atrazine
Desethylterbutylazine	30125-63-4	3.35	2.23	149	201.7	0.327	TP of terbutylazine

	CAS number	pKa[1]	log K_{OW}[1]	K_{OC}	MW	S_w	Compound group
Desisopropylatrazine	1007-28-9	3.41	1.36	69	173.6	-	TP of atrazine
Desphenyl chloridazon	6339-19-1	-	-1.38	85	159.5	-	TP of chloridazone
Diazinon	333-41-5	4.18	3.81	3034	304.3	0.06	insecticide
Diclofenac	15307-86-5	4.15	4.51	245	296.1	0.002	pharmaceutical
Difenoconazole	119446-68-3	2.24	4.40	5889	406.3	0.015	fungicide
Diflufenican	83164-33-4	10.27	4.90	3186	394.3	$5e^{-5}$	herbicide
Dimethoate	60-51-5	-	0.78	13	229.3	39.8	insecticide
Diuron	330-54-1	-	2.68	400	233.1	0.035	herbicide
Epoxiconazole	133855-98-8	2.26	3.58	1073	329.8	0.007	fungicide
Ethofumesate	26225-79-6	-	2.70	150	286.3	0.05	herbicide
Fenpropimorph	67564-91-4	8.49	4.93	3134	303.5	0.004	fungicide
Fenuron	101-42-8	-	0.96	42	164.2	3.85	herbicide
Fipronil	120068-37-3	-	4.00	5923	437.2	0.003	biocide (insecticide)
Flufenacet	142459-58-3	-	3.20	401	363.3	0.056	herbicide
Flurtamone	96525-23-4	3.64	2.87	329	333.3	0.010	herbicide
Flusilazole	85509-19-9	2.32	3.81	1664	315.4	0.041	fungicide
Imidacloprid	105827-78-9	-	0.57	970	255.7	0.61	insecticide
Irgarol	28159-98-0	5.68	4.07	1240	253.1	0.007	biocide (herbicide)
Isoproturon	34123-59-6	-	2.87	122	206.1	0.07	herbicide
Lenacil	2164-08-1	6.60	3.09	165	234.3	0.002	herbicide
Linuron	330-55-2	11.94	3.20	340	249.1	0.063	herbicide
MCPA	94-74-6	3.73	-0.81	74	200.6	29.39	herbicide
Mecoprop	7085-19-0	3.11	-0.19	47	214.6	250	herbicide
Metamitron	41394-05-2	2.78	0.83	78	202.2	1.77	herbicide
Metazachlor	67129-08-2	2.34	2.49	54	277.1	0.45	herbicide
Metolachlor	51218-45-2	-	3.13	120	283.8	0.53	herbicide
Myclobutanil	88671-89-0	2.27	2.94	6075	288.8	0.132	fungicide
n-Acetyl-4-aminoantipyrine	83-15-8	4.3	-0.07	240	245.2	500	TP of aminopyrine
p-Toluenesulfonamide	70-55-3	10.46	0.82	66	171.2	3.2	industrial chemical
Pendimethalin	40487-42-1	10.52	5.20	5615	281.3	$3.3e^{-4}$	herbicide

	CAS number	pKa[1]	log K_{OW}[1]	K_{OC}	MW	S_w	Compound group
Pethoxamid	106700-29-2	-	3.39	154	295.8	0.4	herbicide
Picolinafen	137641-05-5	11.87	5.37	28300	376.3	$4.7e^{-5}$	herbicide
Picoxystrobin	117428-22-5	-	3.67	965	367.3	0.003	fungicide
Pirimicarb	23103-98-2	4.99	1.70	56	238.3	3.1	insecticide
Prochloraz	67747-09-5	2.75	4.38	2425	376.7	0.026	fungicide
Prometryn	7287-19-6	5.71	3.51	656	241.4	0.033	herbicide
Propamocarb	24579-73-5	9.5	0.84	719	188.3	900	fungicide
Propiconazole	60207-90-1	2.24	3.72	1556	342.2	0.15	fungicide
Propoxycarbazone	145026-81-9	3.39	2.66	29	398.4	42	herbicide
Prosulfocarb	52888-80-9	-	4.65	1693	251.4	0.013	herbicide
Prothioconazole-desthio	120983-64-4	2.26	3.05	523	312.2	-	TP of prothioconazole
Pyraclostrobin	175013-18-0	-	3.99	11000	387.8	0.001	fungicide
Quinmerac	90717-03-6	4.31	-1.41	86	221.6	107	herbicide
Saccharin	81-07-2	2.32	0.91	32	205.1	3.4	sweetener
Simazine	122-34-9	3.23	2.18	147	201.7	0.005	herbicide
Spiroxamine	118134-30-8	9.34	5.51	2415	297.5	0.405	fungicide
Sucralose	56038-13-2	11.8	0.68	10	398.6	283	sweetener
Sulfamethazine	57-68-1	6.99	0.14	174	278.3	1.5	pharmaceutical
Sulfamethoxazole	723-46-6	6.16	0.89	607	253.3	0.61	pharmaceutical
Tebuconazole	107534-96-3	2.27	3.70	1536	307.8	0.036	fungicide
Terbuthylazine-2-hydroxy	66753-07-9	-	-	257	211.3	0.007	TP of terbuthylazine
Terbuthylazine	5915-41-3	3.18	3.40	219	229.7	0.006	herbicide
Terbutryn	886-50-0	5.72	3.74	607	241.4	0.025	biocide (herbicide)
Thiabendazole	148-79-8	4.08	2.47	3983	201.2	0.03	fungicide/preservative
Thiacloprid	111988-49-9	1.62	1.26	615	252.7	0.184	insecticide
Thiamethoxam	153719-23-4	-	-0.13	64	291.7	4.1	insecticide
Triethyl citrate	77-93-0	11.82	0.71	20	276.3	65	plasticiser
Trifloxystrobin	141517-21-7	2.37	4.50	2377	408.4	$6.1e^{-4}$	fungicide

[1] calculated using Calculator Plugins, Instant JChem 2012, ChemAxon (www.chemaxon.com); pK_a values only given for $1 < pK_a < 12$.

Table B.2: Detected organic micropollutants in *G. pulex* samples (concentrations in ng g^{-1} wet weight). Method detection limits (MQLs) in ng g^{-1}.

	MQL	**st15**	**st17**	**st22**	**st25**	**st28**	**st31**	**st36a**	**st36b**	**st38**	**st42**
Insecticides											
Imidacloprid	1.11	+	+	1.13	+	1.26	2.46	3.14	2.02	1.79	3.22
Thiacloprid	0.03	0.47	1.51	1.35	1.67	1.64	1.75	2.30	1.39	1.44	2.42
Fungicides											
Flusilazole	0.24			+							+
Propiconazole	0.05		3.85	3.49	2.27	2.94	2.92	2.13	3.06	1.74	2.69
Spiroxamine	0.09	+	0.19	0.16	0.14	+	0.12	0.18	+		
Tebuconazole	1.00		+	+	+	+	+	+	+	+	+
Herbicides											
Atrazine	1.22							+		+	
Diflufenican	0.71								+		
Fenuron	0.11	0.46	0.26	0.19		0.27		1.11	0.40		0.23
Pendimethalin	0.88			0.92				2.10			
Prosulfocarb	0.82				0.97	+	+	13.08	2.80	+	+
Terbutryn	1.18		+	+			+	+	+		
Terbuthylazine	1.07										1.45
Wastewater											
Carbamazepine	0.29		2.48	2.01	1.53	1.54	2.65	2.79	2.83	1.69	2.19
CBZ-diol[a]	1.14		+	+	+	+	+	1.23	+	+	+
1H-Benzotriazole	3.85		+	+	+	+	+	3.92	+	+	+
5MBT[b]	0.03		1.52	0.79	0.63	0.88	1.75	1.78	1.30	0.48	1.11

[a] CBZ-diol = 10,11-Dihydroxy-10,11-dihydrocarbamazepine

[b] 5MBT = 4-/5-Methyl-1H-benzotriazole

+ Symbol means pollutants detected but under the MQL.

Table B.3: Detected organic micropollutants in sediments samples (concentrations in ng g^{-1} TOC). Method detection limits (MQLs) in ng g^{-1} TOC.

	MQL	**st14t**	**st19t**	**st23t**	**st28**	**st31**	**st33t**	**st36a**	**st41t**
Insecticides									
Acetamiprid	4.6		10						
Diazinon	2.7					942	696	722	903
Fipronil	7.7					16		9.9	+
Pirimicarb	3.7	27		11	9.0	6.4	9.0	5.7	3.7
Thiacloprid	6.0	28		10	9.9	9.7	10	7.4	+
Fungicides									
2-Aminobenzimidazole	3.2				1197	4854		14066	1072
Azoxystrobin	7.8	24		14	8.3	+	7.8	+	+
Boscalid	6.4	55		63	23	23	16	16	8.0
Carbendazim	54.0		+		+	+		+	
Difenoconazole	4.7	49		20	25	22	14	17	7.2
Epoxiconazole	4.2	82	15	67	35	31	21	26	12
Fenpropimorph	6.4	54	12	27	38	53	29	28	14
Flusilazole	2.5	40	14	55	19	17	17	17	15
M04[a]	23.8	47		+	+	+	+	+	+
Prochloraz	5.0			17	13	18	11	12	6.5
Propiconazole	0.7				123	263	68	151	82
Pyraclostrobin	8.7			8.9	+	+			
Spiroxamine	46.1	239	+	52	87	58	+	+	+
Tebuconazole	1.5	23	2.9	29	54	79	12	45	18
Herbicides									
Atrazine	2.1	67		39	39	16	16	37	5.1
Bentazone	2.2								+
Chloroxuron	359.9	+							
Clomazone	6.0	37		18	15	13	12	11	8.5
Desethylatrazine	2.2	51		22	19	16		17	
Desethylterbutylazine	1.0	36	13	11	11	10	11	11	4.5
Diflufenican	5.0			52	8.3	8.3	+	9.9	+
Diuron	3.8	44	10	17	19	20	16	18	7.3
Flufenacet	5.1	27		9.9	9.1	8.2	9.5	6.1	+
Flurtamone	4.0							5.2	
Irgarol	5.3				10	15	30	23	6.6
Isoproturon	3.2	32	7.3	12	11	23	12	14	7.1
Metamitron	54.2			68					
Metazachlor	5.6	38		18	11	8.6	10	8.6	+
Metolachlor	7.2	28		10	10	+	9.0	+	+
MT13[b]	4.3					+		33	
Pendimethaline	8.7			+					
Pethoxamid	7.6							+	+
Prometryn	42.2	930	107	850	263	290	61	187	43
Prosulfocarb	5.4			+		+	+	5.6	+
Simazine	1.6	95	13	92	73	39	28	28	9.5
Terbutryn	3.0	32	15	18	72	229	21	137	22

	MQL	st14t	st19t	st23t	st28	st31	st33t	st36a	st41t
Terbuthylazine	2.4	34		35	13	15	14	44	7.5
Wastewater chemicals									
CBZ-diol[c]	76.6					179		114	
1H-Benzotriazole	9.0			50	577	896	95	1120	198
5MBT[d]	4.5	818		217	2029	1768	375	1821	423
Caffeine	2.6	474	3.2	127	138	221	123	121	35
Carbamazepine	7.2	34		9.9	244	340	73	251	51
DEET[e]	4.0	97		15	13	49	28	39	34
Diclofenac	741.5				+				
NAAP[f]	110.2				+	+		+	+
PTSA[g]	232.2		1263					616	
Triethyl citrate	5.3	2275	12	493	348	244	43	187	252

[a] M04 = Prothioconazole-desthio

[b] MT13 = Terbuthylazine-2-hydroxy

[c] CBZ-diol = 10,11-Dihydroxy-10,11-dihydrocarbamazepine

[d] 5MBT = 4-/5-Methyl-1H-benzotriazole

[e] DEET = N,N-Diethyl-meta-toluamide

[f] NAAP = n-Acetyl-4-aminoantipyrine

[g] PTSA = *p*-toluene-sulfoamide

+ Symbol means pollutants detected but under the MQL.

Table B.4: Detected organic micropollutants in water samples (concentrations in ng L^{-1}). Method detection limits (MQLs) in ng L^{-1}.

	MQL	**st14t**	**st15**	**st17**	**st19t**	**st22**	**st23t**	**st25**	**st28**	**st31**	**st33t**	**st36a**	**st36b**	**st38**	**st41t**	**st42**
Insecticides																
Diazinon	0.3			0.5					14.9	1.7		1.3	0.93	1.3	0.6	1.1
Dimethoate	1.0								8.1							
Fipronil	0.6			4.9		3.12		3.4	4.6			4.4	4.6	4.6	2.7	4.1
Imidacloprid	2.4			3.8					44.2	7.5		5.0	3.6	5.7	3.2	5.3
Thiacloprid	0.6								1.3							
Thiamethoxam	1.0								10.2							
Fungicides																
2-Aminobenzimidazole	1.0			2.1		1.3		1.3	14.1	2.7		2.0	2.4	2.2	1.0	1.4
Azoxystrobin	1.0						2.9									
Boscalid	1.8			4.6		3.0	2.9	2.7	9.5	4.0		2.6	2.9	3.5		3.8
Carbendazim	0.8	0.8	1.0	8.2	4.2	6.4	1.7	5.5	4.7	5.2	1.4	6.8	6.9	6.7	3.0	4.9
Difenoconazole	1.0	13.4														
Myclobutanil	0.8	2.0														
Prochloraz	1.0								4.7							
Propiconazole	0.8			50.5		25.4	2.2	21.7	9.2	19.8	1.0	36.4	32.5	33.3	13.6	29.9
Prothioconazole-desthio	1.0										1.0					
Tebuconazole	0.7			35.1	0.9	20.9	3.9	20.2	12.5	18.1	1.0	22.7	23.4	21.3	10.4	21.3
Thiabendazole	0.8			2.8		1.6		2.6	7.4	2.8	1.8	2.6	2.6	2.2		1.8
Herbicides																
2,6-Dichlorobenzamide	1.0			5.2		2.3		2.1		3.7						
Atrazine	0.5	24.3	5.1	4.9	1.1	3.2	9.9	4.9	5.7	4.5	1.0	4.0	4.2	3.7	2.1	3.7
Chloridazone	2.0			3.7												
Chloroxuron	1.0	1.5														
Clomazone	0.8			2.0	1.0	2.6		1.8		1.5	1.5	4.0	5.2	4.6	2.4	4.3
Deisopropylatrazin	1.0	1.4	3.5	2.9		3.0	6.5	2.4	5.9	4.1		3.8		3.8	2.6	
Desethylatrazine	1.5	9.8	5.4	5.0		3.9	8.2	4.9	8.2	5.5		4.3	4.3	6.3	3.0	3.9
Desethylterbutylazine	1.0						1.0		1.1		6.3	1.1	1.3	1.5	1.0	1.0

	MQL	st14t	st15	st17	st19t	st22	st23t	st25	st28	st31	st33t	st36a	st36b	st38	st41t	st42
Desphenylchloridazon	10.0				23.6											
Diflufenican	1.2						1.7									
Diuron	1.5			5.0		2.7		2.8	12.2	5.0		2.4	4.0	4.4		3.2
Fenuron	1.0	7.3		8.6		2.9		3.2	1.7	2.4		7.3	3.2	4.1	3.0	7.0
Flufenacet	1.0				1.7	1.5	1.5	1.2	2.3	1.9	2.1	5.4	5.1	5.3	3.4	3.8
Flurtamone	0.7													0.8		
Irgarol	0.4										0.8					
Isoproturon	0.5			2.4	0.9	1.9	0.7	3.5	5.5	2.4		2.8	2.9	2.8	2.2	2.2
Lenacil	1.5										3.0					
MCPA[a]	1.0			2.1		2.2	2.6		37.9		1.1	19.2	18.0	19.2	6.4	19.7
Mecoprop	1.5						19.3		3.8			7.8	6.1	7.3	3.3	8.7
Metazachlor	0.5	0.9		1.0	0.5	2.3	4.0	1.3	17.9	2.8	1.2	3.9	4.8	4.1	2.6	3.6
Metolachlor	0.6										1.8	0.7				
Pethoxamid	0.7	6.7	1.3									0.8	0.8	1.0		
Prometryn	0.4			0.6	0.9	0.7	2.4	0.6	4.1	0.8		0.9	0.8	0.7	0.5	0.9
Prosulfocarb	8.0							22.2		10.2	9.6	23.5	24.7	25.9	12.5	22.6
Quinmerac	2.5					2.7			7.8					3.7		2.7
Simazine	0.5	4.2	4.3	4.2	0.6	4.2	8.9	5.2	12.1	5.5	1.7	5.4	5.7	5.1	2.6	6.0
MT13[b]	0.6		1.2	4.1	3.3	11.5	4.8	3.2	17.9	4.9	2.5	12.7	13.0	12.1	5.4	11.8
Terbutryn	0.4	0.7	1.2	7.4	1.7	4.9	1.8	4.6	71.7	12.3	0.6	10.6	9.7	9.0	4.3	8.7
Terbutylazine	0.4					0.5	1.0		1.5	0.6	4.8	0.8	0.9	0.8	0.4	0.9
Wastewater chemicals																
CBZ-diol[c]	2.5			605.2		379.7		393	3910	771.5	33	647.7	632.5	633.9	339.8	592.1
1H-Benzotriazole	10.0		10	1039.8	21.3	528.2	118.1	657.6	5239.3	1370.1	58.9	987.2	1050.2	975.8	389.3	667.1
5MBT[d]	2.5	4.3	17.8	808.2	3.4	402.4	25.4	527.6	3999.0	1112.9	24.4	765.7	780.9	744.9	286.6	493.2
Acesulfame	4.0	79	66	895	191	531	150	648	2287.0		1532	799	828	822	667	666
Acetylsulfamethoxazole	3.0			7.9				6.3	24.1	8.1		5.3		7.0		5.9
Caffeine	5.0	19.6	68.6	109.8	14.5	218.2	150.6	109.6	13.3	99.1		104.5	120.6	81.1	44.0	95.6
Carbamazepine	0.5	3.9	2.0	380.2		219.2	1.0	238.2	2343.7	503.6	55.8	395.5	378.9	373.4	210.7	365.8

	MQL	st14t	st15	st17	st19t	st22	st23t	st25	st28	st31	st33t	st36a	st36b	st38	st41t	st42
Cotinine	2.0	33.5	33.6	24.8	64.4	42.9	52.3	42.0	47.5	34.0	67.7	43.7	44.3	57.0	14.9	44.2
Cyclamate	16.0	42	83	43		497	77	88	141		152	144	142	141	249	109
DEET[e]	0.4			9.0		3.8	1.5	3.1	82.2	14.8	1.9	12.0	11.7	15.8	6.6	11.7
Diclofenac	2.5	6.0	15.2		24.8	522.1	83.5	490.0	5717.2		16.3	1003.8	1118.3	880.7	364.3	643.0
NAAP[f]	1.5	12.2	21.9	941.6	27.8	612.6	44.2	591.1	1635.9	839.0	13.6	743.3	754.4	717.3	373.9	650.5
p-Toluene-sulfonamide	10.0		14.0	34.7		44.7		55.8	473.4	90.7	96.3	114.2	117.4	115.3	29.6	105.0
Saccharin	15.0			28.4	72.8	91.3	66.7	31.9	57.1		136.6	41.9	46.1	32.4	29.5	33.7
Sucralose	18.0		56.7	1535.0		891.6		962.4	5877.5		391.7	1340.3	1388.4	1375.1	712.9	834.1
Sulfamethoxazole	1.5			26.9		13.5		13.9	386.8	43.2		31.5	27.4	27.2	16.6	19.0
Triethyl citrate	5.0	12.5	9.9	34.0	17.1	50.2	49.1	46.2	22.1	26.6	15.3	49.8	56.6	51.5	19.4	58.0

[a] MCPA = 2-methyl-4-chlorophenoxyacetic acid,

[b] MT13 = Terbuthylazine-2-hydroxy

[c] CBZ-diol = 10,11-Dihydroxy-10,11-dihydrocarbamazepine,

[d] 5MBT = 4-/5-Methyl-1H-benzotriazole,

[e] DEET = N,N-Diethyl-meta-toluamide

[f] NAAP = n-Acetyl-4-aminoantipyrine

Table B.5: All compounds grouped by classes, mean water, sediment and biota concentrations in ng L^{-1}, ng g^{-1} TOC and in ng g^{-1} wet weights respectively, octanol-water partitioning coefficient (K_{OW}), organic carbon-water partitioning coefficient (K_{OC}), and mean molecular weight (MW). Minimum and maximum values are shown in brackets.

Pollutants	**Concentration**			K_{OW}			K_{OC}		
	Water	**Sediment**	**Biota**	**Water**	**Sediment**	**Biota**	**Water**	**Sediment**	**Biota**
Pesticides									
Herbicides	5.3 (0.4-71.7)	52.0 (4.1-930)	1.8 (0.19-13)	2.4 (-1.4-4.9)	3.1 (0.8-4.9)	2.6 (0.9-5.2)	424	531	1294
Fungicides	10.0 (0.8-50.5)	39.7 (2.9-263)	1.8 (0.12-3.8)	2.8 (1.5-4.4)	3.9 (2.5-5.5)	4.3 (3.7-5.5)	1674	2557	1862
Insecticides	5.5 (0.5-44.2)	172.5 (3.7-942)	1.8 (0.47-3.2)	2.6 (-0.1-4.0)	2.1 (0.8-4.0)	0.9 (0.5-1.2)	3072	1413	761
TP pesticides	4.9 (1.0-23.6)	980.1 (4.5-14066)		1.8 (-1.3-3.3)	1.9 (0.8-3.0)		154	216	
Mean (± SD)				2.4 (0.4)	2.8 (0.9)	2.6 (1.7)			
Wastewater chemicals									
Pharmaceuticals	244 (1.0-5717)	114 (3.2-474)	2.07 (0.6-2.8)	1.6 (-0.07-4.5)	1.3 (-0.07-2.4)	2.4	1919	3595	83
TP pharmaceuticals	524 (5.3-3910)	147 (114-179)	1.2 (1.2-1.2)	0.1 (-0.2-1.2)	-0.2	-0.2	134	29	29
Sweeteners	562 (28.4-5877)			-0.4 (-1.6-0.9)			14		
Industrial chemicals	444 (3.4-5239)	701 (12-2275)	1.4 (0.4-3.9)	1.1 (0.7-1.7)	1.2 (0.7-1.7)	1.6 (1.4-1.7)	94	94	145
Mean (± SD)				0.6 (0.9)	0.8 (0.8)	1.3 (1.3)			

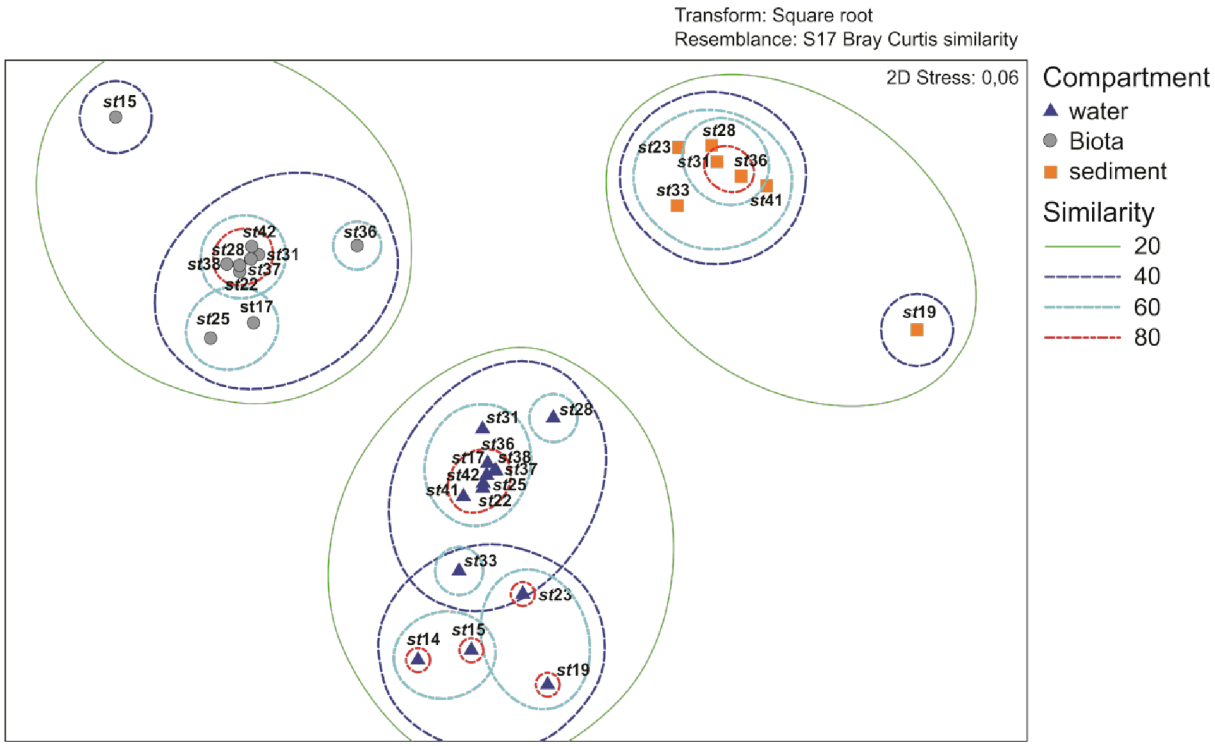

Figure B.1: Two-dimensional ordination of the environmental compartments from non-parametric multidimensional scaling (MDS) applied to a Bray-Curtis similarity matrix based on C^{fd} data. The environmental compartments clustered significantly (*post hoc* Dunn's-test $p<0.05$).

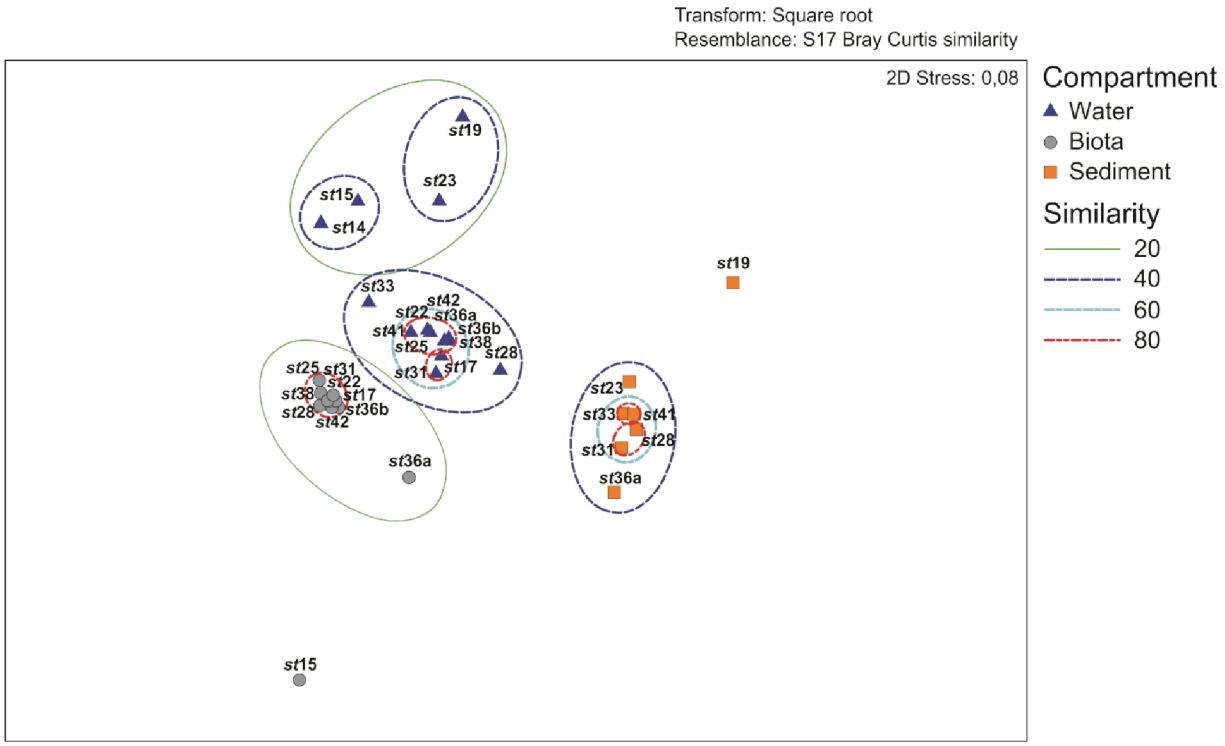

Figure B.2: Two-dimensional ordination of the environmental compartments from non-parametric multidimensional scaling (MDS) applied to a Bray-Curtis similarity matrix based on chemical activity data. The environmental compartments clustered significantly (*post hoc* Dunn's-test $p<0.05$).

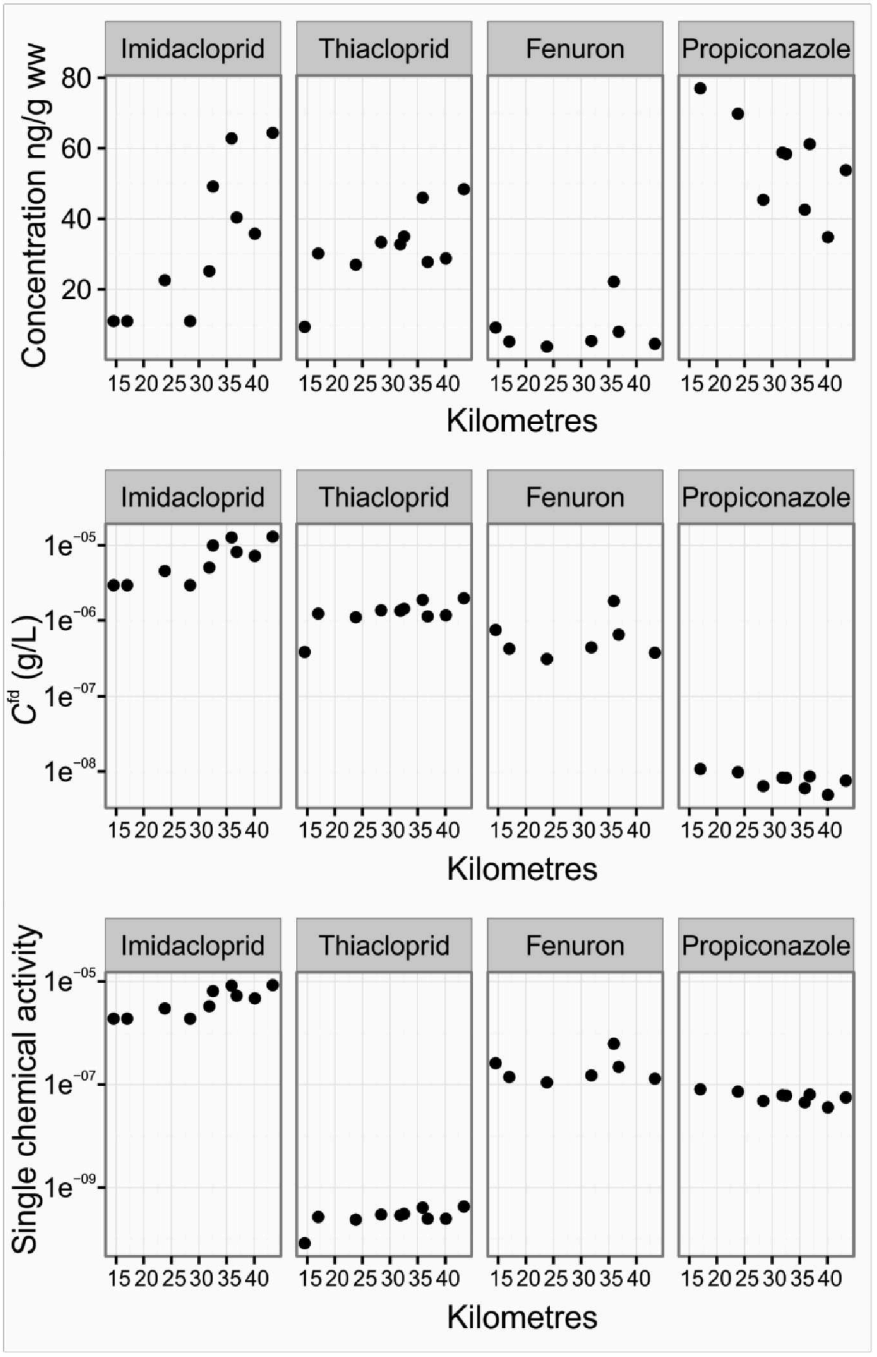

Figure B.3: Individual pesticide chemicals in gammarids. Total concentrations in ng/g normalised by lipid content (upper chart), C^{fd} in g/L (middle) and chemical activity (bottom).

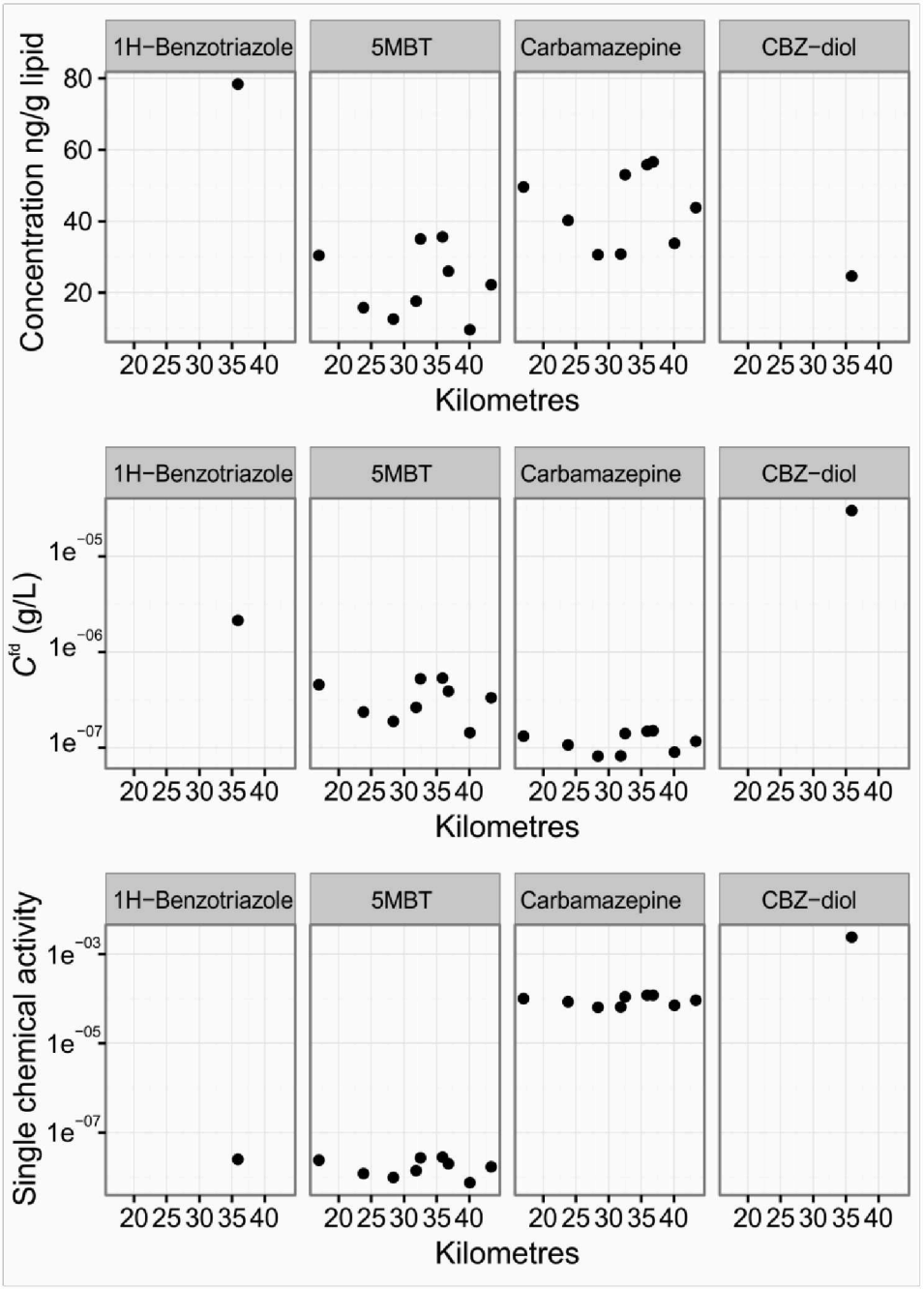

Figure B.4: Individual pharmaceutical and industrial chemicals in gammarids. Total concentrations in ng/g normalised by lipid content (upper chart), C^{fd} in g/L (middle) and chemical activity (bottom).

5MBT=4-/5-methyl-1H-benzotriazole
CBZ-diol=10,11-dihydroxy-10,11-dihydrocarbamazepine

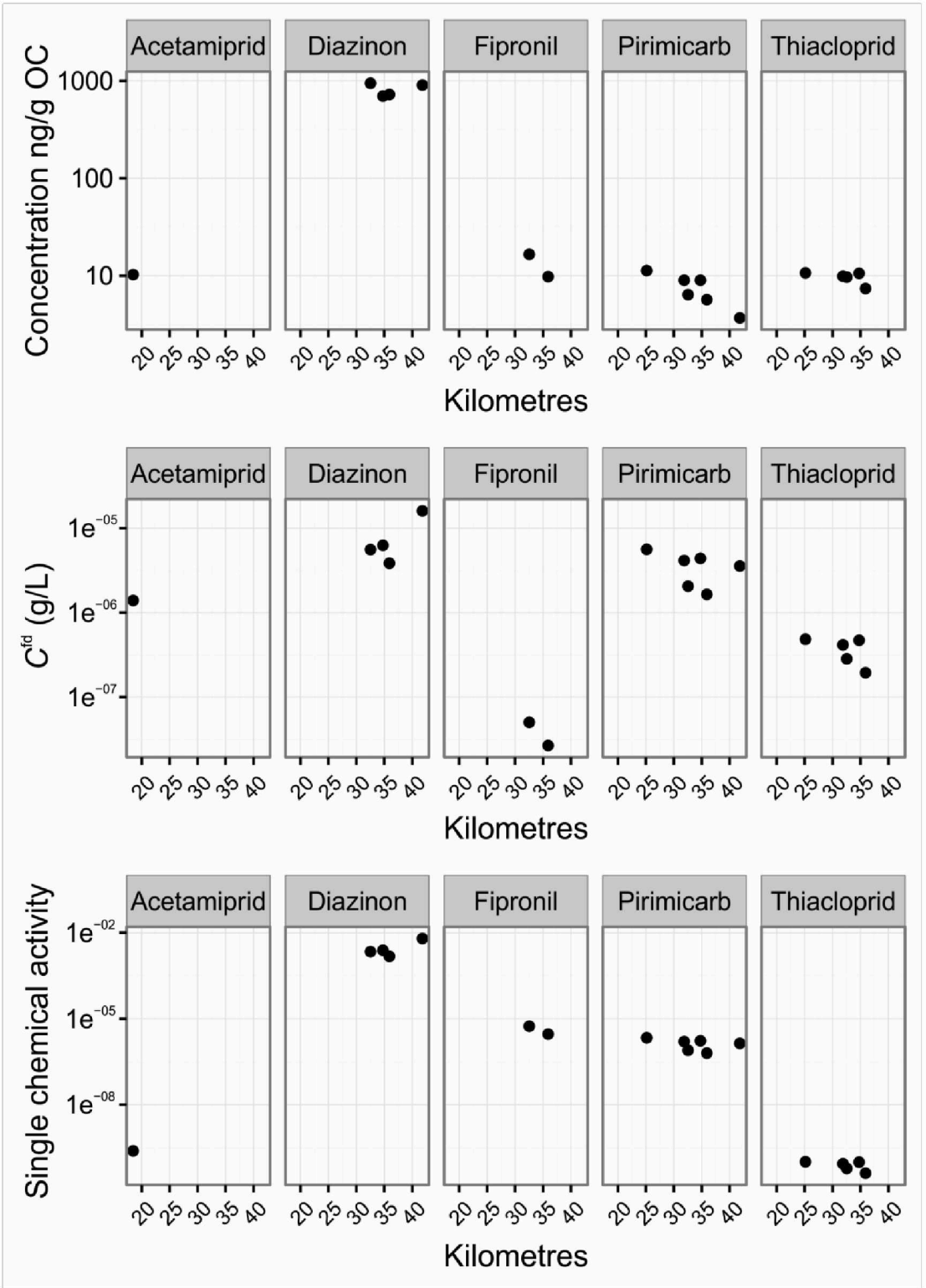

Figure B.5: Individual insecticides in sediments. Total concentrations in ng/g normalised by lipid content (upper chart), C^{fd} in g/L (middle) and chemical activity (bottom).

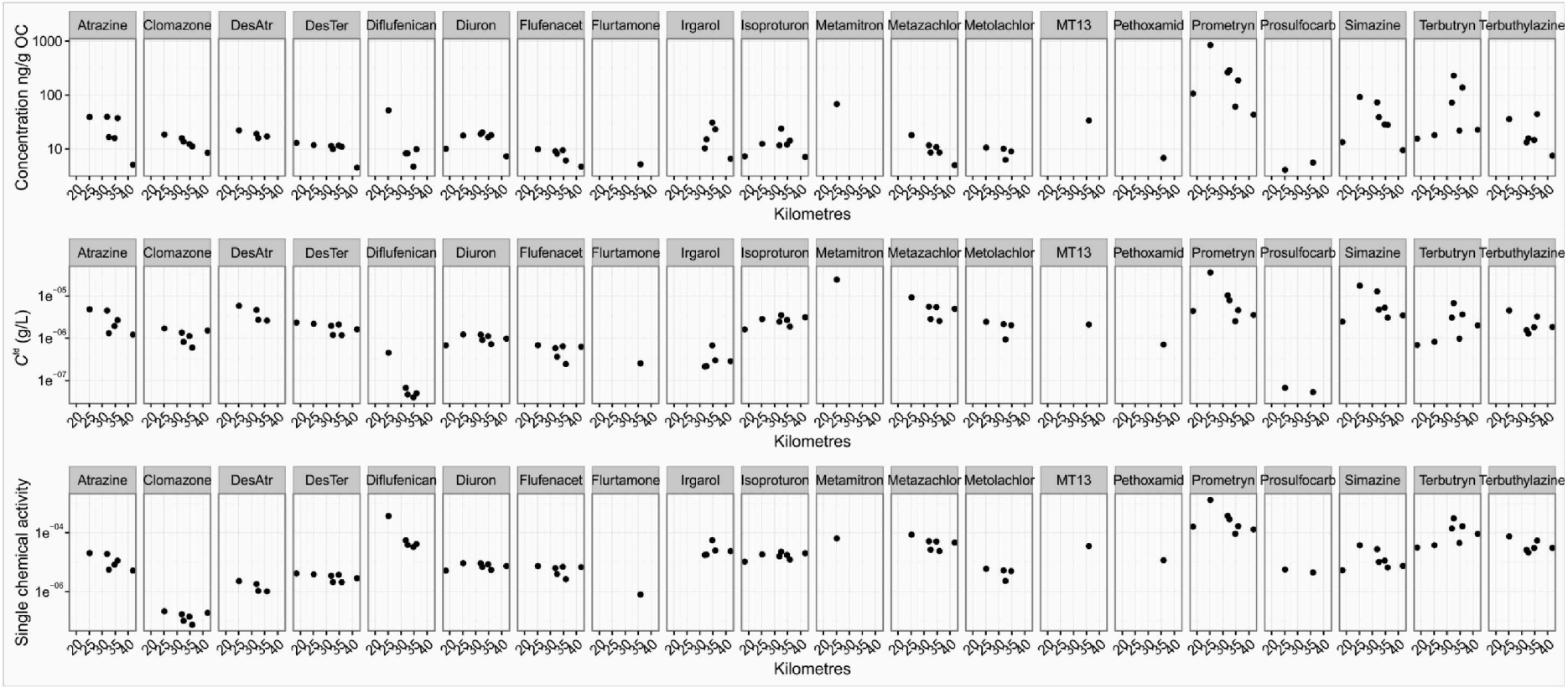

Figure B.6: Individual herbicides in sediments of the River Holtemme. Total concentrations in ng/g normalised by organic carbon content (upper chart), C^{fd} in g/L (middle) and chemical activity (bottom).
DesAtr=Desethylatrazine
DesTer=Desethylterbuthylazine
MT13=Terbuthylazine-2-hydroxy

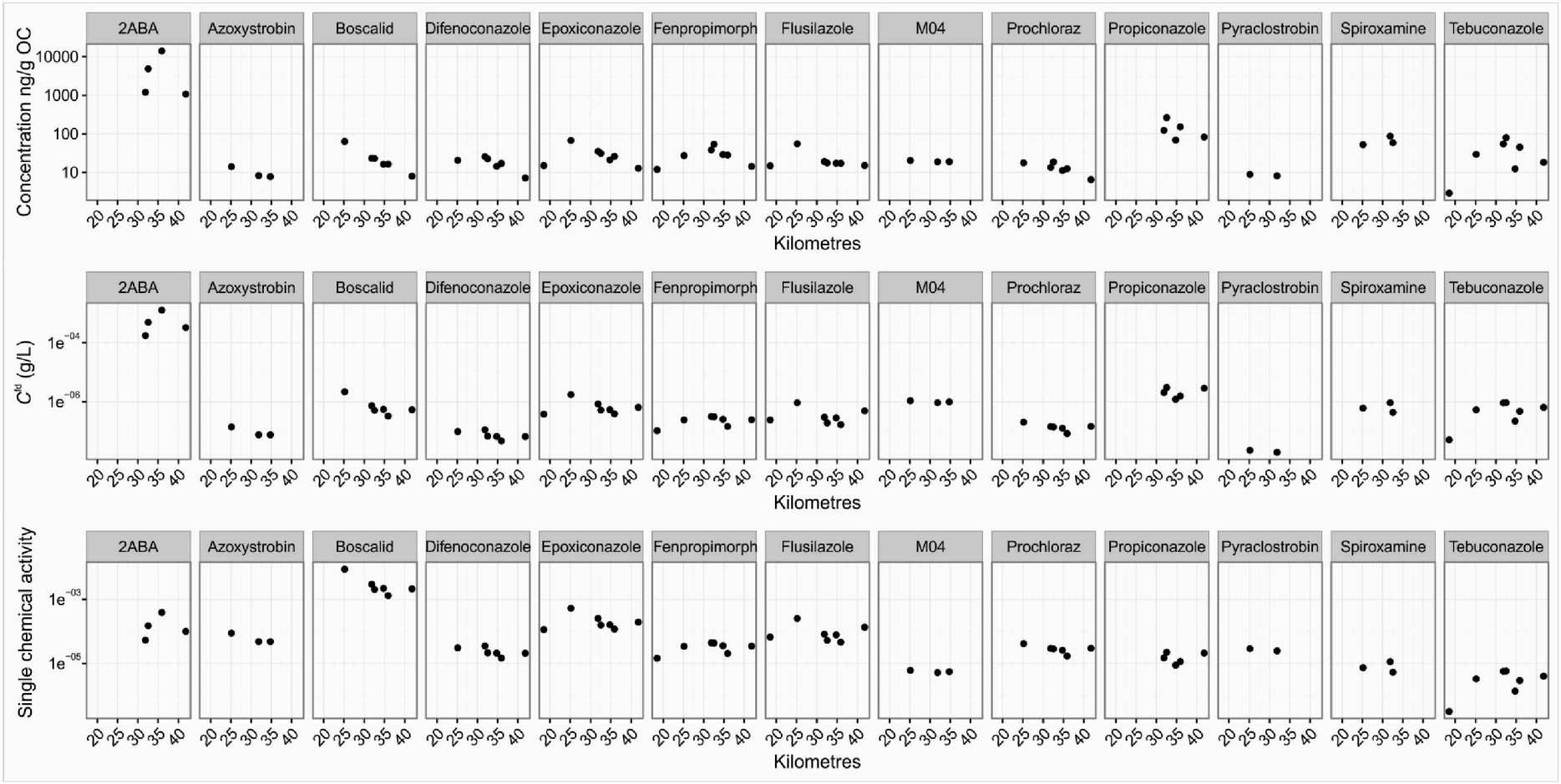

Figure B.7: Individual fungicides in sediments of the River Holtemme. Total concentrations in ng/g normalised by organic carbon content (upper chart), C^{fd} in g/L (middle) and chemical activity (bottom).
2ABA=2-Aminobenzimidazole
M04=Prothioconazole-desthio

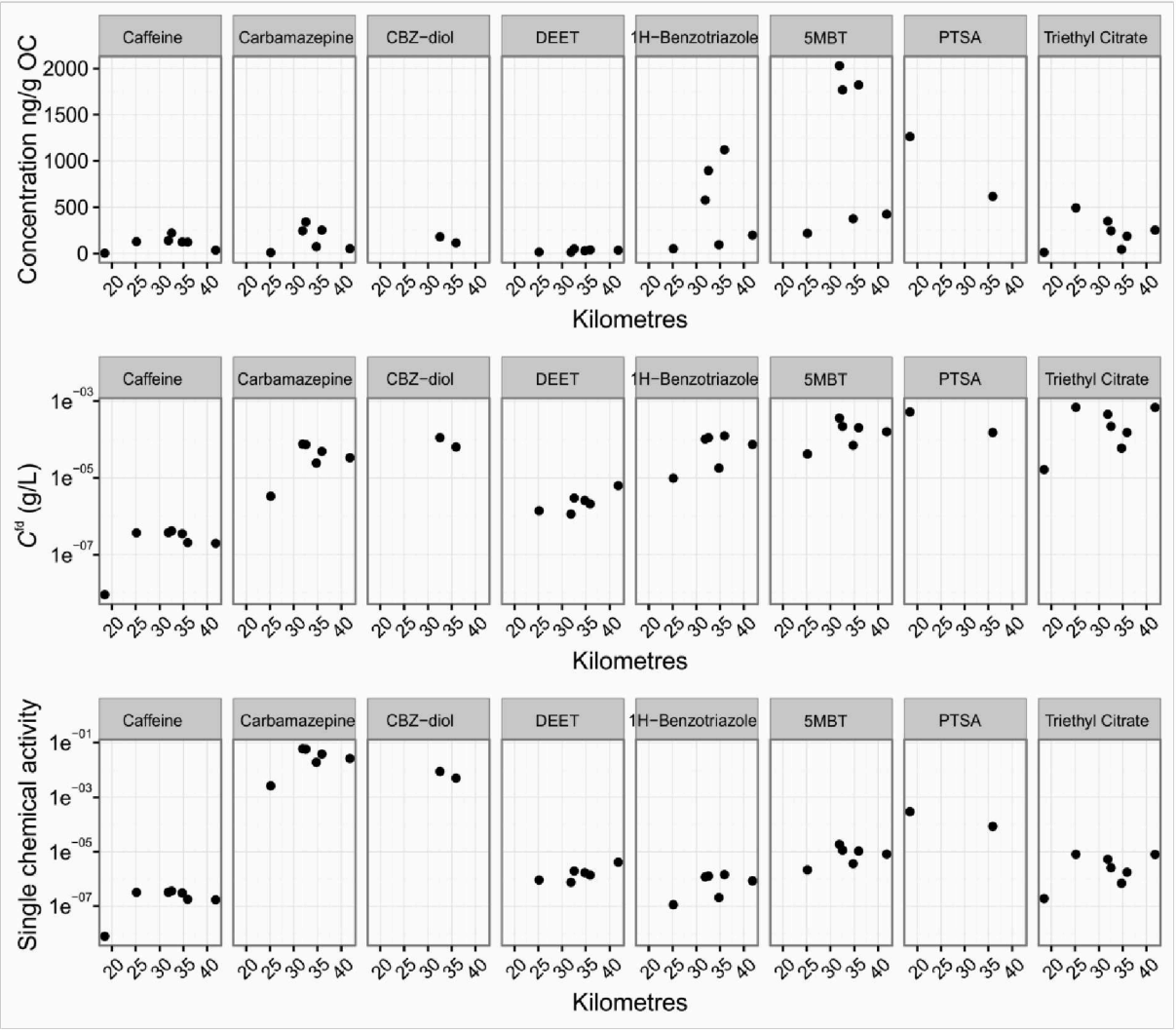

Figure B.8: Individual pharmaceuticals and industrial chemicals in sediment. Total concentrations in ng/g normalised by organic carbon content (upper chart), C^{fd} in g/L (middle) and chemical activity (bottom).
5MBT = 4-/5-methyl-1H-benzotriazole
CBZ-diol=10,11,dihydroxy-10,11-dihydrocarbamazepine
DEET = N,N-diethyl-meta-toluamide
PTSA=*p*-toluene-sulfoamide

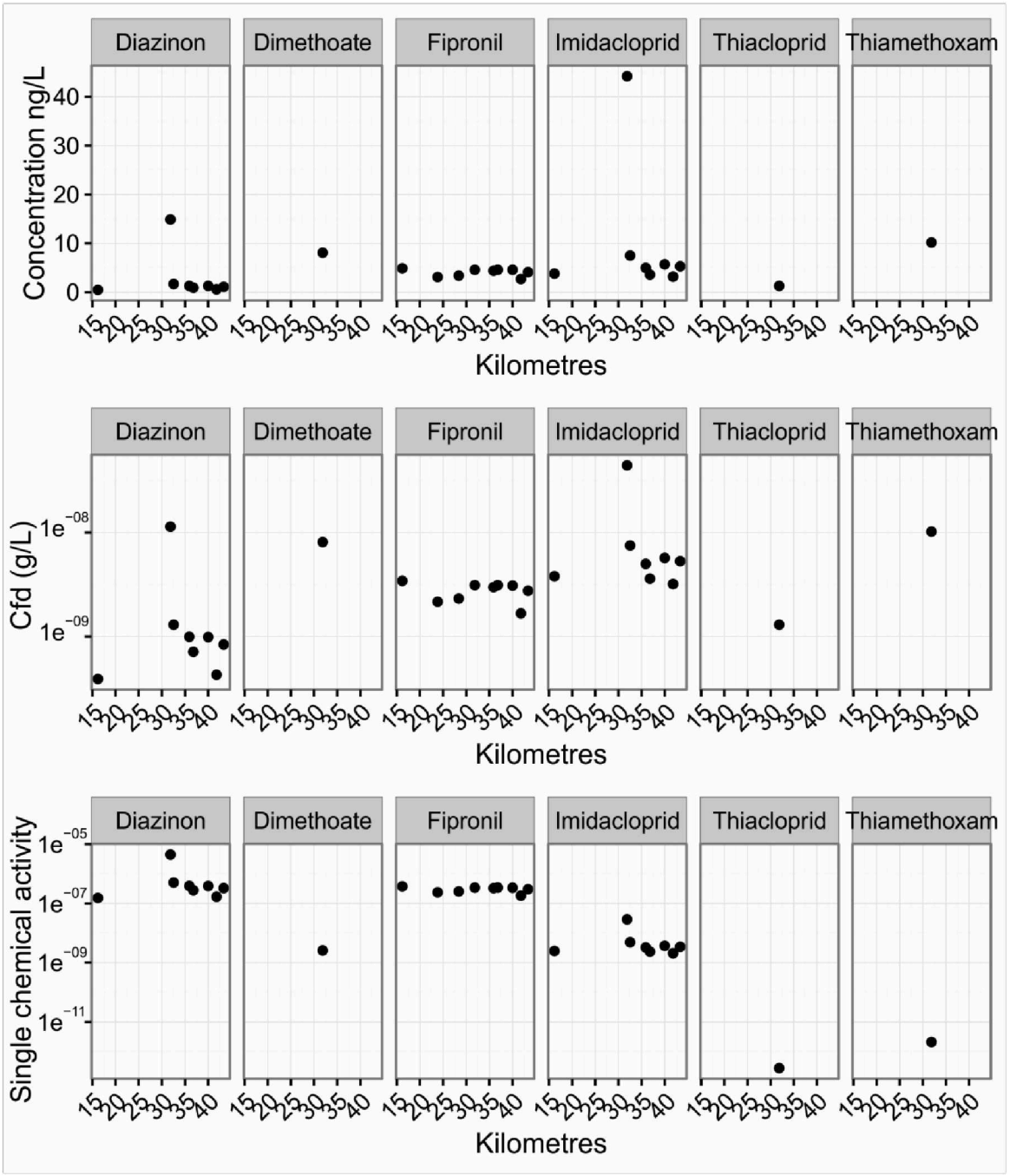

Figure B.9: Individual insecticides in water samples. Total concentrations in ng/g normalised by organic carbon content (upper chart), C^{fd} in g/L (middle) and chemical activity (bottom).

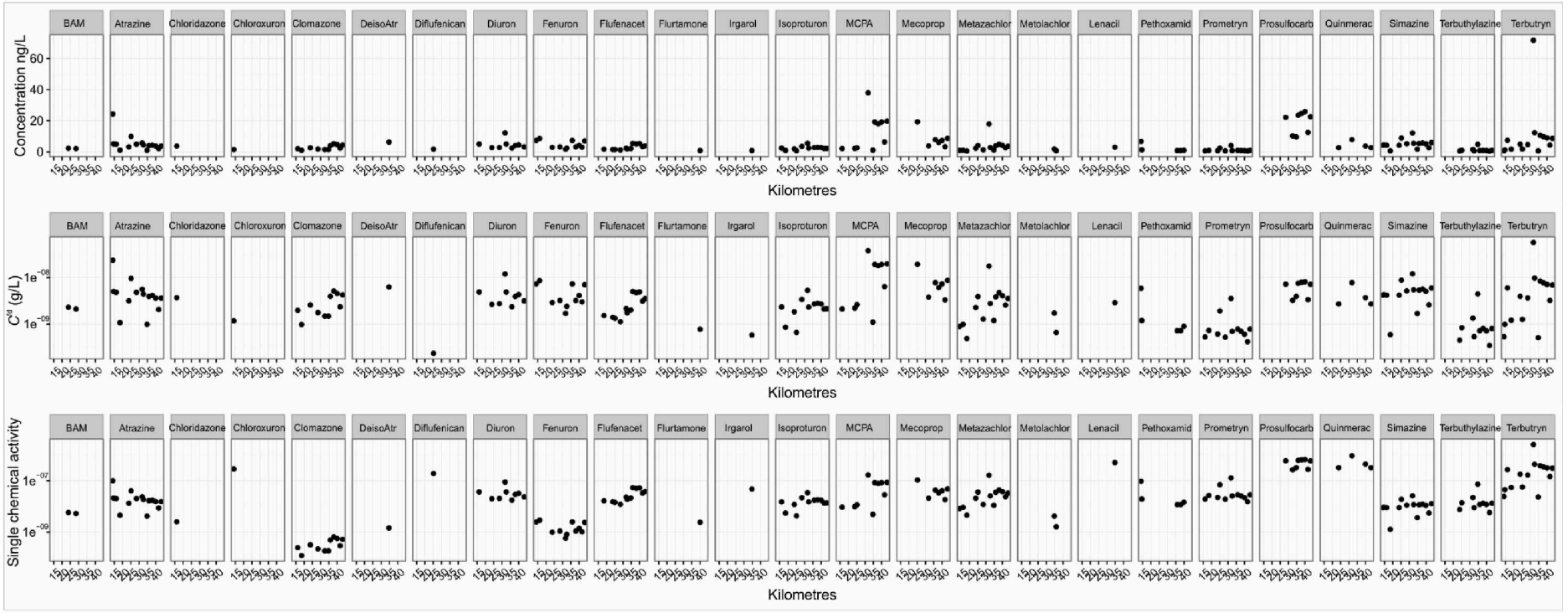

Figure B.10: Individual herbicides in water samples. Total concentrations in ng/L (upper chart), C^{fd} in g/L (middle) and chemical activity (bottom).
BAM=2,6-dichlorobenzamide
DeisoAtr=Deisopropylatrazine
MCPA=2-methyl-4-chlorophenoxyacetic acid

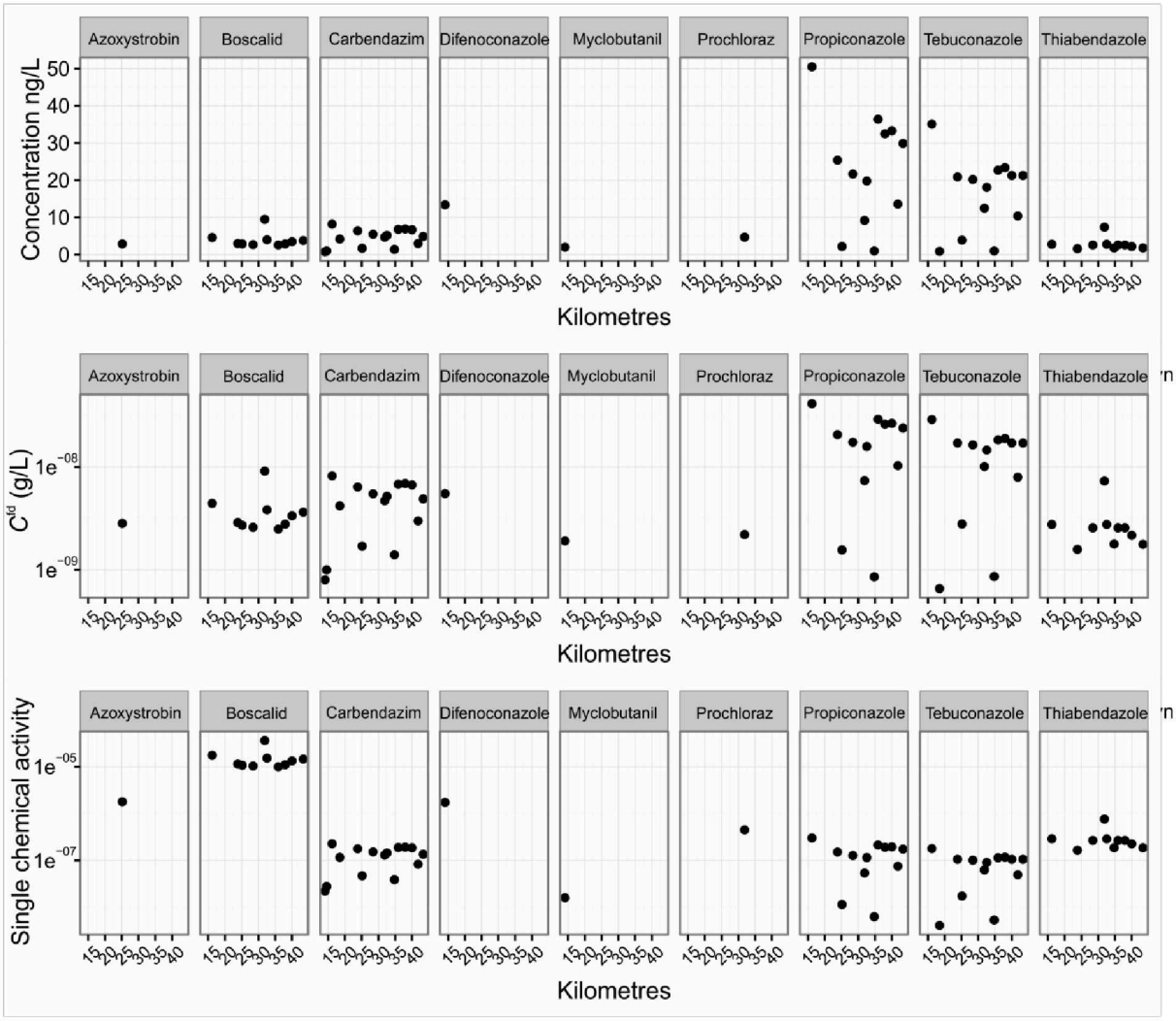

Figure B.11: Individual fungicides in water samples. Total concentrations in ng/L (upper chart), C^{fd} in g/L (middle) and chemical activity (bottom).

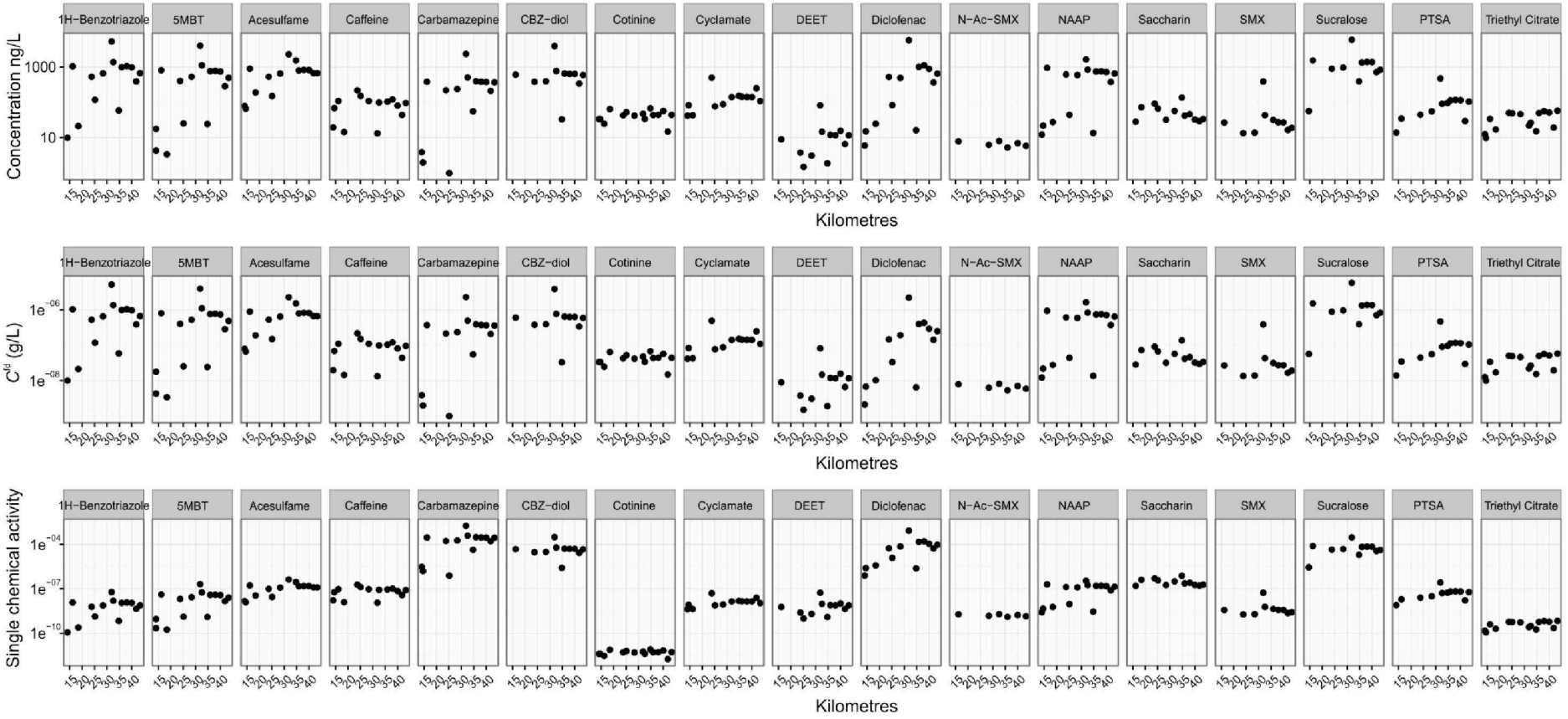

Figure B.12: Individual pharmaceuticals and industrial chemicals in water samples. Total concentrations in ng/L (upper chart), C^{fd} in g/L (middle) and chemical activity (bottom).
5MBT=5-Methyl-1H-benzotriazole
CBZ-diol=10.11-Dihidroxy-10,11-dihydrocarbamazepine
DEET=N,N-Diethyl-meta-toluamide
N-Ac-SMX = N-Aceytylsulfamethoxazole
NAAP = n-Acetyl-4-aminoantipyrine
SMX = Sulfamethoxazole
PTSA = *p*-Toluene-sulfoamide

APPENDIX C

Supplementary information for Chapter 4

Table C.1: Hypothetical freely dissolved concentrations (μg/L) in *G. pulex* transformed to toxic units in the River Holtemme. *m*TU represents maximum toxic unit value.

	st15	**st17**	**st22**	**st25**	**st28**	**st31**	**st36a**	**st36b**	**st38**	**st42**	***m*TU**
Insecticides											
Imidacloprid			6.08		6.78	13.24	16.90	10.87	9.63	17.33	-0.07
Thiacloprid	0.51	1.66	1.48	1.83	1.80	1.92	2.52	1.52	1.58	2.66	-2.11
Fungicides											
Spiroxamine		1.2×10^{-5}	1.0×10^{-5}	9.0×10^{-6}		7.0×10^{-6}	1.1×10^{-5}				-5.65
Propiconazole		0.015	0.013	0.009	0.011	0.011	0.008	0.012	0.007	0.010	-5.72
Herbicides											
Fenuron	1.009	0.570	0.417		0.592		2.434	0.877		0.504	-3.36
Pendimethalin			1.2×10^{-4}				2.7×10^{-4}				-6.03
Prosulfocarb				4.3×10^{-4}			0.006	0.001			-4.98
Terbuthylazine										0.012	-6.26
Wastewater chemicals											
Carbamazepine		0.176	0.143	0.109	0.109	0.188	0.198	0.201	0.120	0.155	-4.93
CBZ-diol							39.897				
1H-Benzotriazole							2.847				-3.64
5MBT		0.607	0.315	0.251	0.351	0.698	0.710	0.519	0.192	0.443	-4.09

Table C.2: *P*-values for bottleneck detection under each model (IAM: Infinite allele model; TPM: two-phase mutation model and SMM: stepwise mutation model). Parameters for the TPM include 95% step-wise mutation and 20% variance on multi-step mutations and recommended defaults setting in parenthesis according to Cornuet & Luikart (1996). Significant results are represented by asterisks.

Sample	**IAM**	**TPM**	**SMM**
st15	0.84 (0.84)	0.98 (0.84)	0.98 (0.98)
st17	0.50 (0.50)	0.75 (0.50)	0.84 (0.84)
st22	0.21 (0.24)	0.75 (0.32)	0.75 (0.75)
st25	0.08 (0.08)	0.67 (0.10)	0.71 (0.71)
st28	0.12 (0.12)	0.32 (0.15)	0.45 (0.45)
st31	0.01* (0.01)*	0.10 (0.01)*	0.15 (0.15)
st36a	0.01* (0.01)*	0.28 (0.01)*	0.41 (0.41)
st36b	0.15 (0.15)	0.71 (0.17)	0.78 (0.78)
st38	0.50 (0.50)	0.84 (0.63)	0.87 (0.87)
st42	0.41 (0.41)	0.67 (0.50)	0.82 (0.82)

Table C.3: Presence/absence and degree of influence scale explained Table 4.1. A matrix was built including main stressors identified along the River Holtemme. Main stressors were agriculture landscapes (Agriculture), presence of rain water drainage (RW), wastewater treatment plant (WWTP) and presence of weirs (Weir).

Pop ID	Agriculture	WWTP	Weir	sMS
*st*15	1	0	0	1
*st*17	1	0.88	0	1.88
s*t*22	0.66	0.71	0	1.37
*st*25	0.33	0.54	0	0.87
*st*28	0	0.54	0	0.54
*st*31	0.33	0.94	0	1.27
*st*36a	1	0.99	1	2.99
*st*36b	1	1	0.66	2.66
*st*38	0.66	0.60	1	2.26
*st*42	1	0.70	0.66	2.36

Table C.4: Results of the permutation test for RDA axes responses using 10^4 random permutations.

	d.f.	**var.**	**F-ratio**	***P*-value**
RDA1	1	0.0052	220.08	0.001
RDA2	1	0.0013	54.66	0.001
RDA3	1	0.0002	9.36	0.001
Residual	234	0.0056		

d.f.=degrees of freedom; var.=variance

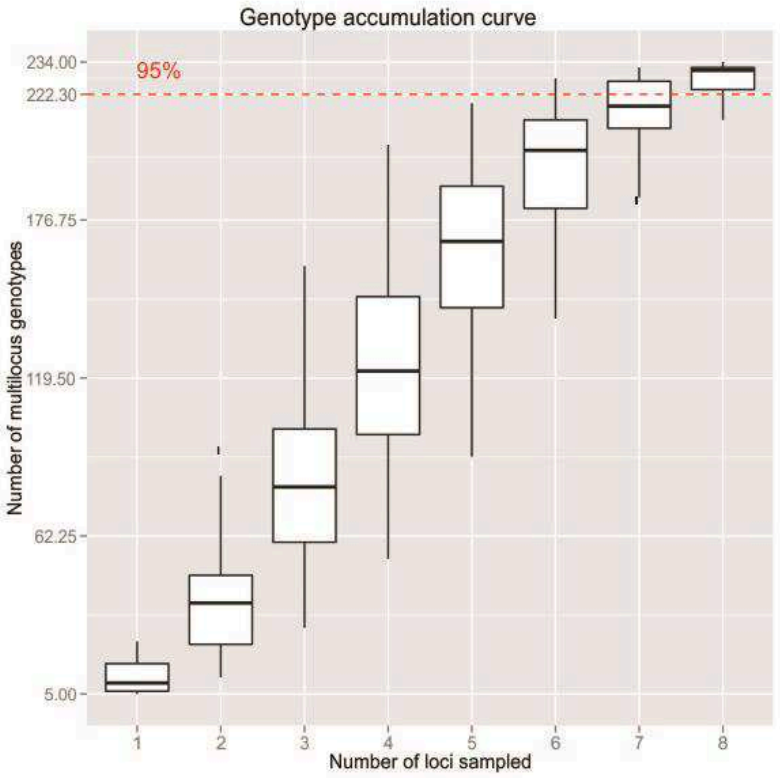

Figure C.1: Genotype accumulative curve for *G. pulex* samples from the River Holtemme. The vertical axis denotes the number of observed multi-locus genotypes. Number of loci is indicated on the horizontal axis, randomly sampled without replacement. Each boxplot contains 10^3 random samples representing different possible combinations of *n* loci. The red dashed line represents 95% of confidence of genotypes resolution.

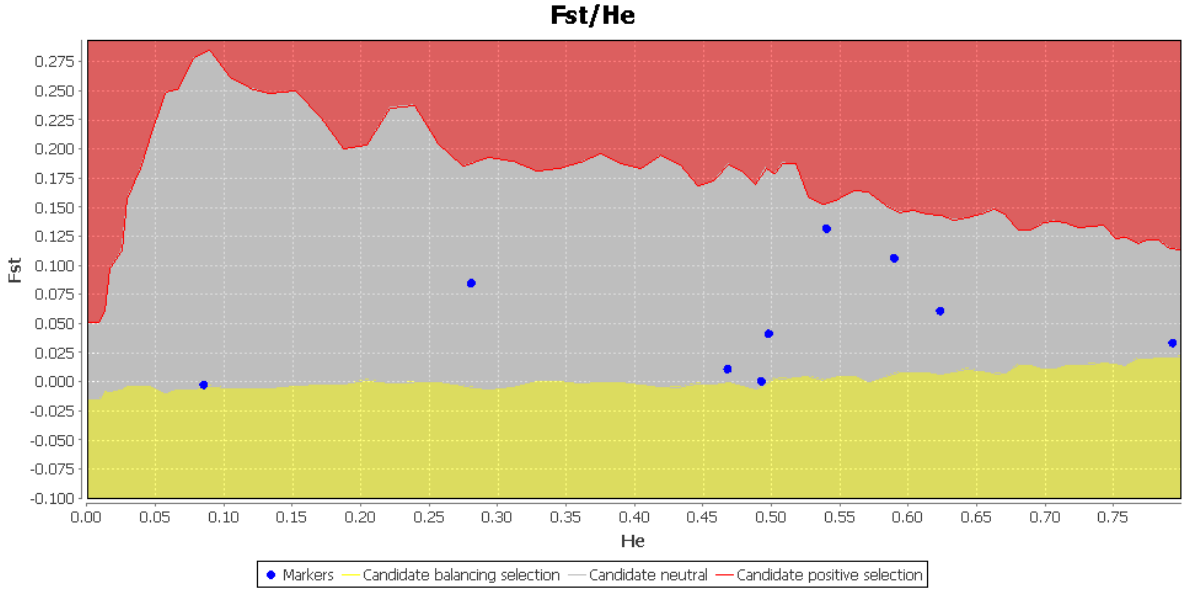

Figure C.2: Outlier markers detection using LOSITAN for nine microsatellite markers from the Holtemme system. Yellow area shows candidate markers under balancing selection, gray areas show candidate markers under natural selection and red areas show candidate markers under positive selection.

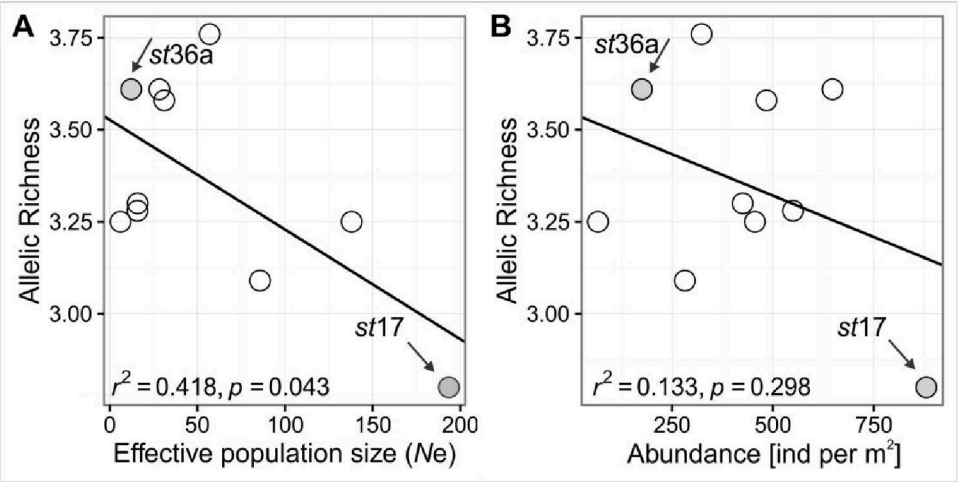

Figure C.3: Relationship between (A) effective population size (N_e) and allelic richness and (B) abundance and allelic richness. Black arrows represent sampling sites with significant drop in genetic diversity along the River Holtemme (sampling site *st*17 after the first wastewater treatment plant and *st*36a upstream of the first weir).

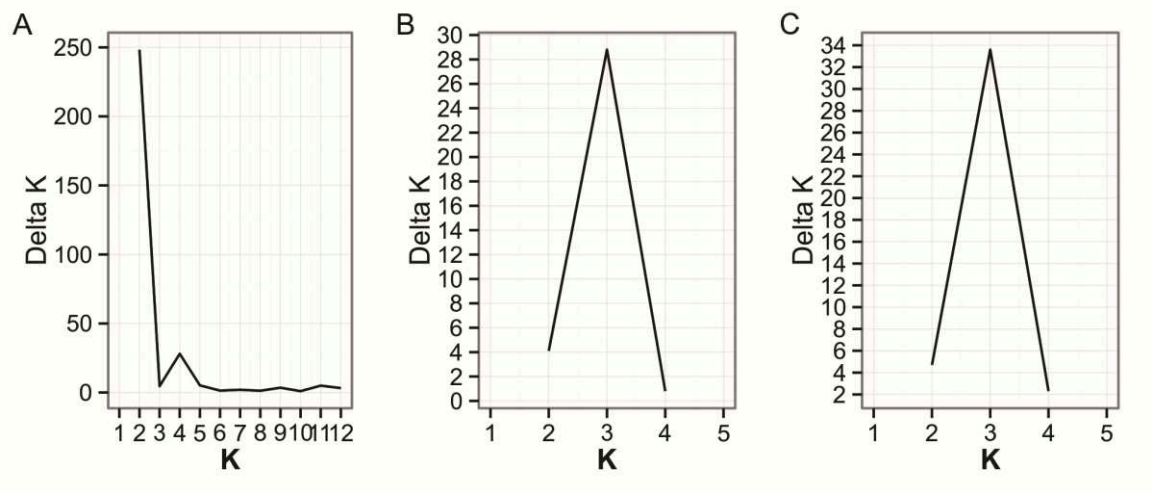

Figure C.4: The Evanno method carried out in STRUCTURE HARVESTER proposes that the most likely number of genetic clusters for *G. pulex* based on 9 microsatellite markers from (A) all the sampling sites in the River Holtemme is *K*=2, (B) number of clusters from sites st15, st17, st22 and st25 is *K*=3 and (C) number of clusters from sites st36a, st36b, st38 and st42 is *K*=3.

ACKNOWLEDGMENT

I would like to express my gratitude to PD Dr. Werner Brack for give the opportunity to develop my PhD under his supervision and advised my PhD work all these years. Prof. Dr. Henner Hollert is greatly acknowledged for accepting me as an external PhD student in RWTH Aachen University and for evaluating this dissertation.

This PhD work was financed by the Chilean government throughout the fellowship program CONICYT-BECASCHILE (Folio 72110798). This work was further supported by the European Union Seventh Framework Programme (FP7) under the Project SOLUTIONS with the grant agreement No. 603437, The Helmholtz Research Program "Chemicals in the Environment" and the Helmholtz Interdisciplinary Graduate Scholl for Environmental Research – HIGRADE.

I further want to express especially my gratitude to Dr. Helge Norf for his continuous supervision and encouragement all the time, as well as to Prof. Dr. Markus Weitere for his permanent support along my PhD. Thanks to all members of the Department of Effect-Directed Analysis, especially to Dr. Martin Krauss for his supervision related with all the chemical analysis performed in my PhD and his continuous advices. Dr. Paula Martinez thanks for all the advices and corrections since the beginning of my PhD. Dr. Tetyana Gilevska for showing me how to explain my biological results in an understandable and wider way to my chemist colleagues. To Romy Wild for her disinterested help and advices. To Dr. Peter von der Ohe for his supervision the first year of my PhD as well as to Dr. Sabine Kleinsteuber and Ute Lohse for their advice and help with the molecular analysis and to Dr. Stefan Michalski for his advices about microsatellite analysis.

Thanks to my entire friends that I met in this enriching experience. Especially, I want to thank Natalia, for all your enormous support even before to come to Germany. Paula, I want to deeply thank you for each single moment that you gave me, your words and encouragement were the key in the worse moment of my PhD and always you had a wise advice that motivated to keep moving on, as well Karla for all those journeys and support. Bremen/Oldenburg friends Paula, Gerdhard and Oscar thanks my dear friend for all your care and being there to hear my troubles and give always that especial energy that I needed to come back to Leipzig and keep working on my PhD. Not less important I also want to thank perhaps one of the main results of my PhD, they are not number or new conclusions. They are Tania, Romy, Lino, Riccardo, Melis, Edoardo, Anna, Florian, Ioana, Harry, Matthias, Steffi, Giovanni, Enrique, Yaru. My special thanks to Megacephalos friends Mauricio, Iván, Matias, Juan, Negro, Tito and Roberto and Tomás that supported me from Chile.

At the end, I would like to give my gratitude to my family. Thank you so much for supporting me since the beginning and always being there for me. Especially Pablo, thank for understand and support me from afar.

LIST OF PUBLICATIONS

The thesis is based on the following publications:

- **Inostroza, P.A**., Massei, R., Wild, R., Krauss, M., Brack, W., (*In preparation*). Freely dissolved concentration, chemical activity and baseline toxicity: insights of a multi-compartment analysis in a freshwater system.
- **Inostroza, P.A**., Vera-Escalona, I., Wicht, A-J, Krauss M., Brack, W., Norf H. (2016). Anthropogenic stressors shape genetic structure: insights from a model freshwater population along land use gradient. Environmental Science & Technology 50(20):11346-11356.
- **Inostroza, P.A**., Wicht, A-J, Huber, T., Nagy, C., Brack, W., Krauss, M. (2016). Body burden of pesticides and wastewater-derived pollutants on freshwater invertebrates: method development and applicability in the Danube River. Environmental Pollution 214:77-85.

Platform presentations:

- **Inostroza, P.A**., Wicht, A-J., Norf, H, W. Brack. 2016. How do anthropogenic pollutants affect the genetic structure of a model invertebrate freshwater population? Society of Environmental Toxicology and Chemistry (SETAC), Nantes, France.
- **Inostroza, P**.**A**., Vera-Escalona, I., Brack, W., H. Norf. 2016. Anthropogenic stressors shape genetic structure: insights from a model freshwater population along land use gradient. Conference Conservation Genomics (ConGenOmics), Porto, Portugal.
- **Inostroza, P.A**., Norf, H., Brack, W. 2015. Genetic diversity of a non-model freshwater population along Holtemme River: insights from a field study. 9th Symposium for European Freshwater Sciences (SEFS), Geneva, Switzerland.
- **Inostroza, P.A**., Michalski, S., Brack, W., H. Norf. 2015. Seasonal variability of amphipod populations in differently impacted stream ecosystems: insights from a field study using microsatellites. Association for the Sciences of Limnology and Oceanography (ASLO), Granada, Spain.
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Curriculum Vitae

Name:	Pedro Antonio Inostroza Bustos
Date of Birth:	30.10.1982
Place of Birth:	Concepción
Citizenship:	Chilean

Education

2012-2016	PhD Student	Helmholtz Centre for Environmental Research – UFZ Department Effect-Directed Analysis RWTH Aachen University Institute for Environmental Research
2007	Dipl.	Marine Biology with major in Oceanography and Environmental Quality University of Concepción, Chile
2004	BS	Marine Biology University of Concepción, Chile

Research Interests

Effects of organic micropollutants on genetic/genomic level in aquatic environments
Identification of pollutants under body burden approach
Investigate chemical activity in a multi-compartment scenario
Molecular tools for genome-level alterations in aquatic populations

Research Experience

2012-2016 PhD student in the Department of Effect-Directed Analysis at Helmholtz Centre for Environmental Research – UFZ in the projects:
- IP Healthy Aquatic Ecosystems (POF III)
- Homogenisation of ecosystem functioning between temperate and Neotropical streams due to agricultural land use (HECTARE)
- Solutions for present and future emerging pollutants in land and water resources management (SOLUTIONS)

2011-2012 Research assistant at the University of Concepción in the project:
- Monitoring Program of the Marine Environment CFI Nueva Aldea (PROMNA)

2008-2012 Research assistant in the Marine Research Program of Excellence (PIMEX) at the University of Concepción in the projects:
- Empirical investigation of the contamination of the Valdivia watershed by steroid hormones.
- Steroid oestrogen concentration estimation on raw sewage plants effluents at the Maule and Los Ríos regions by means of predictive modelling.
- Assessment of the presence and origin of endocrine disrupting compounds in the aquatic ecosystem of central-southern Chile.

2005-2008 Technician in the Centre for Oceanographic Research in the Eastern South-Pacific (COPAS) at the University of Concepción in the project:
- Community structure, metabolisms and biogeochemistry on the Eastern South Pacific (RP-5).

2004 Technician in the Laboratory of Fishery Oceanography and Larval Ecology (LOPEL) at the University of Concepción.

Publications

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Conferences

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