Population genetics of the indicator amphipod species *Gammarus pulex* (Linnaeus, 1758) in relation to anthropogenic organic micropollutants in rivers

Dissertation
zur Erlangung des Doktorgrades
der Naturwissenschaften

vorgelegt beim Fachbereich Biowissenschaften (FB 15)
der Johann Wolfgang Goethe-Universität
in Frankfurt am Main

von

Vid Švara
aus Ljubljana, Slowenien

Frankfurt, 2022
(D 30)

vom Fachbereich 15 (Naturwissenschaften) der

Johann Wolfgang Goethe-Universität als Dissertation angenommen.

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Datum der Disputation: 24.1.2023

“The world and the universe is an extremely beautiful place, and the more we understand about it, the more beautiful does it appear.”

Richard Dawkins

Acknowledgments

My doctoral thesis as a result of all the effort, work, and research over the last years would not be possible without significant contributions of many fantastic people with whom I had a privilege to work in the frame of this PhD project. First, I would like to express my greatest gratitude to Prof. Dr. Werner Brack, my main supervisor, who gave me the opportunity to join his department and guided me throughout the PhD project. You were extremely open and supportive to new ideas and research during the project, you provided great help, feedback, and your expertise whenever I needed it. Your support was one of the most crucial and irreplaceable parts of this thesis. Thank you!

I would also like to thank my second supervisor, Prof. Dr. Henner Hollert, who provided support from the Goethe University in Frankfurt. Thank you for supporting me and being open for interesting discussions from the beginning of the PhD project.

This thesis would have never even started without Dr. Till Luckenbach who was the key person for me to visit Leipzig and start working at the UFZ. I am most thankful to you for this opportunity and especially because we could spend last five years working together, discussed many research and life related topics, and that our collaboration led us to many places of the world, from Leipzig, to Slovenia, and Lake Baikal. I hope we can continue this fantastic journey in the future.

I would like to express my gratitude to incredible mentors and colleagues I had an opportunity to work with and learn from during this project. First, I would like to thank Dr. Martin Krauss, who helped me through the world of environmental chemistry and always found time for my questions despite his packed schedule. I would also like to thank Dr. Tobias Schulze for his support with analysis of environmental data. Finally, I would like to express my thankfulness to Dr. Stefan Michalski and Dr. Helge Norf for their incredible support in the molecular laboratory and for making my research visits at the UFZ in Halle and in Magdeburg interesting, productive, and fun.

In addition to my mentors, this interdisciplinary project would not be possible without other researchers and UFZ members whom I worked with and who contributed significantly to

a successful result. I would like to thank Prof. Dr. Rolf Altenburger for his advice on exposure data analysis and tips on biking possibilities in the region, Prof. Dr. Matthias Liess for his support and knowledge on the laboratory exposure experiments, Dr. Eberhard Küster for his help on toxicokinetics experiments, Dr. Stefan Scholz for supporting the work in the Biotox department, Dr. Sabine Kleinsteuber for letting me work in her lab and for her advice on microsatellite analysis, and Dr. Naeem Shahid, Dr. Renato Russo, and Ayesha Siddique for their advice on the laboratory exposure experiments. I would like to thank to all the technicians and team assistants from the departments Wana, Biotox, Ökotox, and UMB who made my days in the lab and in the field unforgettable and successful. Margit, Nicole, Hubert, Ute, Janette, Jörg, Anke, Christina, Alice, Monique, Peggy, Ola, Oliver, Roman, Marion, Ingrid, and Lisa, thank you so much for all your great support and fun hours in and around the lab!

In the time of my work at the UFZ, I met and worked with many fantastic PhDs students and colleagues, who supported my research, mental well-being, and made sure that we enjoy our PhD years. Basti, Olli, Afolarin, Stephan, Bettina, Gianina, Stefan, Polina L., Polina D., Yuliya, Dasha, David, Eric, Andreas M., Andreas S., Christoph, Liza, Alexander, Riccardo, Melis, Dana, Wibke, Mechtild, Nils, Saskia, Tamara, Erik, Emil, Caroline, Pavel, Janek, and Tim, you made sure that my PhD project and time in Leipzig resulted in many (statistically significant) positive experiences to remember. Hope to spend many more good times with you in the future!

Finally, I would like to thank my family, especially my mum Dini and grandma Helena. Thank you for your support and patience throughout the years, preparedness to share your knowledge and ideas, and willingness to support my academic path. I would like to thank my brother Anže for many hours very well spent on discussing science, projects, places, ups and downs, and to encourage each other to come all the way to this point. It is something special to have a twin, but having one, that defends his PhD thesis almost at the same time as myself is even more special. Without you, this whole journey would definitely not be as fascinating as it has been. Finally, I would like to thank my wife Elena. Thank you for your unconditional support, being positive and optimistic, sharing your many great ideas with me, and helping me be my best every single day!

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Abstract

Chemical pollution is one of the main contributors to the degradation of lotic ecosystems and their biodiversity. Among chemicals driving lotic biodiversity decline are anthropogenic organic micropollutants (AOM), which affect the survival and functioning of freshwater organisms. Continuous exposure of freshwater organisms to AOM leads to adverse effects that sometimes cannot be traced with standard toxicity methods such as standard toxicity testing or biodiversity indices. Among these effects of AOM are selective or mutagenic effects that cause impaired species genetic diversity. Thus, the correlation between different levels of AOM and genetic diversity of species is still poorly understood. However, it can be explored by applying population genetics screening.

In Chapter 1 of this thesis, background information on environmental pollution, genetic screening, and the detection of evolutionary-relevant AOM effects in freshwater organisms are described and the thesis goals are identified. The main goal of the thesis is to study whether AOM exposure occurring in European rivers causes a significant evolutionary footprint in freshwater species and leads to a selection of more tolerant geno- and phenotypes. Therefore, population genetics indices together with high-resolution chemical exposure screening of a widespread indicator invertebrate species, *Gammarus pulex* (Linnaeus, 1758), living in polluted and pristine European rivers were investigated.

In Chapter 2, the development of a genetic screening method for *G. pulex* (microsatellites) is described. Due to genetic differentiation and the presence of morphologically cryptic lineages, the available sets of target loci do not enable a reliable population genetic characterization of *G. pulex* from central Germany. Thus, a novel set of microsatellite loci for a high-precision assessment of population genetic diversity was here applied. Eleven loci were first identified and thereafter amplified in *G. pulex* from three rivers. The new loci reliably amplified and indicated polymorphisms in the studied amphipods. The amplification resulted in the successful identification of genetically distinct populations of *G. pulex* from the analyzed rivers. Moreover, the microsatellite loci were amplified in other genetic lineages of *G. pulex* and another *Gammarus* species*, G. fossarum,* promising a broader applicability of the loci in related amphipod species.

In Chapter 3, the effects of AOM on species genetic differentiation and sensitivity to toxic chemicals in a typical central European river with pristine and AOM-polluted sections was investigated. The river's site-specific concentrations of AOM were assessed by chemical analysis of *G. pulex* tissue and water samples. To test, whether different levels of AOM in the river select for pollution-dependent genotypes, the genetic structure of *G. pulex* from the river was analyzed. Finally, the toxicokinetics of and sensitivity to the commonly used insecticide imidacloprid were determined for amphipods sampled at pristine and polluted sections to assess whether various levels of AOM in the river influence sensitivity of *G. pulex* to imidacloprid. The results indicated that different levels of AOM did not drive genetic divergence of *G. pulex* within the river but led to an increased sensitivity of exposed amphipods to imidacloprid. The amphipods living in polluted river sections were more sensitive to the insecticide due to chronic exposure to toxic levels of AOM.

In Chapter 4, the relationship between site-specific pollution levels of AOM and genetic diversity parameters of *G. pulex* was analyzed at the regional scale within six rivers in central Germany. The genetic structure of *G. pulex* in the studied area was tested for relatedness to the waterway distance between sites. *Gammarus pulex g*enetic diversity parameters, including allelic richness and inbreeding rate, were tested against environmental pollution parameters using linear mixed-effect- and structural-equation models. According to the results, *G. pulex* genetic diversity parameters were significantly associated with the detected AOM levels. At sites with high concentrations of AOM and toxicity potential *G. pulex* showed reduced genetic diversity and increased rates of inbreeding. These results suggest that AOM play a major role in shaping the genetic diversity of *G. pulex* in rivers.

According to the findings presented here, the applied microsatellites can be used to successfully detect changes in genetic patterns in freshwater amphipods facing increased levels of AOM. The findings indicate that levels of AOM representative for European rivers do not lead to the separation of genotypes among *G. pulex* as the connectivity between sites majorly contributes to species' genetic structure. However, the chronic exposure to increased levels of toxic AOM leads to a reduction of species genetic diversity and increases the sensitivity of *G. pulex* to the toxic chemical effects.

Zusammenfassung

Untersuchungen zur Populationsgenetik des Flohkrebses *Gammarus pulex* (Linnaeus, 1758) aus mit anthropogenen organischen Mikroschadstoffen belasteten Flüssen

Die Lebensräume vieler Organismen sind stark durch die Aktivitäten des Menschen beeinflusst. Zu den Ökosystemen, die in besonderem Maße von diesen Aktivitäten betroffen sind, gehören Süßwasserökosysteme wie Flüsse und Bäche. Diese Ökosysteme sind stark von der Belastung mit anthropogenen Schadstoffen geprägt. Eine Gruppe von Umweltschadstoffen, die schädliche Auswirkungen auf Wasserorganismen haben, sind die sogenannten anthropogenen organischen Mikroschadstoffe (AOM). AOM umfassen Chemikalien wie Pestizide, Pharmazeutika, Industrie- und Haushaltchemikalien, die in Konzentrationen von Nano- bis Mikrogramm pro Liter Flusswasser in der Umwelt auftreten. AOM können bereits in solch niedrigen Konzentrationen schädliche Effekte auf Organismen und Ökosystemen bewirken. Toxische Effekte von AOM können spezifisch sein. So können z.B. bestimmte Wirkweisen auf der Bindung der Chemikalien an bestimmte zelluläre Rezeptoren beruhen; durch diese speziellen Wirkweisen können dann ausschließlich bestimmte Taxa betroffen sein. Außerdem können AOM auch unspezifisch, z.B. narkotisch, wirken.

Bisher wenig untersucht wurde die Auswirkung von AOM auf die genetische Vielfalt von Wasserorganismen. Diese könnte über unterschiedliche Mechanismen beeinflusst werden. So könnten mutagene AOM im Wasser zur Entstehung von neuen Genvarianten in einer Population von Wasserorganismen führen, was die genetische Vielfalt einer exponierten Population erhöhen würde. Außerdem kann durch toxische AOM ein Selektionsdruck auf Populationen ausgeübt werden, so dass Individuen mit entsprechenden genetischen Anpassungen gegenüber weniger resistenten Individuen im Vorteil sind. Dies würde zu einem erhöhten Anteil an Individuen führen, die besser an eine Exposition gegen Giftstoffe angepasst sind und in Folge zu einer geringeren genetischen Vielfalt der entsprechenden Population. Mit populationsgenetischen Analysen lassen sich solche Veränderungen untersuchen.

Die Wirkung von AOM auf die genetische Vielfalt von natürlichen Populationen in mit AOM belasteten Süßgewässern wurde bisher an verschiedenen Arten untersucht. Der Rückgang der genetischen Vielfalt durch erhöhte AOM-Belastung des Wassers wurde für die Modellart *Daphnia magna* Straus, 1820 gezeigt. Die Inzuchtrate war erhöht, was auf eine Verkleinerung des Genpools durch die Selektion resistenter Individuen hindeutete. Trotz Studien, die sich mit dem Thema der genetischen Vielfalt in Süßwasser-Makroinvertebraten beschäftigt haben, bleibt die Frage, wie AOM auf das genetische Muster einer Art entlang eines Belastungsgradienten in einem Fluss wirkt, offen. Es ist noch nicht bekannt, ob toxische AOM die genetische Vielfalt von exponierten Wasserorganism en reduzieren, zur Selektion spezifischer Genotypen führen und zur Entstehung an die Wirkung der AOM angepassten Populationen beitragen. Vor diesem Hintergrund wurde in dieser Dissertation die genetische Vielfalt der weitverbreiteten Indikator-Flohkrebsart *Gammarus pulex* (Linnaeus, 1758) von unterschiedlich mit AOM belasteten Standorten an Fließgewässern im mitteldeutschen Raum untersucht.

In Kapitel 1 der Dissertation werden Hintergrund und offene Fragen des Themas vorgestellt. Kapitel 2 geht auf die hier angewandte Methode zur Bestimmung der genetischen Diversität und Struktur von *G. pulex* ein und beschreibt die Entwicklung neuer Marker. Die Methode wurde in den Kapiteln 3 und 4 angewandt, um die genetische Vielfalt von *G. pulex* in den analysierten Flüssen zu bestimmen. In Kapitel 3 wurde analysiert, inwieweit die genetische Struktur der Art in einem typischen mitteleuropäischen Fluss durch AOM verändert wird und ob solche Veränderungen sich in der Empfindlichkeit der Flohkrebse gegen AOM widerspiegeln. In Kapitel 4 wurde der Zusammenhang der genetischen Vielfalt von *G. pulex* mit dem Grad der Belastung eines Gewässers mit AOM auf regionaler Skala analysiert. Schließlich wird in Kapitel 5 auf die wichtigsten Schlussfolgerungen dieser Dissertation eingegangen.

Die in dieser Arbeit verwendeten genetischen Marker für die Bestimmung der genetischen Vielfalt von *G. pulex* waren sogennante Mikrosatelliten, die DNA Abschnitte, die tandemartig wiederholt und normalerweise nicht-kodierend sind. Die Anzahl der Wiederholungen und damit die Länge der Mikrosatelilten variiert stark zwischen Individuen und Populationen. Durch Veränderungen der Längen von Mikrosatelliten-Loci

spiegeln sich die evolutionären Effekte von Mutationen, Selektion, Inzucht oder genetischen Flaschenhalsereignissen in einer Population wider.

Für *G. pulex* wurden bereits in früheren Arbeiten Mikrosatelliten entworfen. Es gibt aber genetisch deutlich unterschiedliche *G. pulex*-Linien, deshalb werden die vorhandenen Mikrosatelliten nicht in allen Linien problemlos amplifiziert. Daher wurden im Rahmen dieser Arbeit neue Mikrosatellitenmarker entwickelt, die für die genetische Polymorphismusanalyse von *G. pulex* aus dem Einzugsgebiet des Flusses Saale optimiert sind (Kapitel 2). Acht DNA-Proben wurden mittels Hochdurchsatz-Sequenzierung analysiert. Die identifizierten Mikrosatellitenabschnitte wurden für weitere *G. pulex*-Individuen aus demselben Einzugsgebiet ermittelt und auf Polymorphismen innerhalb von Proben von unterschiedlichen Standorten im Saaleeinzugsgebiet untersucht. Die untersuchten Tiere gehörten alle zur selben *G. pulex*-Linie. Von den amplifizierten Loci waren 14 polymorph. Elf Loci wurden erfolgreich in drei Multiplexreaktion amplifiziert und in der Populationsanalyse verwendet. Im Vergleich des genetischen Musters der untersuchten Individuen von drei Standorten konnten die Tiere mittels Hauptkomponentenanalyse und Analyse von molekularen Varianzen drei genetisch unterschiedlichen Populationen zugeordnet werden. Zusätzlich wurden die Loci erfolgreich in Proben einer weiteren *G. pulex*-Linie und einer weiteren *Gammarus*-Art amplifiziert, nämlich *G. pulex*, Linie C, und *Gammarus fossarum* Koch, 1836. Die erfolgreiche Amplifikation der Mikrosatelliten aus den DNA Proben bestätigte die Eignung der neuen Mikrosatelliten für populationsgenetische Untersuchungen von *G. pulex.*

In Kapitel 3 wurden die Auswirkungen der AOM-Belastung eines Fließgewässers mit der populationsgenetischen Struktur und der Empfindlichkeit von *G. pulex* für das Insektizid Imidacloprid untersucht. Es wurde von zwei möglichen Szenarien ausgegangen: Populationen aus einem stärker mit AOM belasteten Habitat sind weniger sensitiv für die toxische Wirkung von AOM, weil sich vorrangig die weniger empfindlichen Tiere fortpflanzenr oder sind die Tiere aus stärker mit AOM belasteten Habitaten sensitiver, weil die chronische AOM-Belastung zu erhöhtem Hintergrundstress führt. In dieser Studie wurde der Frage nachgegangen, (1) ob sich die Amphipoden aus den mit AOM belasteten Abschnitten eines Flusses an diese Belastung durch Adaptation oder Akklimatisierung

angepasst haben, oder (2) ob Amphipoden, die chronisch-toxischen AOM in ihrem Habitat exponiert sind, empfindlicher auf Insektizidexposition reagieren. Die Studie wurde an einem typischen mitteleuropäischen Fluss durchgeführt, wo *G. pulex* sowohl in den belasteten als auch in den unbelasteten Flussabschnitten lebt. Die Belastung des Wassers mit AOM wurde durch die Analyse von 60 Chemikalien und deren toxischem Potenzial für *G. pulex* charakterisiert. Um die genetische Anpassung zu testen, wurde die genetische Struktur von *G. pulex* sowohl über einen Sequenzvergleich des Cytochromoxidase I-Gens (COI), als über einen Vergleich der Mikrosatelliten bestimmt. Die Senitivitäten von *G. pulex* von unterschiedlich belasteten Standorten wurden durch Toxizitäts- und Aufnahmetests mit dem Insektizid Imidacloprid bestimmt.

Die Ergebnisse zeigten, dass die höhere und toxischere AOM-Belastung keine signifikante Trennung in der genetischen Struktur von *G. pulex* verursacht. Die genetische Struktur von *G. pulex* ist v.a. durch Migration von Individuen zwischen den Standorten beeinflusst. Deswegen ist eine spezifische Anpassung von *G. pulex* an eine erhöhte AOM-Belastung in den entsprechenden Bereichen des Flusses auszuschließen. Die Empfindlichkeit von *G. pulex* von belasteten Standorten, gemessen als Zeitraum bis zum Tod von 50% der Tiere, war bis zu 54% gegenüber Tieren von unbelasteten Standorten erhöht und die Rate immobiler Tiere war bis zu 65% erhöht. Entsprechend wurde auch gefunden, dass nach Exposition von *G. pulex* gegen Imidacloprid die Eliminationsraten der vom Gewebe aufgenommenen Substanz bei Individuen von belasteten Standorten geringer waren. Diese Befunde zeigen, dass die chronische Exposition von *G. pulex* gegen AOM in ihrem Lebensraum die Sensitivität der Tiere für toxische Stoffe erhöht, weil ein hohes Angebot an Nahrung in den belasteten Habitaten das Überleben ermöglicht.

In Kapitel 4 wurde der Zusammenhang zwischen AOM-Konzentrationen und der genetischen Vielfalt von *G. pulex* in sechs Flüssen auf regionaler Skala untersucht. Bislang war bekannt, dass die genetische Vielfalt von *Gammarus sp.* in Flüssen mit anthropogenen Einflüssen stark variiert. Es war aber noch nicht bekannt, ob die genetische Vielfalt der Populationen durch eine selektive Wirkung von AOM geringer ist oder durch die mutagene Wirkung von AOM erhöht wird. Um dies zu beurteilen, wurden der Grad der Belastung durch AOM und deren Toxizität in Wasserproben und in

Gammarus-Gewebeproben von 34 Standorten aus sechs Flüssen in Mitteldeutschland untersucht. Die genetische Struktur und Vielfalt von *G. pulex* wurde mittels 16 Mikrosatellitenloci ermittelt. Ein Zusammenhang zwischen den genetischen Mustern von *G. pulex* und der AOM-Belastung und deren Toxizität für die Tiere wurde mittels sogenannter linear mixed-effect models (LME) bestimmt.

In den Flüssen wurden unterschiedliche Belastungen mit AOM festgestellt. Pharmaka und Industrie-Chemikalien haben am stärksten zur AOM-Gesamtkonzentration beigetragen. Obwohl Insektizide in relativ niedrigen Konzentrationen nachgewiesen wurden, war ihre Toxizität für *G. pulex* von allen AOM-Gruppen am höchsten. Trotz des toxischen Potentials der AOM wurde kein Zusammenhang der genetischen Struktur von *G. pulex* mit dem Grad der Belastung festgestellt. Unterschiede in der genetischen Struktur von *G. pulex* standen v.a. mit den Entfernungen der Probenstandorte voneinander in Zusammenhang. Die verwendeten Statistikmodelle zeigten jedoch, dass die Belastung mit AOM zu einer Veränderung der genetischen Vielfalt von *G. pulex* beitrug. Die genetische Vielfalt war an Standorten mit erhöhten Konzentrationen von AOM insgesamt geringer, auch wenn Kläranlagenausflüsse mit einer Erhöhung der genetischen Vielfalt in Zusammenhang standen. Erhöhte Toxizität durch AOM in den Flüssen korrelierte mit höheren Raten von Inzucht bei *G. pulex*. Insgesamt zeigen diese Ergebnisse, dass AOM die genetische Vielfalt von *G. pulex* innerhalb insgesamt vermindern können.

In Kapitel 5 werden die allgemeinen Ergebnisse dieser Arbeit und die Anwendungsmöglichkeiten populationsgenetischer Methoden für die Bewertung von toxischen und selektiven Effekten von AOM diskutiert. Die Belastung von Flusswasser mit AOM kann als ein sehr bedeutender multipler Stressor betrachtet werden, der aber nicht zu einer Aufteilung von einer *G. pulex*-Population in einem Fluss führt. Die Belastung eines Fließgewässers mit AOM kann die Populationsgenetik einer Art trotzdem signifikant beeinflussen, was sich in der reduzierten genetischen Vielfalt einer exponierten Population von Organismen widerspiegelt. Die in der Arbeit angewendeten Methoden haben ein großes Potential, Effekte von AOM aus Punktquellen auf betroffene Populationen zu identifizieren, ermöglichen aber auch den Nachweis von Genfluss durch Migration zwischen Populationen.

Abbreviations

ADaM	Aachner Daphnien Medium
AFLP	amplified fragment length polymorphisms
AICc	Akaike information criterion
AMOVA	analysis of molecular variance
AOM	anthropogenic organic micropollutants
COI	partial cytochrome oxidase subunit one gene
ddRADseq	double digested restriction site associated DNA sequencing
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
DOC	dissolved organic carbon
EC_{50}	concentration of the compound, causing effect in 50% of exposed organisms
EEA	European Environmental Agency
EPA	Environmental Protection Agency
F_{is}	inbreeding coefficient
F_{st}	fixation index of population differentiation
GC	gas chromatography
H_o	observed heterozygosity
H_e	expected heterozygosity
HC5	median hazardous concentration for 5% of species
HRMS	high resolution mass spectrometry
LC	liquid chromatography
LC_{50}	concentration of the compound, lethal for 50% of organisms included in the experiment
LME	linear mixed-effects models
$LogK_{OW}$	octanol-water partitioning coefficient
LogD	logarithm of the distribution coefficient used to measure the lipophilicity of ionizable compounds
LT_{50}	time of exposure, in which lethal effect for 50% of organisms included in the experiment is observed
m/z	mass-to-charge ratio

N_e	effective population size
PAHs	polycyclic aromatic hydrocarbons
PCoA	principal Coordinate Analysis
PCR	polymerase chain reaction
RAPD	random amplification of polymorphic DNAs
RFLP	restriction fragment length polymorphisms
SE	standard error
SSRs	simple sequence repeats
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
SNPs	single nucleotide polymorphisms
TP	total phosphorus
TU	toxic unit
WWTP	wastewater treatment plant

Chapter 1

Thesis introduction and background

1.1 Threats of anthropogenic pollutants to freshwater biodiversity

Freshwater environments, including rivers, streams, lakes, and groundwater, comprise one of the most diverse and essential ecosystems for human health, social and economic progress, and the integrity of global biodiversity (UNEP, 2016). Due to the rapid growth of human population and economic development of countries worldwide, human demand for water and freshwater ecosystem services has been constantly increasing (UNEP, 2016). Accordingly, across the planet, this rapid growth results in an increased exploitation of freshwater sources mainly associated with urbanization, industrialization, and the expansion and intensification of agriculture. This intensification of water use worldwide results in deteriorating conditions in freshwater ecosystems and contributes significantly to reduced ecosystem integrity that is reflected by reduced diversity of freshwater biological communities (Reid et al., 2018). The function in the altered ecosystems, such as for instance the trophic transfer of nutrients, may be significantly impacted by increased environmental pressure, as some species representing an important link in the trophic chain may not be able to survive. Among the main drivers deteriorating the state of freshwater ecosystems, their functioning, and freshwater biodiversity is also anthropogenic pollution (Dudgeon et al., 2006).

Among the most severely impacted freshwater ecosystems are lotic ecosystems, which include rivers and streams. Altogether, freshwater from rivers and streams represents only 0.006% of all available freshwater on the planet (Shiklomanov, 1993). Yet, a large portion of these freshwater habitats has already been negatively altered by human activity. Human pressure on rivers and streams has been consistently increasing (Vörösmarty et al., 2013) and will, together with climate change, continue to affect freshwater ecosystems under the impact of human activity (Bunn, 2016). A major driver of negative ecosystem trends in rivers and streams are anthropogenic pollutants (Strayer & Dudgeon, 2010). The most important contaminants in freshwater ecosystems include metals (Nriagu, 1996), organic nutrients, such as phosphorus and nitrate from untreated wastewater discharges and from artificial fertilizers, and anthropogenic organic micropollutants (AOM), a wide range of chemical compounds that have been increasingly in use in the recent decades and that end up in waters as contaminants (Richardson & Kimura, 2017). The widespread

presence of the latter in freshwater ecosystems has become more and more evident with the development of novel analytical tools and techniques for the detection of thousands of anthropogenic organic compounds that enter freshwater ecosystems (Krauss, 2010). However, due to the immense quantity and diversity of AOM entering rivers and streams worldwide, some impacts on freshwater biota, as for example impacts on genetic diversity of freshwater species, still remain to a large degree unknown.

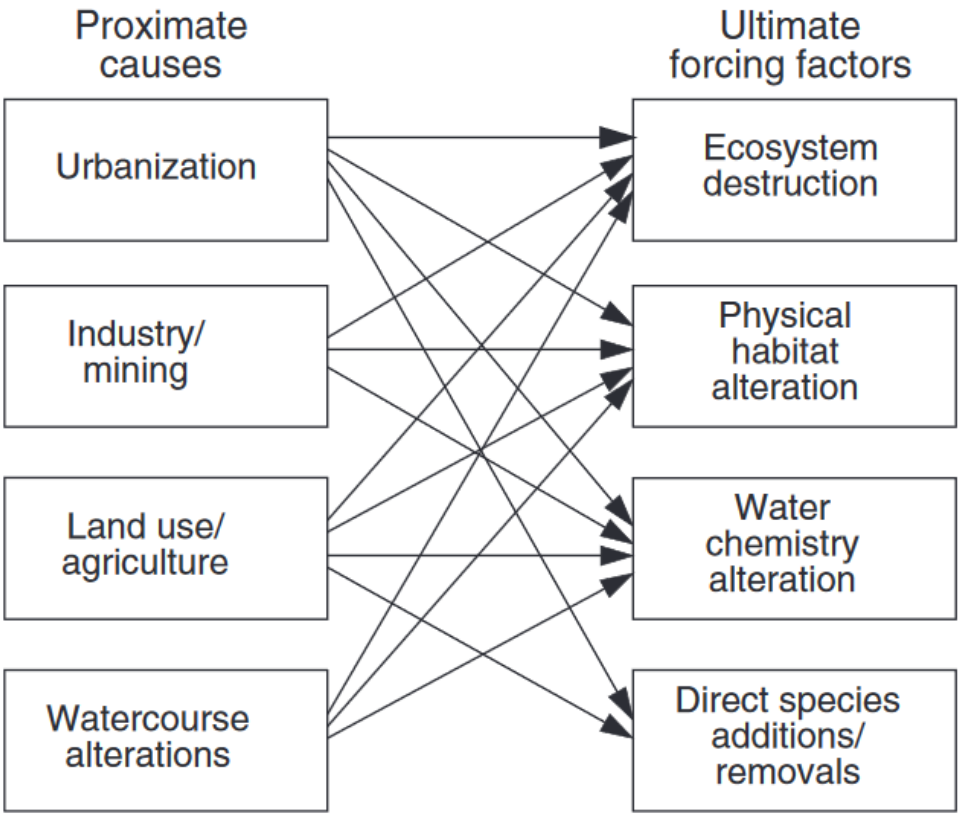

Figure 1.1 Causes of ecosystem disturbance in streams and rivers. The four main anthropogenic causes for freshwater ecosystem changes (proximate causes) with the link to the factors (ultimate forcing factors) that lead to changes in freshwater biodiversity and ecosystem functions (Malmqvist & Rundle, 2002).

1.2 Anthropogenic organic micropollutants with potential to affect biodiversity in lotic ecosystems

Anthropogenic organic micropollutants can, due to human use and application, enter natural environments and occur above natural background levels (Stamm et al., 2016). European surface waters contain up to several thousand different AOM, among them pesticides, biocides, pharmaceuticals, surfactants, personal care products, and other groups of chemicals with a myriad of transformation products (Brack et al., 2019). These compounds, used by humans, can be of a synthetic origin (e.g. the neonicotinoid insecticides) or of natural origin (e.g. antibiotics) with a wide range of sources in the environment including agricultural activities, household applications, industry, and transportation. Anthropogenic organic micropollutants are commonly discharged into rivers at point sources, such as wastewater treatment plant (WWTP) discharges, or through diffuse sources, such as run-off from agricultural sites containing e.g., pesticides. AOM usually represent only a small portion of chemical compounds in rivers and streams; they are commonly detected at concentrations, lower than 1 µg/L of water (Fig. 1.2). Nonetheless, even at such low concentrations, AOM can have significant negative impacts on ecosystems. The main reason for their significant potential to elicit biological effects in the environment is related to their applicative purpose. Most AOM are applied due to their high potency to elicit a very specific biological action. For example, to prevent vast damage of pests in the crop production, a pesticide that suppresses the occurrence of the pest is applied. In a similar manner, pharmaceuticals are applied or a specific disease treatment and chemicals (e.g., sweeteners) are added to food products in order to achieve a very specific effect with consumers. As AOM often target a specific function or molecular pathway in organisms, these chemicals mostly affect only specific groups of organisms or a specific organism function in the environment (Busch et al., 2016; Connon et al., 2012). The effects of specific AOM in a target species can therefore be, to a certain degree, predicted. However, due to non-target effects of AOM, the effects can often occur in non-target groups of organisms. For instance, insecticides do not only affect insects, but can also threaten the survival of crustaceans in freshwaters; due to evolutionary

similarities, crustaceans poses receptors with affinity for the same chemicals as insects (Barbee & Stout, 2009; Damasceno et al., 2021).

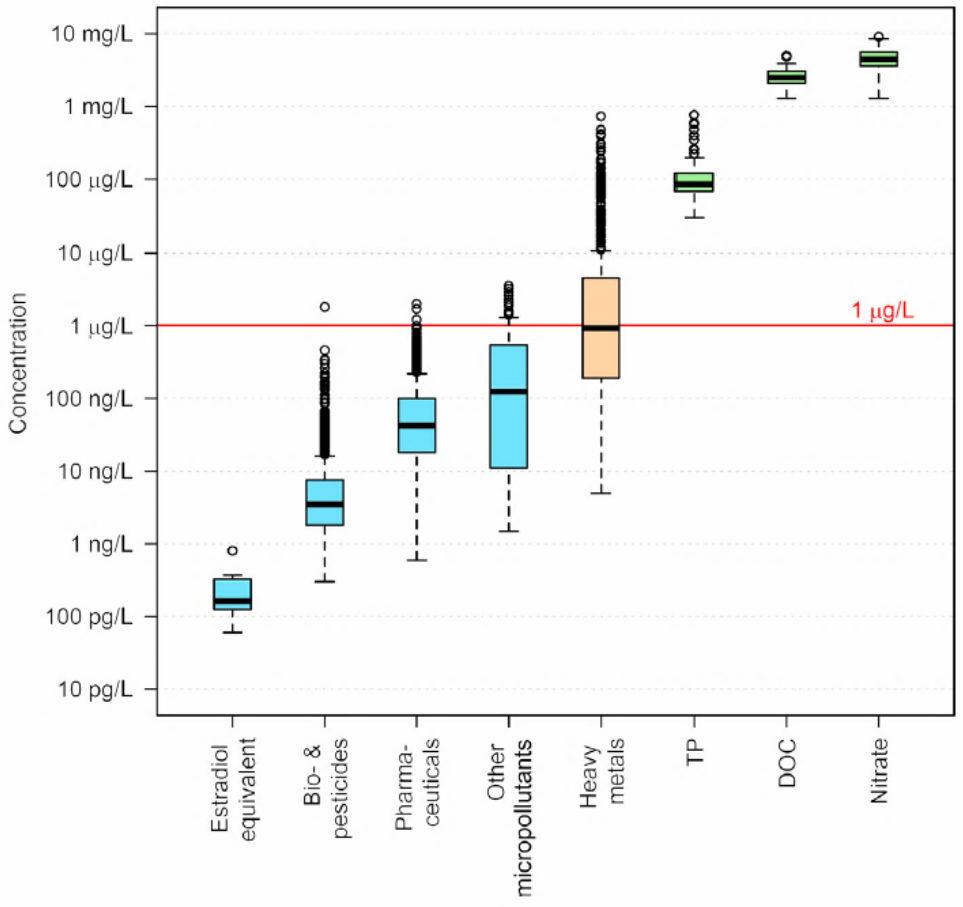

Figure 1.2 Boxplots indicating common detected ranges of the measured concentrations of different environmental contaminants from 24 freshwater sites in Switzerland. Blue boxes represent different groups of AOM. TP represents total phosphorus, DOC represents dissolved organic carbon. The red line indicates the upper concentration range of commonly detected AOM in freshwater ecosystems (Stamm et al., 2016).

1.3 Analytical methods for the detection of AOM in freshwater ecosystems

In order to detect the diverse AOM affecting the diversity of freshwater organisms, integrative, multidisciplinary approaches of AOM assessment coupled with stress ecology

have been developed (Van den Brink, 2008). These approaches enable the detection of complex AOM mixtures of hundreds of chemicals in freshwater ecosystems in an automated and efficient way. This is essential, to be able to comprehensively identify AOM that may threaten survival of exposed species. The approaches that can characterize AOM occurring in the environment comprise two main methods for the screening of AOM (Krauss et al., 2010). These two methods enable the detection of AOM from different matrices including water, sediment, and the tissue of freshwater organisms. The method selection depends largely on the solubility and volatility of the analyzed compounds in different environmental media (Hogenboom et al., 2009). To assess the levels of AOM directly from the liquid medium, liquid chromatography coupled with high resolution mass spectrometry (LC-HRMS) is the most commonly applied method (Krauss et al., 2010). This method is suitable for the assessment of polar water-soluble compounds (Hogenboom et al., 2009). In contrast to LC-MS, gas chromatography coupled with mass spectrometry (GC-MS) is applied when assessing non-polar, hydrophobic compounds.

The LC-MS analysis involves several steps from sample collection to data analysis. First the samples need to be collected without any contamination. Therefore, sufficient negative controls need to be included when collecting and analyzing environmental samples. The samples have to be kept cool or frozen until the analysis. Before measurement with the LC-MS device, samples need to be prepared using internal standards to be able to characterize compounds present in the samples. Sample preparation is followed by an automated injection in the LC, where compounds are separated based on their different hydrophobicities and the properties of the separation columns (Tadeo, 2008). Other parameters, such as solvent pH, temperature, composition, and flow rate also significantly contribute to the successful separation of the compounds. Compounds that pass the separation column of the LC enter the MS system. In the MS, which consists of an ion source, mass analyzer, and a analyte detector, samples are ionized by high temperature, impacts of energetic electrons, ions, or photons (Gross, 2011). The most commonly used technique of ionization is the electrospray ionization (Tadeo, 2008). The compounds are separated based on their mass-to-charge ratio with a mass being analyzed and detected based on this ratio and the compound abundance. The method has been applied to the

analysis of different water samples from rivers and streams, as well as for the analysis of invertebrate tissues (Beckers et al., 2020; Munz et al., 2017; Muschket et al., 2018).

In order to screen AOM compounds from environmental samples, three different approaches can be applied. These include non-target screening, suspect screening, and target screening of compounds (Krauss et al., 2010). Non-target screening can be applied for a non-biased detection of a myriad of chemical signals without *a priori* information on the chemical mass and is only restricted by limitations of the analytical method, such as minimum detection limit (Altenburger et al., 2019; Krauss et al., 2010). The identification of compounds is based on the exact mass, isotope, adduct, and fragmentation information. One of the major drawbacks of non-target screening is that the characterization of detected peaks can be challenging and reference toxicity data for the majority of characterized chemicals are lacking. Therefore, applying this approach for the analysis of the effects of specific AOM in specific organisms is rather laborious and time consuming (Hollender et al., 2017). The method has nevertheless been applied in the screening of pollutants in environmental water samples (Beckers et al., 2020; Ruff et al., 2015).

Suspect screening, in contrast to non-target screening, relies on the known molecular formulas and structures for which no reference standards are available. The application of different filters based on the exact compound mass, peak shape, or ionization can help narrow down the selection of suspect chemicals and allows for more efficient AOM detection (Moschet et al., 2013). The method can be useful when the detection of a broad range of micropollutants is needed in environmental screening, but the compounds are not part of an established screening infrastructure.

The third method, target screening, is, in contrast to previous methods, limited by pre-selected compounds with known masses and available reference standards. The method allows the analysis of up to several hundred chemicals in parallel through a multitarget approach (Beckers et al., 2018; Munz et al., 2018; Münze et al., 2017). The chemicals are selected based on the known information on the occurrence and potential hazard in the assessed environment. Although the method is limited by the selection of targeted

compounds, it can be extremely useful when assessing the effects of a limited number of compounds for which toxicity data is actually available. The method does not screen the total mixture of AOM in the sample, yet it is, in comparison to the previous two approaches, more convenient when assessing specific compounds known to cause adverse effects in organisms or in an ecosystem. The method has so far been applied in chemical assessments based on different types of samples, including the compounds found in the water column, as well as those that are found in the tissues of freshwater organisms (Inostroza et al., 2016b; Munz et al., 2018).

The target screening method is especially useful for the characterization of AOM mixtures from specific sources with known or expected target compounds. For instance, sources of contaminants, such as the effluents of WWTPs that can contain pharmaceuticals, household chemicals, and industrial chemicals used in the households, hospitals, and local industry can be efficiently monitored and compared using target screening method (Munz et al., 2017). Target screening can also be applied for the detection of compounds expected to occur in freshwaters. For instance, the impact of AOM in the polluted streams can be assessed by contaminant screening that enter rivers from agricultural fields where known mixtures of pesticides are applied (Beckers et al., 2018). Therefore, this method is especially useful for monitoring pollution patterns in pollution management or in research on the targeted effects of specific AOM or defined AOM groups in freshwater biota.

1.4 Effects of AOM on organisms in freshwater ecosystems

The impact of AOM affecting the survival of organisms and ecosystem integrity can be detected across different levels of biological organization, from genes to ecosystems across the whole planet (Schwarzenbach et al., 2006). The occurrence and effects of AOM at different scales is, first, a consequence of the global presence of the human population and a widespread use of organic compounds and, second, due to AOM transport by water, air, and organisms (Stamm et al., 2016). In lotic ecosystems, AOM pollution occurs in different ways, from dramatic point events, such as spills, to consistent, almost undetectable, leakage of AOM into rivers and streams through diffuse sources.

Large spills often result in severe and immediate effects on the ecosystem and freshwater inhabitants, such as extensive mortality of fish and other river organisms (e.g., Güttinger & Stumm, 1990). Despite severe consequences, such catastrophes are easier to recognize and action to prevent their effects can be taken (Malle, 1994). In comparison, small persistent leakages often result in the accumulation of AOM in the environment at low concentrations that are often difficult to detect. Many chemicals tend to accumulate in the tissue of biota or in various organic or inorganic matter in the species' habitat (e.g. sediment) (Neff, 1984). Such species can be continuously exposed to toxic micropollutants, which can cause chronic effects, affecting species survival and performance. Thus, changes in species traits, such as altered activity of organisms, reduced longevity, or altered genetic composition of affected species, can be considered in toxicological studies of chronic AOM effects.

The effects of AOM on a single organism can lead to changes across whole populations; these effects can be extremely diverse and observed at different biological levels. AOM can act at the molecular level of genes and proteins, for instance with receptor-mediated effects, and at the cell level, with cytotoxic effects acting at cellular and tissue levels (Escher et al., 2014). Effects on the molecular and cellular level can translate into effects on the functioning of the whole organism and can have consequences on species performance, population dynamics, and ecosystem integrity (Baird & Van den Brink, 2007). Depending on the type of toxicity screening method applied, from *in vitro* standard bioassays to other non-standardized *in vivo* or *in situ* screenings, these effects of AOM can be observed across different levels of biological organization. Modern screening methods can provide information on morphological damage, changes at the genetic level such as changed allelic frequencies (Weston et al., 2013) and gene expression rates (David et al., 2014), altered behavior, survival (Nyman et al., 2013), feeding rates (Englert et al., 2017; Graça, 2001), and species reproduction (Alves da Silva et al., 2018).

The potential for toxicity for a chemical usually has to be assessed on a per-species basis, as the effects of AOM largely differ among species. While some species show sensitivity to a certain compound, others do not respond at all and can tolerate almost any concentration of a specific chemical that appears in the freshwater ecosystem. For

example, in contrast to arthropods that show high sensitivity in exposure to the pesticide diazinon (median hazardous concentration for 5% of species (HC5) is 0.36 µg/L), the rest of the non-arthropod macroinvertebrates for which data is available show comparatively low sensitivity to environmentally relevant levels of this pesticide (HC5 229 µg/L) (Maltby et al., 2005). A species' sensitivity to a compound depends on its physiological adjustments to its habitat (e.g. demand for oxygen, nutrients, volume to surface ratio), presence or absence of specific physiological pathways (e.g. binding sites for specific AOM), and behavior in the environment (i.e. feeding type, mobility), but also on the physio-chemical properties of the compound. For instance, due to their ecological niche and function, organisms such as oligochaetes living in the sediment might be mostly exposed to compounds that tend to accumulate in the sediment (Smutná et al., 2008)

These differences in the sensitivities of species to AOM are evident at the ecosystem scale. At this level, the effects of AOM mixtures become evident in changes of freshwater species communities (Beketov et al., 2009; Friberg et al., 2010; Münze et al., 2017) and in a reduced performance of ecosystem functions (Karrasch et al., 2019; Schäfer et al., 2012). Altered species communities and their functioning can be accompanied by changes at the individual and population level through physiological or different evolutionary adjustments due to exposure to AOM. In particular, pesticides were shown to drive changes in the physiology, survival, and genetic composition of freshwater organisms. The consequences of the adverse effects of pesticides in non-target organisms were shown for different groups of pesticides. Biocides were shown to act on the species composition of freshwater microbiomes (Foissner, 1997). Herbicides were associated with reduced biomass of macrophytes in freshwater ecosystems (Mohr et al., 2007) and altered periphyton communities (Tlili et al., 2017). Insecticides were associated with reduced macroinvertebrate diversity (Burdon et al., 2019; Münze et al., 2017), mostly due to increased mortality rates of macroinvertebrates that had the same receptors as terrestrial insects (Reiber et al., 2021). Finally, pesticides can also affect the survival of fish (Reid et al., 2016). The drastic effects of pesticides in natural environments occur because pesticides target specific species of pests, but additionally adversely impact non-target species with a similar evolutionary background.

Many of the direct adverse effects of single compounds are well-studied, especially in model organisms. On the other hand, the sub-lethal effects of mixtures of different AOM that occur simultaneously in natural ecosystems at low concentrations and induce toxic stress on non-target organisms can be to a large degree unknown (Cedergreen, 2014). Organisms adjust in different ways to sub-lethal AOM effects. The adjustments may occur as an organism's physiological response over a short time period (e.g. change in the rate of metabolism, increased expression of genes related to a defense mechanism etc.), or by genetic adaptations at the population level over a longer time period. Genetic adaptation can be of a greater benefit for populations that live in habitats with persistent toxic conditions (Muyssen & Janssen, 2004; Sun et al., 2014; Weston et al., 2013). Many of such species responses to environmental pollution have only recently been addressed by researchers. This precise characterization of AOM environmental pollution and species responses was particularly enabled by advances in analytical techniques that allow for a high-throughput assessment of thousands of AOM. The powerful analytical methods have been coupled with standard methods for the assessment of the impact of AOM at the cellular and molecular level and the level of single organisms. These standard approaches include standard toxicity tests for lethal (e.g., *Daphnia* toxicity test) and sublethal effects of AOM *(*e.g., Ames mutagenicity test), and tests of effects at the community and ecosystem levels (e.g., freshwater community-based methods such as the SPEAR index; Liess & von der Ohe, 2005). In addition, recent applications of molecular techniques that allow for studies of genetic and physiological responses of organisms, such as genome-, transcriptome- or proteome-wide analyses, have improved the precision of assessments of AOM impacts. With the right selection and application of the available toxicity assessment methods, the precise characterization of organisms' responses to AOM exposure in laboratory or in field exposures can be performed (Reid et al., 2016; Whitehead, 2012).

1.5 Assessment of toxicity potential of AOM

Toxic AOM in freshwater ecosystems contribute significantly to biodiversity decline (Grizzetti et al., 2017). Anthropogenic organic micropollutants, especially when in combination with other stressors such as high temperatures, cause toxic effects in sensitive organisms and alter species diversity. These toxic effects on the survival of specific organisms can be reflected by drastic changes in freshwater ecosystems (Lemm et al., 2020), such as shifts in species communities and impacts on species performance (Fulton et al., 2021; Peschke et al., 2014; von der Ohe et al., 2009). Therefore, a precise assessment of contaminant toxicity for the organisms inhabiting freshwater ecosystems that are exposed to AOM should be conducted. The assessment of environmental impacts of AOM and the identification of the main drivers of toxic effects in biota can be done by a precise characterization of AOM in the impacted river sections (Brack et al., 2019). The characterization can be performed based on AOM screening of the media where AOM accumulate. Anthropogenic organic micropollutants in rivers and streams in general occur in water, sediment, and tissues of organisms. The accumulation in different media is dependent on compound physiochemical properties (e.g. polarity), which can be assessed based on each compound's octanol-water coefficient (i.e. K_{ow}). For instance, contaminants may enter the water column as freely dissolved compounds. More polar AOM with low K_{ow} may stay in the water column, while non-polar compounds with high K_{ow} may strongly accumulate in sediments or tissues of organisms (e.g. in lipids; Tlili et al, 2012). Due to different accumulation rates, bioavailability, i.e. the ability of compounds to enter a living organism and affect it, and toxicity of single AOM, each compound should be considered in the assessment of AOM effects for every species of interest.

Due to the specific potential of every AOM to cause an effect in a specific organism, the contribution of each compound found in a species' habitat to the total acute toxicity on organisms of interest should first be predicted (Altenburger et al., 2015). To do so, the toxic potential of each compound for a target species based on the detected concentration needs to be assessed. The toxicity of each AOM can be assessed in a standard toxicity test. Based on the detected concentrations of each AOM in the assessed medium (e.g. water, sediment, or tissue) and an estimation of the effect by a standard toxicity test, the

potential toxicity in the environment for the specific species can be estimated. The toxic effects of different AOM can be summed according to the concept of concentration addition to estimate mixture toxicity (Schreiner et al., 2016). Such an approach for AOM mixture toxicity in freshwater organisms was proven to be precise and broadly applicable for an acute toxicity assessment for single species (Belden et al., 2007).

To estimate the toxic contribution of every detected compound from the studied location to the total toxicity potential for a single species, toxic units (TUs) can be applied as estimates of the toxicity for each compound (Ginebreda et al., 2014; Sprague, 1970). The TUs approach is applicable for different types of samples, e.g. water, sediment, and animal tissue (de Castro-Català et al., 2016; Shahid et al., 2018b). However, for the most precise TU estimation, compound concentrations of freely dissolved AOM need to be considered. Such an approach is needed when estimating the value of compounds found in the tissue of organisms, as the reference toxicity data from standard toxicity tests mostly refers to experiments in which freely dissolved concentrations of the assessed AOM were measured. The final result of the total toxic potential for adverse effects of AOM in the assessed species consists of summarized TUs derived from all the available detected compounds and standard toxicity data for the given taxa. With this method, the main AOM compounds from the environment that pose a risk for adverse and potentially selective toxic effects (e.g. mortality, immobility) in the assessed species can be identified as indicated by various field studies using, e.g., freshwater amphipods (Inostroza et al., 2016a; Munz et al., 2018). However, it should be noted that the capacity of the TUs analysis to precisely predict the potential for adverse effects in the selected taxa strongly depends on several factors, including the available data from standard toxicity tests for detected AOM, the detection sensitivity for each AOM, and the extent of AOM targets that are comprised in the target analysis (Malaj et al., 2014).

1.6 Evolutionary ecotoxicology as a novel discipline in the assessment of AOM impacts

One of the major drivers of negative trends in freshwater biodiversity are AOM that can, due to their continuous presence in rivers, exert constant stress, cause acute effects, and lead to chronic sub-lethal effects in exposed organisms. Standard monitoring approaches based on pollutant screening and toxicity assessment contribute significantly to the discovery of toxic effects of screened contaminants and their environmental impact. However, analyzing the lethal and sub-lethal effects of AOM and changes in species composition by traditional toxicological approaches might not be sufficient for the preservation of species and their functions in a polluted environment in the long run (Straub et al., 2020). In ecosystems impacted by anthropogenic changes, indigenous species need to be able to adjust to novel, likely sub-optimal environmental conditions (Noyes et al., 2009). To allow species to adapt to locally and globally occurring changes under increasing human pressure on lotic ecosystems, the preservation of genetic diversity of vulnerable species is key (Keller & Waller, 2002). Current and future scenarios of biodiversity under environmental stress and the impact of chemical pollution can be addressed by the adoption of population genetics methods (Pauls et al., 2013) coupled with traditional ecotoxicological assessments. Thus, impacts on species fitness can be assessed with a combination of analytical methods, estimation of toxicological effects, and estimation of genetic diversity parameters to identify the main chemical drivers of selective pressure and determine the environmentally hazardous compound concentration limits that can still be tolerated without critical genetic diversity erosion (Bickham, 2011).

The increased selective pressure in freshwater populations exposed to AOM can contribute to a decrease in species fitness. For example, AOM can be the main drivers of reduced survival and reproduction in species. Species can respond to such pressure in different ways, however, responses to same stressor over multiple generations will likely result in an evolutionary change within exposed populations (Whitehead et al., 2017). Therefore, in evolutionary ecotoxicology, ecotoxicological methods are applied to species under exposure with appropriate reference populations (i.e. non-impacted sites and

populations), followed by an evaluation of genetic change based on principles of evolutionary theory and conservation biology (Bickham, 2011).

Specific AOM compound groups can be expected to drive genetic changes in populations. Among these groups are mutagenic compounds exerting direct genetic changes by the promotion of point and frameshift mutations (Rinner et al., 2011). Additionally, compounds classified as genotoxic can, in addition to mutations, induce larger structural changes in chromosomes and affect inheritance mechanisms (Theodorakis et al., 2001). In addition to direct changes, compounds not classified as mutagenic or genotoxic may also lead to changes in the genetic diversity in an organism or a population (Coors et al., 2009; Coutellec et al., 2013; Weston et al., 2013). These AOM consist in general of all compounds that do not directly alter chromosomes or DNA bases, but can affect individual survival and drive alterations in mating and developmental success within exposed species. These effects may cause changes in the demographics of species and allele frequencies in the exposed populations. Four main evolutionary processes affecting a species' genetic diversity can be expected under exposure to toxicants driving genetic changes (Fig. 1.3). These include the selection of survivorship loci, alteration in genetic diversity, changes in gene flow and dispersal rates, and differences in mutation rates (Bickham, 2011). The important assumption when addressing the altered genetic diversity of species by toxicants is that AOM do not only cause acute effects that drive genetic changes, but can be strongly associated to non-acute, sub-lethal evolutionary mechanisms that result from multiple stressors that occur in human-altered habitats.

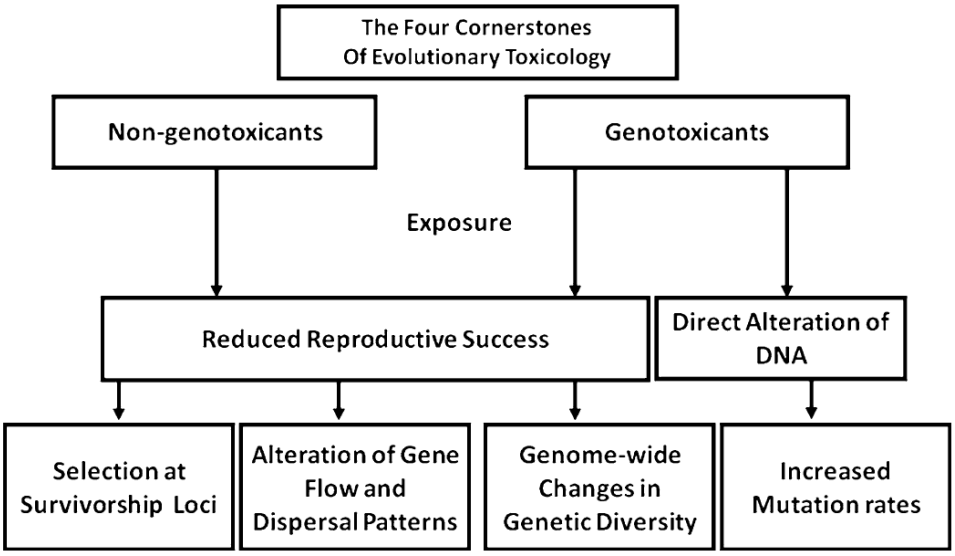

Figure 1.3 The four cornerstones of evolutionary toxicology. According to Bickham (2011), the four cornerstones of evolutionary toxicology include the evolutionary processes of selection, alteration of geneflow, genetic diversity change, and changes in mutation rates. Figure acquired from (Bickham, 2011).

1.6.1 Effects of AOM reflected by genetic diversity parameters

Evolutionary change may be driven by the four processes of genetic alteration (Fig. 1.3) simultaneously or as single processes. The impact and extent of genetic change in the affected species is independent of the number of processes happening simultaneously and can be especially evident in the case of single processes. One of the most obvious effects of AOM at acutely toxic levels on freshwater organisms may be a drastic decline in population size that accompanies selective processes. A decline in abundance and population size can induce a loss of genetic variability by the means of genetic drift (Nei & Tajima, 1981). Genetic drift occurs genome-wide and can be indicated by neutral genetic markers through changes in genetic diversity parameters such as allelic richness, nucleotide diversity, and heterozygosity. Genetic diversity loss is, in such cases, the most common effect and it was, for instance, detected in parallel to increased pollution in marsh frogs, *Pelophylax ridibundus* (Pallas, 1771), living in highly contaminated water reservoirs near Sumgayit in Azerbaijan (Matson et al., 2006).

The change of genetic diversity in a population exposed to toxic pollution can be also strongly influenced by migration. In contaminated areas, AOM can negatively affect species genetic diversity, however, the genetic diversity can nonetheless be maintained by high migration rates from non-affected populations to exposed ones. Still, the population affected by selective pressure would act as a sink of genetic diversity and would not be able to preserve the same levels of genetic diversity without contributions from undisturbed populations (Theodorakis et al., 2001). The study of frogs migrating from pristine to contaminated lakes in Azerbaijan showed such population dynamics (Matson et al., 2006). In addition to sinks of genetic diversity, genotoxic contaminants can also cause genetic damage in exposed organisms, as shown in a study on kangaroo rats (*Dipodomys merriami* Mearns, 1890) (Theodorakis et al., 2001). Radioactively contaminated areas in this case acted as a sink of species genetic diversity due to radioactive toxicity and as a source of genotoxic damage that was detected among migrants.

The genetic diversity of species may also be altered by genotoxic and mutagenic contaminants. These can, on the one hand, impact the reproduction success of organisms, but, on the other hand, increase the genetic diversity of the affected populations due to increased rates of mutations producing novel alleles at the affected loci (Devaux et al., 2011; Eeva et al., 2006). Genotoxic and mutagenic effects can be detected in populations when there is an increased frequency of novel alleles at contaminated sites. Due to proximity, the occurrence of novel alleles within populations more adjacent to the pollution source is more probable than in the distant ones (Bickham, 2011). Yet, the prevalence of such alleles in the adjacent populations is expected to be lower than in the source population. Usually, such *de-novo* mutations originating from contaminated areas indicate only one mutational step from the common genotypes in the affected population. The mutations induced by contaminants in the population affected by toxic WWTP contamination was indicated for both the mosquitofish (*Gambusia holbrooki*, Girard, 1859) and marsh frogs (Matson et al., 2006; Rinner et al., 2011) in highly contaminated freshwater areas.

1.7 Population genetics methods for genetic diversity estimation in AOM-contaminated environments

Changes in the genetic diversity of populations exposed to environmental pollutants can be assessed by the evaluation of genetic polymorphisms using different molecular markers. Genetic markers enable the quantification of genetic diversity parameters, migration rates among populations, inbreeding rates, and can identify populations and site specific genotypes of a species in the landscape (Kirk & Freeland, 2011). So-called neutral genetic markers, which are usually not under selective constrains, can provide insight into recent population changes and local population adjustments. Three classes of genetic markers exist and are classified according to the DNA analysis technique: 1) single nucleotide polymorphisms (SNPs), 2) hybridization of nucleic acid based on complementary bases, and 3) DNA amplification-based methods (Hoshino et al., 2012).

With the first technique, SNPs, polymorphisms in bases at the same position in the genome across individuals are analyzed. This technique offers great predictive power for the detection of changes in a population's genetic parameters when sufficient number of individuals or loci are studied (Morin et al., 2004). With the application of novel sequencing methods and sufficient computational power, several thousand polymorphisms across the genome of each individual can be detected and compared simultaneously (McCormack et al., 2012). This enables the detection of evolutionary processes with high precision and identifies specific genomic regions that are under selective constraints. On the other hand, this technique is much costlier in comparison to the other techniques and is therefore sometimes more difficult to apply to large scale multi-site analyses and genotyping. Association population genetics studies including changes in populations under pollutant pressure were carried out mostly with species for which assembled genome data were available, as in such cases, the analysis offers a great resolution in the prediction of effects bound to specific gene regions. For example, a study on populations of Gulf killifish (*Fundulus grandis*, Baird & Girard, 1853) from the Gulf of Mexico indicated genetic adaptations to oil pollution in chronically exposed fish populations (Oziolor et al., 2019).

The second genetic technique is based on the determination of restriction fragment length polymorphisms (RFLP): a DNA sample is fragmented using restriction enzymes and DNA fragment lengths are quantified. For this, DNA fragments are separated by an agarose gel with each specific fragment of a different length representing a specific allele regardless of the region the fragment originates from. The method is, due to challenges associated with the manipulation of high throughput allele sampling, not commonly applied in population genetics studies anymore (Hoshino et al., 2012).

The final technique, PCR-based amplification of DNA, encompasses several different approaches. Among them, the most commonly applied approaches are random amplification of polymorphic DNAs (RAPD), amplified fragment length polymorphisms (AFLP), and microsatellites or simple sequence repeats (SSRs). The main advantages these methods have in common that make them widely applicable are their affordability and reproducibility across many samples. In connection to environmental pollution, the RAPD method was, for instance, used in studies looking for genetic markers associated with survivorship in mosquitofish in polluted ponds in the USA (Theodorakis et al., 2006). The AFLP method has also been incorporated in studies uncovering changes caused by pollution. For example, this method was applied to uncover the effects of arsenic on the genetic divergence of earthworms living in polluted and pristine soils (Kille et al., 2013). Microsatellites have so far been frequently used in many environmental population genetics studies, including studies on the effects of water pollution on the genetic structure of macroinvertebrates, comparing populations living in polluted and pristine river sections (Inostroza et al., 2016a; Weiss & Leese, 2016).

1.7.1 The application of microsatellites for population genetics assessment

Microsatellites are tandemly repeated DNA motifs that vary in length and are mostly distributed throughout eukaryotic nuclear genomes. Nevertheless, they can also occur in other genomes, such as in the mitochondrial genome (Soranzo et al., 1999). Most microsatellites are considered to be located in non-coding regions of the genome. Thus, microsatellites exhibit a high mutation rate and are specific for defined populations.

Microsatellite PCR products can easily be genotyped or sequenced using the Sanger method. Microsatellite loci offer high species and amplification specificity and a relatively low cost for their development and application, which makes them one of the most informative PCR-based molecular markers for population genetics studies. Due to these characteristics, microsatellites have also proved to be very useful in high resolution population genetics studies. After the selection of loci in a species, the microsatellite method can often be applied in another related target species. The main advantage of microsatellites over other PCR-based techniques is the ability to distinguish between both heterozygotic and homozygotic samples. Thus, microsatellites are one of the most widely applied methods in population genomics studies (Hoshino et al., 2012).

In studies on how environmental change impacts population genetics, microsatellites have been used in various studies spanning from analyses of metal pollution to AOM effects in terrestrial organisms, fish, and freshwater macroinvertebrates. For example, the genetic diversity of mussels exposed to increased pollution pressure from the Adriatic sea was found to increase in a study examining eight microsatellite loci (Štambuk et al., 2013). Another study, applying ten microsatellites in mice that lived in sites polluted with heavy metals in Belgium revealed the genetic differentiation of populations living at the most polluted sites and populations at sites with low levels of metal pollution (Berckmoes et al., 2005). Finally, a study on freshwater amphipods from a river in central Germany suggested strong differentiation and changes of genetic diversity between amphipods living in anthropogenically polluted and pristine river sections (Inostroza et al., 2016a). All in all, microsatellites so far have proved to be robust, reliable, cost-effective, and sensitive for the assessment of a species' genetic structure, diversity, and effects of contaminants on genetic diversity parameters in organisms under pollution pressure.

1.8 Amphipods as indicator organisms for pollution in freshwater ecosystems

1.8.1 Amphipod diversity and ecosystem function in freshwater ecosystems

Amphipods (peracarida, amphipoda) are among the most commonly studied freshwater organisms in ecotoxicological research. The crustacean order of amphipods is extremely rich and includes mainly aquatic, but also terrestrial crustaceans. Globally more than 10,000 species have been described to date (Väinölä et al., 2008). Approximately 20% of these species live in freshwater ecosystems, half of which can be found in the Palearctic. Amphipods inhabit a wide diversity of freshwater habitats, among which the most typical are lakes, rivers, and streams. Some amphipod species have adapted to extreme environments, such as great water depth, as in the case of amphipods from lake Baikal (Jeffery et al., 2016), or constant darkness, as in the case of the subterranean genus *Niphargus* (Fišer, 2012). Amphipods are often the dominant macroinvertebrate group in rivers and streams and therefore an important indicator of freshwater quality (Kirkpatrick et al., 2006).

Due to their high reproduction rates and relatively simple breeding and maintenance in captivity, several amphipod species are important model organisms in biology. For instance, *Parhyale hawaiensis* Dana, 1853, is one of the most important model organisms for arthropod development, regeneration, immunity, and lignocellulose digestion (Kao et al., 2016). Several other species, such as amphipods from the freshwater genera *Hyallela, Gammarus,* and species from the terrestrial family of Talitridae are regularly used in environmental toxicology studies (Ashauer et al., 2010; Costa et al., 2021; Zhao & Newman, 2006).

Amphipods living in freshwater ecosystems are especially important as secondary consumers and shredders of leaves and other organic material that they can break down to smaller pieces that are then made available for other debris-feeding organisms (Kelly et al., 2002). Additionally, they are an important part of the food chain as scavengers, predators of other freshwater macroinvertebrates, and as fish prey. Due to their ecological functions and abundance in various ecosystems, they are often used in aquatic

biomonitoring (e.g. saprobic and SPEAR indices (Beketov et al., 2009), in the determination of toxicological pressure on an ecosystem, and in toxicological studies on a species' ecological performance and function (e.g. leaf shredding, reproduction).

Due to ongoing alterations of their habitats, amphipods are facing rapid changes in their distribution and community structures worldwide. The main drivers of such changes in species composition are alterations of river morphology and invasions of non-native aquatic species (Alther & Altermatt, 2018; Hellmann et al., 2017; MacNeil & Platvoet, 2005). It is known that water pollution also affects freshwater amphipods, altering their abundance, however, as most studies on the composition of an amphipod population and the distribution of the amphipods focused on factors other than pollution, e.g., multiple stressors, species invasions, river barriers etc. (Alp et al., 2012; Gergs et al, 2019; Weiss & Leese, 2016), effects of the pollution on the amphipods' distribution and diversity in natural populations are often still poorly understood.

1.8.2 The genus *Gammarus* – indicator for anthropogenic impacts in rivers and streams

The amphipod genus *Gammarus* is one of the most studied genera of freshwater organisms in environmental studies in central Europe. It is a widespread genus of amphipods living in the Palearctic region and consists of more than 100 described species from freshwater habitats (Karaman & Pinkster, 1977). Species belonging to the *Gammarus* genus inhabit marine as well as freshwater habitats. Amphipods from this genus are morphologically similar, often with relatively high intraspecific variation in morphology (Altermatt et al., 2019). The most commonly found *Gammarus* species in central European area are *Gammarus pulex*, *Gammarus fossarum* s.l., and *Gammarus roeselii* Gervais, 1835 that inhabit streams, small to mid-sized rivers, and lakes. All three species are known for their large genetic variation and cryptic diversity (Altermatt et al., 2019). Amphipods from the genus have been often used in ecological and environmental experiments. The main advantages and reasons that species from the *Gammarus* genus are used in environmental studies is their rapid, so called "r", reproductive strategy and

relatively easy maintenance in laboratory culture. These traits also make *Gammarus* amphipods a suitable subject for testing the effects of environmental disturbances and stressors in freshwater ecosystems in laboratory experiments. Moreover, amphipods from this genus are widespread in many European rivers, allowing *in situ* testing and the transfer of results and hypotheses derived from laboratory studies to populations living in impacted habitats in natural environments.

Figure 1.4 Sites of rivers with *G. pulex* habitats, to various degrees impacted by human activities; (a) the Saale River in the vicinity of Rudolstadt, Thuringia; (b) the Spittelwasser Stream in the vicinity of Jeßnitz, Saxony-Anhalt; (c) Effluent of the WWTP Rudolstadt in the Saale River; (d) the Rolandbach Stream in an agricultural landscape in the vicinity of Glauchau, Saxony.

Amphipods from the *Gammarus* genus have been used as indicator organisms in various environmental studies that focus on anthropogenically induced changes in freshwater environments. The response of freshwater organisms to increasing temperature in light of global warming was studied using *Gammarus pulex* from Mediterranean climates (Foucreau et al., 2014). Native populations of *G. pulex* and *G. fossarum* were investigated

for their adjustment to the invasion of non-native amphipod species (Gergs et al., 2019; Little & Altermatt, 2018). The performance of *Gammarus* amphipods under the impact of multiple stressors was studied by examining, for example, their feeding rates (Galic et al., 2018) and reproduction (Coulaud et al., 2015; Geffard et al., 2010).

One of the major environmental stressors that has been investigated using *Gammarus* is freshwater pollution by anthropogenic contaminants. The effects of anthropogenic pollutants, including nutrients (Henry et al., 2017), metals (Vigneron et al., 2019), and organic compounds (Ashauer et al., 2010; De Lange et al., 2006), have been studied using *Gammarus* amphipods in ecotoxicological studies performed in the laboratory and in natural exposure set-ups. Among the studied endpoints, the ones most frequently studied include survival with different lethal descriptors, such as lethal time or lethal concentration that causes the effect in half of the exposed individuals (LT_{50}, LC_{50}, respectively) (Nyman et al., 2013; Stuhlbacher & Maltby, 1992). In addition, sublethal descriptors were also frequently observed. Among these, the most common ones include immobility rate increase (Nyman et al., 2013), feeding and shredding rates (Englert et al., 2017), and the rate of reproduction and its success (Crane, 1994). Previous studies indicated that amphipods from this genus are highly suitable for stress research as numerous endpoints can be observed. Based on these, the effects of environmental stressors on the freshwater macroinvertebrate fauna can be predicted.

1.8.3 Genetic diversity in the genus Gammarus in relation to AOM

Many of the amphipod species from the genus *Gammarus* have a wide distribution, often spanning across continents. However, the actual genetic diversity of these species has only been revealed by recent advances in molecular biology methods. With the novel methods, genetically divergent, independently evolving clades were indicated within morphologically indistinguishable, widely distributed species. The name “cryptic species” has been used for these morphologically similar, but genetically, and often ecologically distinct species (Bickford et al., 2007). Cryptic species often have a narrow geographic distribution and are found in habitats with distinct environmental parameters (Eme et al.,

2014). Due to evolutionary independence, cryptic species often acquired different environmental adaptations through their evolutionary history. Genetic adaptations to different environments may be evident in differences in the sensitivity of such cryptic species to contemporary pollutants. Within the *Gammarus* genus living in Central Europe, the presence of cryptic species was shown for *G. fossarum* (Grabner et al., 2015), while genetic diversity at the level of cryptic species was also indicated for *G. pulex* (Lagrue et al., 2014) and *G. roeselii* (Grabowski et al., 2017). However, all of these species complexes have been used in studies of species response to environmental change and only in rare cases has the genetic lineage, to which the tested organisms belonged to, been indicated (Feckler et al., 2012; Feckler et al., 2014; Gergs et al., 2019; Leese et al., 2016).

Cryptic species within *Gammarus* are usually adapted to a narrow ecological niche. Although they do not differ morphologically and can even occur in sympatry, differences in mate selection (Lagrue et al., 2014) and sensitivity to environmental pollutants (e.g. pesticides) have been shown (Feckler et al., 2012). The species with higher tolerance to pollutants were shown to occur in areas with increased human impact but, on the other hand, were more prone to parasite infections. These results imply that the genetic background is important to precisely determine the toxicity of chemicals for species and to provide correct toxicity information for species that are not standard laboratory test organism with known genetic background.

In addition to genetic diversity at the species level, genetic diversity within a species can vary largely among geographical areas and can also contribute to organisms having different sensitivities to environmental stressors. In some species, rapid reproduction and survival in stressful conditions might select for different degrees of adjustment to environmental stressors that would originate from regional genetic diversity pool. In contrast, adjustment to certain environmental stressors might also have a non-genetic origin in the form of acclimation that results in a plastic physiological adjustment (Vigneron et al., 2019). Genetic markers commonly applied for barcoding and phylogenetic relationships, such as the mitochondrial cytochrome oxidase subunit I (COI) marker may not be able to detect genetic differences of distinct populations living in specific conditions

within a species' distribution range. To determine the genetic diversity at the subspecies levels, specific highly-variable genetic markers, such as microsatellite loci, need to be investigated. These loci can, despite their location in non-coding regions of the genome, be important for gene expression regulation and could therefore be related to adaptation in coding regions of the genome and can be affected by species fitness in exposure to environmental stressors (Kashi & King, 2006).

Several studies have shown that genetic differences and adjustments to contaminants were associated with AOM at different taxonomic levels. Differences in sensitivities to environmental pollutants in urban and rural environments have been found within the same genetic lineage of the model freshwater invertebrate *D. magna* (Coors et al., 2009). Although belonging to the same lineage, the *Daphnia* crustaceans showed differences in sensitivities to pollutants that were related to the genetic diversity of the populations at the study sites. Such changes in genetic diversity can be driven by mutations with substitutions, deletions, or insertions of bases in the affected gene coding DNA region. In amphipods, a point mutation has been indicated as the main reason for the reduced sensitivity of populations belonging to different lineages of the North American species *Hyalella azteca* (Weston et al., 2013). In some toxicological studies, genetic adaptation and differences in genetic diversity are assumed to be the main reasons for differences in sensitivities to compounds based on spatial distinctness (Shahid et al., 2018a; Stuhlbacher & Maltby, 1992). However, as shown by other examples, without clearly indicated genetic information on the distinctness of genetic lineages or beneficial genetic predisposition in the affected genetic regions, physiological non-genetic adjustments can also be a reason for the reduced sensitivity of freshwater organisms to increased pollution levels (Muyssen & Janssen, 2004; Vigneron et al., 2019).

1.8.4 *Gammarus pulex* – indicator for toxic effects of AOM and changes in the genetic diversity of freshwater species

In studies examining the effects of environmental pollutants in Europe, one of the most commonly used amphipod species is *G. pulex*. It is a widespread Palearctic species that

lives in different freshwaters, including smaller to mid-sized rivers with semi-strong water currents and lakes (Altermatt et al., 2019). *G. pulex* has been regularly investigated in studies on the toxic effects of anthropogenic contaminants (Berghahn et al., 2012; Russo et al., 2018; Stuhlbacher & Maltby, 1992). There are several reasons, why *G. pulex* has been frequently used in the assessment of toxic effects in environmentally relevant settings. The species is often a dominant aquatic macroinvertebrate in rivers and streams and is especially abundant in habitats where sufficient amounts of nutrients are present, such as in river sections downstream of WWTP discharges. The main ecological role of *G. pulex* is the shredding of organic material; *G. pulex* is the key species in freshwater systems that transforms organic material, such as leaves, that enter from the terrestrial environment. The amphipods shred leaves and make the organic material available for decomposition by other freshwater organisms such as fungi and bacteria. Besides shredding organic debris and leaves, *G. pulex* feeds on other invertebrates (e.g. oligochaetes living in sediment) and is an ubiquitous predator (Kelly et al., 2002). Thus, *G. pulex* is constantly exposed to pollutants entering and accumulating in its habitat and food (Englert et al., 2017; Inostroza et al., 2016a; Munz et al., 2017). Ecologically, *G. pulex* is one of the most important sources of food for fish. Therefore, the species is an essential link in the food chain as well as in the pollutant's uptake and transfer from the environment to higher trophic levels in freshwater habitats. *G. pulex* is therefore an optimal freshwater study organism that has the potential to indicate the effects of environmentally relevant toxic compounds occurring in freshwater habitats and their transfer to other trophic levels.

Gammarus pulex accumulates pollutants through various sources and pathways. Firstly, it acquires pollutants passively in exchange with the water column (passive transport/osmosis). Secondly, amphipods accumulate AOM through their close contact with the sediment, where many AOM accumulate (Masiá et al., 2013); AOM then become enriched in the amphipod exoskeleton and lipids. Thirdly, *G. pulex* takes up contaminants through its food sources, such as leaves, organic plant debris, and other freshwater invertebrates. Due to these different ways of AOM uptake, chemicals entering the river through WWTP effluents and run-off accumulate in the tissue of *G. pulex*. Analysis of the chemicals accumulated in *Gammarus* tissue are representation of the contaminants and

their levels in the species' habitat and potential contamination sources (Inostroza et al., 2016a; Munz et al., 2018).

Anthropogenic organic micropollutants, including pesticides, pharmaceuticals, and industrial chemicals were shown to affect the survival and performance of *G. pulex* upon exposure. *Gammarus pulex* amphipods showed high sensitivity to several insecticides, including neonicotinoids and organophosphates, that are regularly found in their habitats (Ashauer et al., 2007; Nyman et al., 2013). At the low concentrations that are detected in streams and rivers inhabited by amphipods, AOM such as imidacloprid, thiacloprid, or chlorpyrifos already cause sub-lethal effects, such as immobility (Nyman et al., 2013), reduction in feeding behavior (Agatz et al., 2014), significant reduction in *G. pulex* survival, and changes in whole community composition (Shahid et al., 2018b). Thus, the main driver of acute toxic effects in *G. pulex* in freshwater ecosystems in central Europe is, according to a previously published analysis determining TUs, the effect of pesticides (Inostroza et al., 2016a). Pesticides enter freshwater systems mostly by run-off from agricultural fields and urban areas. A large proportion of these contaminants also enters rivers with WWTP effluents (Beckers et al., 2018). Depending on the source type, amphipods can face a pulsed exposure to AOM that can last from a few minutes to a few hours when large amounts of AOM enter the river at the same time. When exposed to insecticides with receptor specific binding, such as neonicotinoids, the insecticides rapidly bind to the nicotinoid acetylcholine receptor and can incur immediate damage to exposed individuals. In such cases, the recovery of amphipods after exposure is slow or non-existent, disturbing the functionality of the specimens and impacting their population (Ashauer et al., 2015). Other compounds that do not bind to the tissue might be eliminated faster, allowing the amphipods to recover after exposure. *G. pulex* might face long-term exposure to compounds that constantly enter its habitat (e.g. via WWTP) or persistently accumulate in sediments in the species' habitat. Although many studies have shown effects of AOM on *G. pulex* in exposures under controlled conditions in the laboratory, there is a lack of studies that characterize the effects of AOM on *G. pulex* under environmentally realistic, multiple stressor scenarios. The effects may not always manifest themselves in mortality, but also in sub-lethal effects that could result in increased species vulnerability, lower reproduction rates, or reduced genetic diversity of the species (Agatz

et al., 2014; Alves da Silva et al., 2018; Bach & Dahllöf, 2012). As species often survive in potentially toxic environments, such studies would be necessary to indicate to which degree single AOM and mixtures of different AOM actually affect individuals and exposed populations. In addition, exposure to many compounds that could lead to sub-lethal or chronic effects in amphipods has not yet been assessed *in situ.* For example, only data for the toxicity of several AOM for *G. pulex* are available in the United States Environmental Protection Agency database up to date (https://cfpub.epa.gov/ecotox/, 1.6.2022) and most of these have not been studied for effects on population of organisms in a natural setting.

The published information on the sensitivity of *G. pulex* to AOM indicate that amphipods living in habitats with different levels and types of pollutants differ significantly. In a study comparing amphipods living in freshwater habitats with high and low diversity of macroinvertebrate communities, *G. pulex* from habitats with more diverse macroinvertebrate communities showed increased sensitivity in exposure to AOM, implying that *G. pulex* in these environments does not adjust to exposure to insecticides (Becker & Liess, 2017). Another study suggests that amphipods in habitats with higher rates of potentially toxic AOM develop resistance to toxicants (Shahid et al., 2018b). Amphipods from these polluted habitats showed lower sensitivity to toxic AOM exposure than amphipods that did not face toxic exposure in their habitats. In both studies, LC_{50} values in a 2-day exposure to insecticides were compared, however, it remains unknown whether genetic or physiological adjustments of *G. pulex* contributed to the increased resistance. In contrast to these studies, it was also found that amphipods previously under toxic exposure showed increased sensitivity to toxic AOM exposure (Ashauer et al., 2017; Russo et al., 2018). Thus, in addition to adjustment to AOM exposure, *G. pulex* can also be adversely affected by increased concentrations of pesticides in its habitat.

The effects of frequently occurring AOM in amphipods can be related to changes in species' genetic diversity and species' genetic structure (Berckmoes et al., 2005; Osterberg et al., 2018; Weston et al., 2013). AOM may affect the organisms' mating ability, cause endocrine disruption, or induce behavioral changes. These effects may change an organism's energy budget and its functional performance. Altogether, these traits may

result in a significantly altered ability of the amphipods to adjust to increased toxic pressure. However, no clear connection has so far been made between a changed sensitivity to AOM and altered genetic diversity of *G. pulex* at the local scale, as well as at the regional scale. For instance, a recent study indicated adjustments in the genetic structure of *G. pulex* from habitats with persistent exposure to AOM (Inostroza et al., 2016a). However, whether the sensitivity of potentially adapted *G. pulex* to toxic AOM resulted from the genetic basis of an adaptation was not studied. It remains largely uncertain to which degree genetic diversity and genetic structure are actually relevant for *G. pulex* to be able to tolerate toxic AOM levels and still thrive in environments with multiple stressors.

1.9 Main objectives of the thesis

The overarching objectives of this thesis are to assess the AOM occurring in rivers in central Germany, study the relationship between AOM and the genetic diversity of a widely distributed amphipod species, *G. pulex*, and link detected AOM to differing sensitivities of amphipods living in polluted and pristine sections of rivers.

Objective 1 of the thesis is to apply and evaluate a genetic method for determination of *G. pulex* genetic population structure and diversity. So far, the methods for AOM screening in water and the tissue of *G. pulex* have already been established and regularly applied. However, the methods for the assessment of the genetic diversity of *G. pulex* have so far been applied to different lineages of *G. pulex* in other regions in Central Europe. For this purpose, novel and existing microsatellite markers for *G. pulex* were tested for a precise determination of population genetic diversity patterns of *G. pulex* locally (within river) and regionally (between rivers).

Objective 2 of this thesis is to investigate to which degree AOM shape genetic structure of *G. pulex* in rivers with a pollution gradient and whether the detected genetic structure at polluted and non-polluted sites in a typical central European river relates to the sensitivity of *G. pulex* to toxic levels of selected AOM. Through the successful application

of the method for the determination of genetic diversity patterns in *G. pulex*, specific impacts of AOM pollution on genetic structure and diversity of the species can be investigated. According to previous studies, the association between different levels of AOM in rivers and the genetic divergence in the genetic structure of populations of freshwater amphipods has not been clearly shown. Fulfilling this objective, information on whether the sensitivity of *G. pulex* to toxic AOM from polluted river sections is reduced due to an adaptation or acclimation to increased levels of toxic AOM or whether it is increased due to persistent toxic stress will be provided.

Objective 3 of this thesis is to understand patterns in the genetic diversity of *G. pulex* with respect to different levels of AOM at the regional scale. The main purpose of this objective is to show whether AOM contamination and population genetic patterns discovered in a single river also occur across a larger geographic area in central Germany and whether altered genetic diversity parameters can be associated with increased levels of AOM in the studied rivers. To assess these patterns, a microsatellite dataset for *G. pulex* from six rivers and 34 sites was analyzed. Different genetic diversity parameters including allelic richness, private alleles, effective population size, and inbreeding were correlated to the site-specific AOM burden descriptors including AOM levels, site-specific AOM toxicity, and the presence/absence of main AOM sources, (i.e. WWTPs) in the studied rivers.

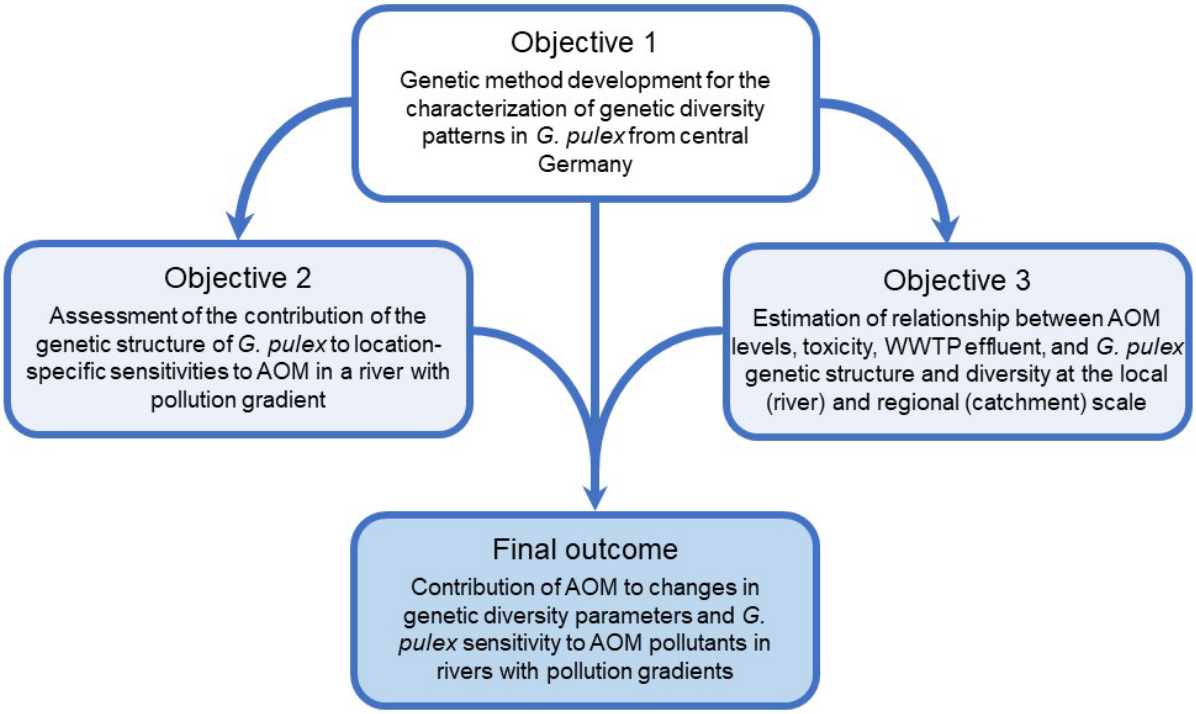

Figure 1.5 Objectives addressed in chapters two to four, leading to the final outcome of the thesis, discussed in chapter five.

To meet objective 1 of the thesis, first, a genotyping tool for a freshwater indicator species in the studied region was applied. Therefore, in Chapter 2, species-specific microsatellite primers were developed for *G. pulex* lineage E, which inhabits rivers in central Germany. To test the applicability of the novel primers, the microsatellites were assessed in a population genetic comparison of *G. pulex* amphipods from three sites from three different rivers. The amplification of the microsatellite loci in a sister lineage of *G. pulex* and another gammarid species, namely, *G. fossarum,* was performed for a broader applicability of the method in the future. The developed method was applied in population genetics analyses in Chapter 3 and Chapter 4.

Objective 2 was addressed in Chapter 3. In this chapter, the effects of anthropogenic organic micropollutants on the genetic structure and sensitivity upon exposure of *G. pulex* to AOM were investigated. The study was conducted in a typical central European river, the Holtemme River, with pristine and with AOM-polluted sections. First, site-specific

patterns of AOM were determined based on the *G. pulex*-tissue sample and water sample analyses. The genetic structure of amphipods living throughout the pollution gradient was assessed based on the conservative mitochondrial COI gene region and non-coding microsatellite loci, mainly established in Chapter 2. The pollution pattern and genetic structure of *G. pulex* was compared among amphipods living at differently polluted sites. Finally, the sensitivity to a commonly-used insecticide, imidacloprid, of amphipods from different river sections was determined to assess whether differences in site-specific AOM concentrations and genetic structure affect the sensitivity of amphipods to imidacloprid.

In Chapter 4, objective 3 was addressed. Thus, genetic diversity descriptors of *G. pulex* at the regional scale in six rivers from different catchments located in central Germany were assessed in relation to the pollution patterns and levels of AOM. The association between genetic diversity descriptors estimated at each sampling site (allelic richness, private allele rates, inbreeding coefficient, effective population size, fixation index), species abundance, and AOM-pollution descriptors (WWTP effluent, AOM concentrations, and AOM toxicity) along with environmental parameters was analyzed to assess whether local genetic patterns correlate with AOM pollution levels and their toxic potential at the local and regional scale. Genetic descriptors were also associated to the distance of the sampling sites from the source, as migration of *G. pulex* downstream at specific site can significantly influence species' genetic diversity.

Finally, in Chapter 5, the results from the previous chapters were summarized and discussed with perspective on the future application of population genetics approaches for assessing the AOM impacts in freshwater macroinvertebrates in rivers.

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Chapter 2

Isolation and characterization of eleven novel microsatellite markers for fine-scale population genetic analyses of *Gammarus pulex* (Crustacea: Amphipoda)

Author's contribution statement 1

Declaration of author contributions to the publication:

Isolation and characterization of eleven novel microsatellite markers for fine-scale population genetic analyses of *Gammarus pulex* (Crustacea: Amphipoda)

Status: Published in Molecular Biology Reports
Material from: Švara et al. (2019) Isolation and characterization of eleven novel microsatellite markers for fine-scale population genetic analyses of *Gammarus pulex* (Crustacea: Amphipoda). *Molecular Biology Reports*, 46(6), 6609–6615. Copyright Springer Nature B.V.

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The contributions of the doctoral candidate and co-authors:

(1) Concept and design
Doctoral candidate: 40%
Co-author 2 and 5: 50%
Other co-authors: 10%

(2) Conducting laboratory analysis
Doctoral candidate: 80% (carried out field work, DNA extraction, analysis)
Co-author 2, 3 and 5: 20% (field work, sample preparation, and DNA analysis)

(3) Compilation of data sets
Doctoral candidate: 80% (raw data evaluation, genotyping, analysis and presentation of the data)
Other Co-authors: 20% (supervised data evaluation and analysis)

(4) Data interpretation
Doctoral candidate: 70% (genotype data interpretation, cross-amplification evaluation)
Other co-author: 30% (genotype data interpretation, cross-amplification evaluation)

(5) Drafting of manuscript
Doctoral candidate: 80%
Other co-authors: 20%

I hereby certify that the information above is correct.

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Date and place Signature doctoral candidate

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2.1 Abstract

The freshwater amphipod species *Gammarus pule*x (Linnaeus, 1758) is widespread across Europe and Asia and is able to live in a broad range of environmental conditions. Yet, it is of great interest to which degree it is able to tolerate and adapt to the current rapid anthropogenic environmental changes affecting its habitat, such as pollution, changes in river morphology, and invasions of alien gammarid species. Microsatellite primers for genetic population studies with *G. pulex* have been developed but due to the existence of several genetically different lineages within the species, the application of these primers is not always successful. In order to investigate the impacts of anthropogenic environmental changes on the spatio-genetic patterns of *G. pulex* clade E in streams in the Saale river catchment in Germany, we designed eleven novel polymorphic microsatellites for this clade using a high-throughput sequencing approach. These microsatellites enabled highly specific characterization of three closely related populations. The results show genetically distinct populations reflected by both a principal coordinates analysis (PCoA) and an analysis of molecular variance (AMOVA). Several of the newly designed microsatellite primers also enabled successful cross-amplification of the respective microsatellites in specimens of *G. pulex* lineage C, while only two microsatellites were amplified successfully and showed polymorphisms for all of the analyzed specimens of *G. fossarum* Koch, 1836. The microsatellites identified here are suitable for future assessments of micro-evolutionary dynamics of *G. pulex* from central Germany.

Keywords: Population genetics, Polymorphic markers**,** High-throughput Sequencing, Amphipoda, Peracarida

2.2 Introduction

Gammarus pulex (Linnaeus, 1758) is a widespread freshwater amphipod species distributed across Europe, Siberia, China, and the Himalayas (Pinkster, 1972) that can be found in a diverse range of habitats in streams, large rivers, and lakes (Altermatt et al.,

2019). The species plays an important ecological role in the decomposition of plant remains and as a prey species for other invertebrates and fish (MacNeil et al., 1997). It is often used as an experimental model in ecological (Dick 1995; Foucreau et al., 2013) and toxicological studies (Ashauer et al., 2011; Nyman et al. 2013; Russo et al., 2018). Recently, *G. pulex* habitats have been subjected to major anthropogenic modifications, such as river regulation and increased pollution (Inostroza et al., 2016a). Furthermore, the species is impacted by competition with non-native amphipod species, such as *G. roeselii* Gervais, 1835 and *Dikerogammarus villosus* (Sowinsky, 1894) (MacNeil & Platvoet, 2005). Thus, a reliable method for determining *G. pulex*'s population genetic structure can be beneficial to detect micro-evolutionary effects of environmental changes in the species' habitats.

Microsatellites, also called short tandem repeats (STRs), simple sequence repeats (SSR), or simple sequence length polymorphisms (SSLP), are short, repetitive, non-coding DNA sequences that show high intra-specific allelic diversity. In population genetic studies of freshwater organisms, microsatellite markers have shown great power in assessments of population structure (Weiss & Leese, 2016), migrations and invasion dynamics (Rewicz et al., 2015), and intra-species modifications of the population structure due to environmental changes (Gergs et al., 2019; Weiss & Leese, 2016). So far, microsatellite markers for *G. pulex* were published in only one study (Gergs et al., 2010). Additionally, several microsatellite primers designed for *Gammarus fossarum* Koch, 1836 were successfully applied to *G. pulex* (Danancher et al., 2009; Inostroza et al., 2016a). Within *G. pulex*, intra-specific genetic differences can be large and can exceed 10% even among sympatrically occurring lineages (Grabner et al., 2015; Lagrue et al., 2014). As existing microsatellite primers were not designed to target particular lineages of the *G. pulex* complex, polymerase chain reaction (PCR) amplification of already described microsatellite primers may not be successful on specimens that are genetically different from the specimens used to design the microsatellite primers (pers. obs.). We thus aimed to establish a reliable testing system for in-depth genetic analysis of closely related *G. pulex* populations facing genetic disturbances in central Germany. We here present eleven novel microsatellite markers designed from *G. pulex* specimens belonging to clade E (Grabner et al., 2015), which is prevalent in the study region where samples were taken

(Saale catchment in central Germany). We amplified the microsatellite loci from several closely related *G. pulex* specimens for fine-scale genetic analysis. We also tested the applicability of these loci for an additional *G. pulex* lineage (i. e. lineage C (Grabner et al., 2015)) and *G. fossarum* specimens from central Germany.

2.3 Materials and methods

2.3.1 Species collection and identification

Gammarus pulex specimens were collected in three rivers in the Saale catchment using Surbers samplers (Holtemme (Hol) - 51.867732 N, 10.873714 E; Parthe (Par) - 51.154140 N, 12.700353 E; Saale (Sal) - 50.720117 N, 11.377208 E, 16 specimens sampled at each location). Additional DNA samples of *G. pulex* from lineage E (two specimens) and C (eight specimens) were kindly provided by Dr. Daniel Grabner and Dr. Alexander Weigand from the collection at the University of Duisburg-Essen (Grabner et al., 2015). The specimens of *G. fossarum* (six specimens) were collected in the Luppa stream in the vicinity of Leipzig (51.335883 N, 12.967850 E). The species were morphologically determined based on a morphological key for central European amphipod species (Altermatt et al., 2019). The specimens were stored individually in 1.5 mL vials with fresh absolute ethanol at -20 °C until further analysis.

2.3.2 DNA extraction and COI sequencing

Genomic DNA was extracted from the individuals' pereopods using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). To prevent dealing with cryptic lineages (Grabner et al., 2015; Lagrue et al., 2014), the genetic lineage of 20 *G. pulex* individuals from the sampling sites was confirmed based on their cytochrome C oxidase subunit I (COI) gene sequence. The gene region was amplified in 20 µL PCR reactions consisting of 1 µL 10 mM dNTPs, 1.6 µL 25 mM MgCl2, 4 µL 5x GoTaq Flexi Buffer (Promega), 0.4 µL GoTaq G2 Flexi DNA Polymerase (Promega), 1 µL of each 10 µM primer (LCO1490 and HCO2198 (Folmer et al., 1994)), 9 µL RNase-free water, and 2 µL DNA template. PCR cycle conditions were as follows: initial denaturation for 2 min at 95 °C, followed by 34

cycles of 1 min at 95 °C (denaturation), 45 s at 51 °C (annealing) and 1 min at 72 °C (elongation), and a final elongation step for 5 min at 72 °C. The acquired PCR products were sequenced on the ABI Prism 3130XL Genetic Analyzer (Applied Biosystems) and assembled in Sequencher 5.4.5. (Tab. SA1). The resulting sequences were compared with published COI sequences of different *G. pulex* specimens in the BLASTn web tool (Altschul et al., 1990). Using ClustalW (Thompson et al., 1994), the acquired COI sequences (one from each sampling location; Genbank accession numbers in Tab. SA1) were aligned with published sequences of specimens from three lineages of *G. pulex* identified in Germany so far (*G. pulex* C - KT075256.1, *G. pulex* D - KT075232.1, *G. pulex* E - KT075230.1), A *G. fossarum* sequence (KF521835.1; (Grabner et al., 2015)) served as an outgroup. A neighbor-joining tree was created based on the Kimura 2-parameter model with 1000 bootstraps using MEGA software (Hall, 2013). The genetic distances were calculated based on the same algorithm in the MEGA software (Tab. SA2).

2.3.3 Primer development

Equimolar DNA extracts from 20 *G. pulex* specimens were pooled in a 50 µL solution at a final DNA concentration of 100 ng/µL, which was used for the preparation of a next generation sequencing library (Illumina TruSeq nano). The DNA was sequenced by Ecogenics, Switzerland on an Illumina MiSeq platform using a Nano v2 500 cycles sequencing chip. The resulting paired-end reads, which passed Illumina's chastity filter, were subjected to de-multiplexing and trimming of Illumina adaptor residuals. Subsequently, the quality of the trimmed reads was checked with the software FastQC v0.117. Paired-end reads were merged to in-silico reform the sequenced molecule using the software USEARCH v10.0.240, resulting in 4,415,897 merged reads from which 100,000 best quality reads were used for the subsequent screening. The reads were screened with the open access software Msatcommander 0.8.2 (Faircloth, 2008) for microsatellite regions of di-, tri-, and tetra-nucleotide repeats, with a minimum of six repeats, and other settings set as default, resulting in 13,915 sequences including microsatellite repeats. Primer pairs for 3,802 sequences could be successfully generated with Primer3 (Rozen & Skaletsky, 2000), implemented in Msatcommander, from which 70

primers were selected for PCR amplification. Each forward primer was tagged with a M13R (5'-GGAAACAGCTATGACCAT-3') or CAG (5'-CAGTCGGGCGTCATCA-3') tag (see Tab. 2.1), and each reverse primer with a pigtail (5'-GTTT-3') to promote adenylation and facilitate genotyping (Brownstein et al., 1996). A touchdown PCR was performed according to the protocol in Schuelke (2000) using specifically labeled M13R or CAG primers (0.25 µM) (Tab. 2.1) with 6-FAM, VIC, NED, or PET labels (Thermo Scientific). An Eppendorf thermocycler was used for the PCR reaction with the following conditions: a primary denaturation step at 95°C for 15 min followed by 20 cycles of 94°C for 30 s, 60 - 50 °C for 30 s (touchdown PCR step; temperature decrease by 0.5°C after each cycle), and 72 °C for 90 s; subsequently 20 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s; the final elongation step was at 72 °C for 10 min. Reactions were diluted 1:10 with RNase-free water and 0.2 µL LIZ 500 size standard (MCLAB) was added to 1 µL of each sample. The mixture was denatured in 8.8 µL Hi-Di formamide (Thermo Scientific) at 95°C for 5 min, followed by immediate cooling on ice. Fragments were then separated on an ABI Prism 3130XL Genetic Analyzer and genotyped using Genemapper 4.0 (Applied Biosystems).

2.3.4 Application of the new primers

From 14 successfully applied primer sets, eleven primer sets could be combined into three multiplex reactions (Tab. 2.1). Eleven amplified loci were used to analyze the variation and differentiation of 48 *G. pul*ex specimens, 16 from each of the three rivers (Holtemme, Parthe, and Saale) in the Saale catchment. Two additional specimens of *G. pulex* lineage E from the Ruhr region were also tested for the amplification of the new microsatellite primers (Grabner et al., 2015). Genotypes were checked for stuttering and null alleles with Microchecker (Van Oosterhout et al., 2004). Allelic diversity and observed (H_o) and expected (H_e) heterozygosity were estimated, and each population's Hardy-Weinberg equilibrium, gametic disequilibrium, and genetic differentiation (F_{st}) were assessed using Fstat 2.9.3.2 (Goudet, 1995) and Arlequin (v. 3.5.1.2) (Excoffier & Lischer, 2010). To visualize differentiation among populations, genetic distances between pairs of individuals were calculated and subjected to a principal-coordinates analysis (PCoA) using a

covariance-standardized method implemented in GenAlEx (Peakall & Smouse, 2010). Results were visualized with R (Fig. 1) (Team R development core, 2016).

2.3.5 Cross-amplification

The developed microsatellite primer pairs were applied to the DNA extracts of eight *G. pulex* lineage C specimens (Grabner et al., 2015) and six *G. fossarum* specimens from the Luppa stream (Tab. 2.3). To avoid potential cross-reactions, the primers were not combined in multiplex reactions but tested individually or in a combination of two primer pairs with different adaptors and fluorescent dyes. The PCR amplification protocol was the same as for the *G. pulex* individuals from the Saale catchment.

2.4 Results and Discussion

Based on their morphology, all of the studied individuals from the Saale catchment were identified as *G. pulex* s. *l.* The COI sequences of all analyzed specimens matched with more than 99% similarity to several published COI sequences of *G. pulex* clade E sensu Grabner et al. (2015) (refer to Tabs. SA1, SA2, and the phylogenetic tree in Fig 2.1a).

The PCR amplification of eight microsatellite loci was successful for all 48 Saale catchment specimens. Null alleles were detected for only two loci (Gp18, Gp37) in two populations (Par, Hol). The average allelic richness per locus was 3.665, 2.850, and 3.721 for populations Hol, Par, Sal, respectively, and the genetic diversity per locus per individual ranged from 0 to 0.798 (Tab. 2.2). The mean observed and expected heterozygosity ranged from 0 (Gp42) to 0.8750 (Gp13). Deviations from Hardy-Weinberg equilibrium ($p<0.01$) were detected for primer pairs Gp37 for population Hol, for Gp31 and Gp37 for population Par, and for Gp37 and Gp42 for population Sal (Tab. 2.2). Observed heterozygosity lower than Hardy-Weinberg expectations can be a consequence of closely related individuals from a restricted sampling area or due to the presence of null alleles (Gergs et al., 2010). Differentiation among sampled populations was significant ($p<0.01$) for all pair-wise comparisons, with F_{st} indices of 0.18628, 0.18553, and 0.22820 for

Hol:Par, Hol:Sal, and Par:Sal respectively (Tab. 2.2). As shown in the PCoA, 19.41, 15.02, and 7.15 percent of variation in genetic distances between individuals was explained by the first three axes, respectively. Analyzed specimens formed three barely overlapping clusters matching each sampled location (Fig. 2.1b), with 20% of variance occurring across populations, 3% among individuals, and 77% within individuals. Based on the significant F_{st} values and the clustering of specimens from the different rivers in the PCoA, we conclude that the specimens from the three sampling locations belong to distinct populations within the *G. pulex* E lineage. The application of the primers to two *G. pulex* lineage E specimens from the Ruhr River in western Germany (Grabner et al., 2015) was successful for all loci indicating the suitability of these microsatellite primers for population genetics analyses of *G. pulex* lineage E.

The application of the novel microsatellite primers for *G. pulex* lineage C and *G. fossarum* specimens was successful for eleven and five loci, respectively. In the case of *G. pulex* lineage C, null alleles were observed for several loci (Tab. 2.3). The here-presented microsatellite primers seem to be promising for application to *G. pulex* lineage C. Nonetheless, it still needs be tested whether the number of functional loci is sufficient for population genetics analysis or if it needs to be supplemented with additional microsatellites. The application of the primers for *G. fossarum* specimens with unambiguous and variable allele calls was acquired in only two cases (Tab. 2.3), indicating that the majority of the microsatellite primers are not suitable for this species that is distantly related to *G. pulex* lineage E.

The use of these microsatellite primers and the delimitation of closely related individuals of *G. pulex* from central Germany can especially be useful in discovering hidden diversity at the population level and will contribute to a better characterization of *G. pulex* populations in the context of environmental changes and the formation of gene-flow barriers.

2.5 Tables and Figures

Table 2.1 Primer set codes, respective forward and reverse primers, repeat motifs, tags used in the PCR reactions, the concentration of each primer in the PCR reaction, numbers denominating the multiplex combination in which the respective primer sets were used, and accession numbers.

Primer code	Sequence	Repeat	Tag	Conc. [µM]	Mult.	Genebank accesion
Gp10	F:TGAAATCGCACCCACTTCG	(AC)^18	M13R	0.06	1	MK512680
	R:AGCTTCCAACAAGATTCCACC			0.24		
Gp11	F:CATGCGCGACTAACCAGAC	(ACT)^14	M13R	0.06	1	MK512681
	R:GGATGACTGCCATGTGTACC			0.24		
Gp13	F:GGGAATTTGGCCTAGCGTATG	(TA)^22	M13R	0.06	1	MK512682
	R:TGCAGTGGAGATGGTAGTCG			0.24		
Gp18	F:GCACCATGGAGTCGATTTAGG	(ATT)^9	M13R	0.06	1	MK512683
	R:AAGTCATTGCTTGACGACGG			0.24		
Gp28	F:TTGTAGACCCGGCACATCC	(AC)^12	M13R	0.06	2	MK512684
	R:TTCCCACGGATCTTGCACC			0.24		
Gp30	F:AAACGACACAGTCTTGACTTC	(AT)^22	CAG	0.06	1	MK512685
	R:CCCTTCTTTATACCAAATAACATTGCG			0.24		
Gp31	F:CCTAACTAGGGGGAATCGGC	(ATAC)^7	M13R	0.03	3	MK512686
	R:TGTCACACGAGACCCTGATG			0.12		
Gp37	F:TGGGTATGTTTCGAATGATGTCTAC	(AT)^14	M13R	0.06	3	MK512687
	R:TCCCTGCTCTAAGAAATTTGCG			0.24		
Gp42	F:GTAAGCTCAACTCCACGGC	(AAT)^8	CAG	0.06	3	MK512688
	R:TCATGGTTGTAATGTTTGGATCAG			0.24		
Gp55	F:CCACATCTGGTCTACACTGGG	(AAC)^11	M13R	0.06	2	MK512689
	R:TGCGGACGCAAAGATGAAC			0.24		
Gp68	F:TAACCTTGGGTGAGTGGCAG	(ACGG)^8	CAG	0.06	2	MK512690
	R:CCACCAGCGATTGTATGCAC			0.24		

Table 2.2 Size range of the amplified loci for each population (Range); observed and expected heterozygosity (H_o, and H_e) within each population; the number of different alleles detected in each population ($N_{alleles}$), and weighted genetic diversity per locus within each sampled population (Gene diversity); null alleles (Null). Asterisks denote F_{is} values that significantly deviate from zero.

Code	Location	Range	H_o	H_e	$N_{alleles}$	Gene diversity	F_{is} (p-value)	Null
Gp10	Hol	162-180	0.750	0.772	5	0.773	0.030	0
	Par		0.625	0.522	3	0.519	-0.205	0
	Sal		0.375	0.339	5	0.338	-0.111	0
Gp11	Hol	328-334	0.438	0.373	3	0.371	-0.180	0
	Par		0.563	0.514	2	0.513	-0.098	0
	Sal		0.188	0.175	2	0.175	-0.071	0
Gp13	Hol	91-99	0.188	0.417	2	0.425	0.559	0
	Par		0.875	0.653	5	0.646	-0.355	0
	Sal		0.563	0.591	4	0.592	0.049	0
Gp18	Hol	111-123	0.375	0.365	3	0.365	-0.029	0
	Par		0.200	0.186	2	0.186	-0.077	1
	Sal		0.563	0.685	3	0.690	0.184	0
Gp28	Hol	271-361	0.688	0.629	5	0.627	-0.096	0
	Par		0.563	0.583	3	0.583	0.036	0
	Sal		0.688	0.738	7	0.740	0.070	0
Gp30	Hol	100-106	0.625	0.611	4	0.610	-0.024	0
	Par		0.250	0.554	4	0.565	0.557*	0
	Sal		0.500	0.494	3	0.494	-0.013	0
Gp31	Hol	129-145	0.625	0.601	4	0.600	-0.042	0
	Par		0.813	0.498	2	0.488	-0.667	0
	Sal		0.313	0.284	3	0.283	-0.103	0
Gp37	Hol	229-331	0.467	0.786	7	0.798	0.415*	1
	Par		0.125	0.433	5	0.444	0.718*	0
	Sal		0.188	0.720	5	0.738	0.746*	0
Gp42	Hol	376-391	0.313	0.373	3	0.375	0.167	0
	Par		-	-	1	0.000	-	0
	Sal		0.000	0.331	3	0.342	1.000*	0
Gp55	Hol	288-333	0.500	0.387	2	0.383	-0.304	0
	Par		0.625	0.506	3	0.502	-0.245	0
	Sal		0.688	0.665	4	0.665	-0.034	0

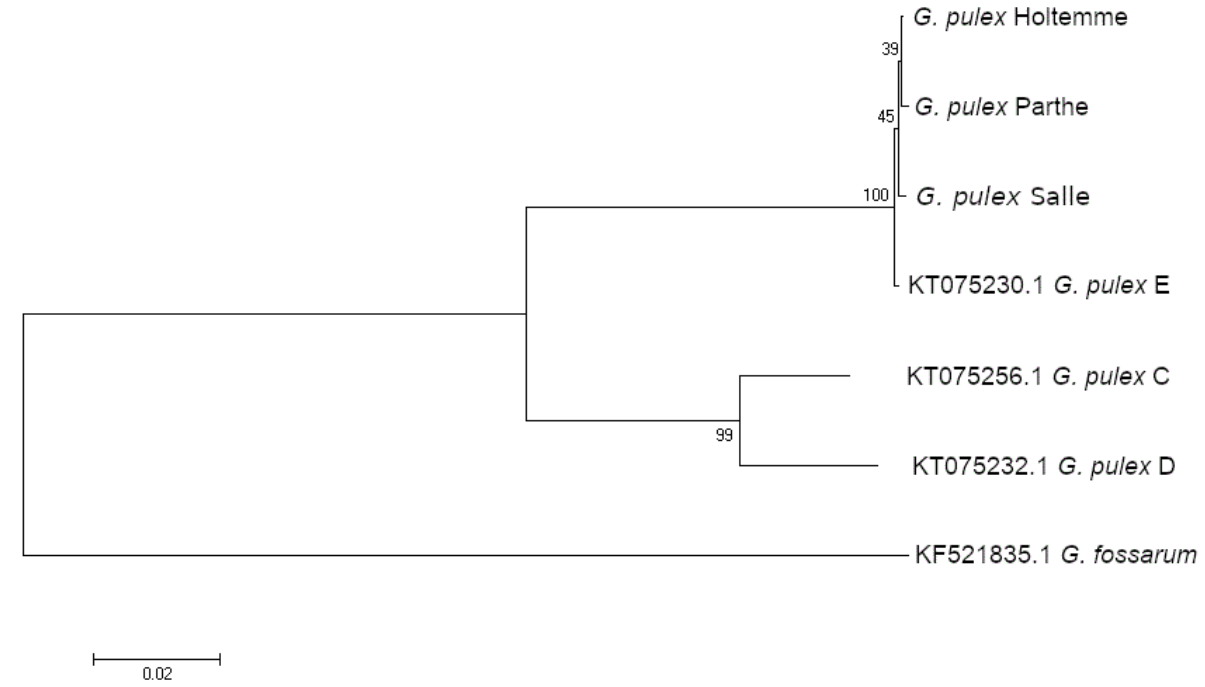
	Hol		0.813	0.522	3	0.513	-0.585	0
Gp68	Par	92-118	0.750	0.484	2	0.475	-0.579	0
	Sal		0.563	0.492	3	0.490	-0.149	0

*Significant values for p>0.01

Table 2.3 Cross-species amplification of developed primers in eight *G. pulex* C and six *G. fossarum* specimens. The amplification success number (S) represents the number of individuals that were successfully amplified using the respective primer. The number of alleles (A) and size range of the amplified fragments are given for each species.

	G. pulex C			*G. fossarum*		
Locus	S	Size range	A	S	Size range	A
Gp10	8	162-176	3	0	/	/
Gp11	5	328-331	2	0	/	/
Gp13	7	88-94	3	0	/	/
Gp18	7	110-128	4	6	110-122	3
Gp28	8	271-350	4	0	/	/
Gp30	8	102-112	4	0	/	/
Gp31	8	128-148	3	6	145	1
Gp37	3	232-236	2	1	246-254	2
Gp42	8	378-384	2	5	391	1
Gp55	8	287-329	2	0	/	/
Gp68	2	92-124	2	6	96-104	2

a)



b)

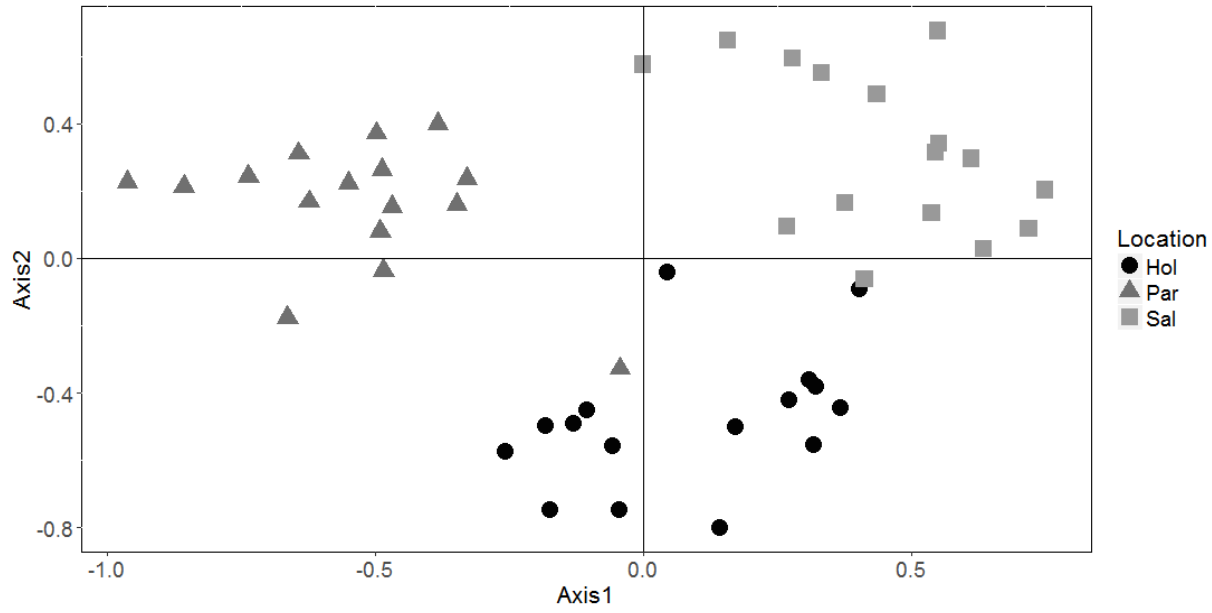


Figure 2.1 a) Neighbor joining tree of *G. pulex* from the Saale catchment and the reference sequences. The scale bar represents genetic distance between the specimens. b) PCoA plot of genotyped individuals sampled from three locations. The first two axes, which cover 19.41 and 15.02 percent of the whole variation respectively, are shown.

Acknowledgments

This work was supported by UFZ - Helmholtz Centre of Environmental Research (POF III-T32). We would like to thank firstly Dr. Alexander Weigand and Dr. Daniel Grabner from the University Duisburg-Essen for providing specimens of *G. pulex* from the Ruhr area, and secondly, the two anonymous reviewers for constructive comments, which contributed to the improvement of the manuscript.

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Chapter 3

Chemical pollution levels in a river explain site-specific sensitivities to micropollutants within a genetically homogeneous population of freshwater amphipods

Author's contribution statement 2

Declaration of author contributions to the publication:

Chemical pollution levels in a river explain site-specific sensitivities to micropollutants within a genetically homogeneous population of freshwater amphipods

Status: Published in Environmental Science & Technology.
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The contributions of the doctoral candidate and co-authors:

(1) Concept and design
Doctoral candidate: 40%
Co-authors 2, 5, 6: 50%
Co-authors 3, 4: 10%

(2) Conducting laboratory analysis
Doctoral candidate: 70% (field work, lab experiments, DNA amplification, sequencing, LC-MS analysis)
Co-author 2, 3, 5 and 6: 30% (helped with field work, supervised sample preparation, supported DNA analysis, supported LC-MS analysis)

(3) Compilation of data sets
Doctoral candidate: 80% (performing genetic, chemical, and toxicological analysis)
Other Co-authors: 20% (supervised and supported data evaluation and analysis)

(4) Data interpretation and presentation
Doctoral candidate: 70% (data interpretation and visualization)
Other co-author: 30% (support of data interpretation and visualization)

(5) Drafting of manuscript
Doctoral candidate: 70%
Other co-authors: 30%

I hereby certify that the information above is correct.

_____

Date and place Signature doctoral candidate

_____

Date and place Signature supervisor

3.1 Abstract

Anthropogenic micropollutants alter chemical and ecological conditions of freshwater ecosystems and impact aquatic species that live along the pollution gradient of a river. Species sensitivity to micropollutants depends on the site-specific exposure, however, it remains unclear to what degree this sensitivity relates to species' genetic structure. Here, we explored the relationship between toxic sensitivity and genetic structure of the amphipod species *Gammarus pulex* (Linnaeus, 1758) along an organic micropollutant gradient in the Holtemme River in central Germany. We determined the river's site-specific micropollutant patterns and analyzed the genetic structure of *G. pulex* using nuclear and mitochondrial genetic markers. Furthermore, we examined the exposure sensitivities and bioaccumulation of the commonly detected insecticide imidacloprid in *G. pulex* from different sites. Our results show that throughout the Holtemme River, *G. pulex* forms a well-connected and homogenous population with no observable pollution–related differences in genetic structure. However, *G. pulex* from polluted sites responded more sensitively to imidacloprid; survival times for half of the amphipods were up to 54% shorter, the percentage of immobile individuals increased up to 65%, and the modeled imidacloprid depuration rate was lower in comparison to amphipods from non-polluted sites. Altogether, these results suggest that the level of sensitivity of *G. pulex* amphipods to micropollutants in the river depends on the degree of pollution: amphipods may thrive in food-rich but polluted habitats, yet their sensitivity is increased when chronically exposed to organic micropollutants.

Keywords: *Gammarus pulex*, anthropogenic pollution, imidacloprid, LC-HRMS, population genetics, microsatellites, selection

Synopsis: *Gammarus pulex* amphipods from river sections with higher levels of organic pollution show increased sensitivity to the pesticide imidacloprid; the amphipods' sensitivity depends largely on the toxic pressure that they are exposed to in their habitat.

3.2 Introduction

Chemical water pollution, river regulation, and invasive species affect river ecosystem functioning and indigenous aquatic species (Grizzetti et al., 2017; Rohr et al., 2006; Vörösmarty et al., 2010). In particular organic micropollutants, bioactive compounds such as pesticides (Pimentel, 2009) and pharmaceuticals (Ginebreda et al., 2010) that are only partially eliminated by wastewater treatment plants (WWTP), are important, but often neglected stressors in rivers (Stamm et al. 2016). These pollutants have been shown to significantly contribute to a deteriorated chemical and ecological river status (Burdon et al. 2019; von der Ohe et al., 2009). Specifically, the type and degree of pollution was demonstrated to influence the aquatic species composition (Beketov et al., 2009; Goodnight 1973). Some species, such as the amphipod *Gammarus pulex* (Linnaeus, 1758), can nonetheless occur along pollution gradients in both pristine and polluted habitats of a river.

In rivers with different levels of pollution, the toxic sensitivity of *G. pulex* differs depending on the degree of pollution in the respective habitat (Khan et al., 2011; Russo et al., 2018; Shahid et al., 2018a; Stuhlbacher & Maltby, 1992). Differences in sensitivities to chemicals of up to three-fold were detected among amphipods from polluted and unpolluted sites (Becker & Liess, 2017; Russo et al., 2018; Shahid et al., 2018a). Such discrepancies in sensitivities may arise due to different mechanisms; sensitivity of amphipods at polluted sites can decrease due to genetic and physiological adjustment to pollution (i.e., adaptation and acclimation, respectively) or can increase due to impairment from chronic chemical exposure (Ghalambor et al., 2007).

Adaptation to pollution can occur as a result of co-acting mutagenic and selective effects of toxic pollutants in exposed populations (Hoffmann & Willi 2008). Mutations increase the rates of new alleles in such populations, while the selective pressure of micropollutants, such as pesticides, increases the frequency of resistant alleles due to higher survival and reproduction rates of the individuals with these alleles (Inostroza et al., 2016a; Reid et al., 2016; Theodorakis et al., 2006). Adaptation due to a mutation in a pyrethroid receptor resulting in reduced sensitivity to the pyrethroid insecticide was shown among genetic

lineages of an amphipod, *Hyallea azteca* (Saussure, 1858), living in polluted habitats (Weston et al., 2013). Environmental pollution can also cause changes in genetic diversity (Giridhar Athrey et al., 2007; Inostroza et al., 2016a; Ungherese et al., 2010). In naturally exposed populations of *Daphnia magna* Straus, 1820 that showed reduced sensitivity to the pesticide carbaryl, reduced allelic richness and observed heterozygosity were detected by neutral genetic markers (Coors et al., 2009). In addition, different sensitivities were shown for different cryptic genetic lineages of *Gammarus* amphipods (Feckler et al., 2012). Some of these lineages occur sympatrically in a river (Lagrue et al., 2014), yet it is unclear to which degree their sensitivities to toxins depend on site-specific pollution and lineage-related genetic differences.

Acclimation, a physiological, behavioral, or morphological response of amphipods to different pollution levels (Biagianti-Risbourg et al., 2013; Zhao & Newman 2006), can similarly to adaptation result in a reduced sensitivity against toxicants. Acclimation can occur within populations under stressful conditions if individuals are able to physiologically adjust to directional selection and still reproduce (Ghalambor et al., 2007). Acclimation is for example illustrated by a study, in which the parental generation (F0) of *Gammarus fossarum* Koch, 1836 amphipods that was acclimated to toxic conditions showed lower sensitivity to cadmium than the F2 generation that was continuously kept in cadmium-free conditions (Vigneron et al., 2019).

In addition to the above-mentioned mechanisms, external factors can also modify sensitivity of amphipods to micropollutants. Thus, sensitivity increased due to a rise of temperature in rivers (Russo et al., 2018), food shortage (Liess et al., 2001), and when exposure to micropollutants occurred in a certain sequence. The latter in particular, was found to increase sensitivity in *G. pulex* to chemical exposure under repeated exposures to two pesticides in a specific order (Ashauer et al., 2007). An explanation for this may be provided by a study finding a carry-over due to slow toxicodynamic recovery from diazinon exposure and an increased mortality under subsequent exposure to propiconazole compared to the sequential exposure in the reversed order (Ashauer et al., 2017).

Despite abundant information on toxic effects of organic micropollutants on *G. pulex*, it remains unclear how the pollution gradient in a river affects the genetic structure of *G. pulex* and how the genetic structure relates to the species' sensitivity to toxicant exposure. We therefore investigated two competing hypotheses: 1) the sensitivity of *G. pulex* to organic micropollutants in polluted river sections is reduced due to the site-specific genetic or physiological adjustment to exposure, i.e., adaptation and acclimation, respectively, and 2) micropollutants in the river increase the sensitivity of *G. pulex* from polluted sites.

We performed a study at the Holtemme River, serving as a landscape model for studies of the effects of anthropogenic pollution on riverine ecosystem functioning (Beckers et al., 2018; Brase et al., 2018; Inostroza et al., 2016a; Karrasch et al., 2019). We analyzed the widespread Palearctic amphipod species *G. pulex*, which occurs in rivers with different degrees of pollution (Foucreau et al., 2013; Graça, 2001; Maltby et al., 2002). It is common in the Holtemme River, where two distinct populations were described in the past (Inostroza et al., 2016a). To test our hypotheses, we 1) determined the degree of organic micropollution pressure on *G. pulex* along the river using a toxic unit scale and 2) compared it to the genetic structure of *G. pulex* in the river. In laboratory exposures, we 3) determined the sensitivities to toxic chemicals of *G. pulex* sampled along the pollution gradient employing the common insecticide imidacloprid, and 4) measured imidacloprid tissue levels in exposed amphipods from different sites to determine if differences in sensitivity can be related to imidacloprid uptake and depuration rates.

3.3 Materials and methods

3.3.1 Sample collection

Samples were taken at eight locations (H1–H8) along a 47 km stretch of the Holtemme River (mean annual discharge: 1.34 m^3s^{-1}; Wollschläger et al., 2017) in Saxony-Anhalt (Germany) (Fig. SB1). The river comprises a micropollutant gradient; the water from the spring in the Harz National Park starts off as a pristine mountainous headwater that becomes increasingly polluted by WWTP effluents and runoffs from agricultural land and urban areas of the towns of Wernigerode and Halberstadt (Beckers et al., 2018; Inostroza

et al., 2016a). Reference samples were collected near the spring of the Parthe River (Saxony, Germany).

At each site, up to 100 *G. pulex* amphipods were collected with a Surber sampler (0.5 mm mesh size) from at least five spots across the entire river width. For DNA analysis, amphipods were stored in absolute ethanol. Amphipods for chemical analysis were rinsed with distilled water and frozen at -20°C until analysis. Concurrently, a water grab sample consisting of 1 mL river water was collected at each site from 10 cm water depth with a sterile pipette and frozen at -20°C until analysis. For detailed information on sampling locations refer to Tab. SB1 in the Supplementary information (SI).

3.3.2 Chemical analysis

Pooled *G. pulex* individuals (900 mg) from each site were extracted with the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method according to Inostroza et al. (2016b). An extract from each site was analyzed by liquid chromatography-high resolution mass-spectrometry (LC-HRMS, Thermo Ultimate 3000 HPLC system coupled to a Thermo QExactive Plus quadrupole-orbitrap instrument). Water samples were analyzed using the same instrument. Details on sample preparation and instrument settings with a target screening method are included in section SB1 in the SI. Subsequently, the levels of organic compounds of anthropogenic origin, comprising pesticides, pharmaceuticals, household and industrial chemicals with a wide range of hydrophobicity known to occur in the Holtemme River (Beckers et al., 2018; Inostroza et al., 2016a) were determined (Tab. SB2).

3.3.3 Micropollutant toxic effect estimation

The toxic capacities of the analyzed pollutants in *G. pulex* tissue were estimated based on the respective toxic units (TUs). According to the finding that chemical levels of several orders of magnitude below EC_{50} values affect freshwater macroinvertebrate communities, log TU values equal or higher than -3 were taken to indicate pollutant levels causing

adverse effects as suggested by Schäfer et al. (2012). From the measured tissue concentrations, the freely dissolved fraction (C^f) of each compound *i* was estimated according to equilibrium partitioning theory:

$$C_i^{fd} = \frac{C_i^{tG}}{f_{LIPID} K_{OW}}$$

where C^{tG} is the total measured concentration [ng/g of wet tissue] in *G. pulex*, f_{LIPID} the lipid fraction value (1.34% of the total body mass; Ashauer et al., 2010), and K_{ow} is the n-octanol-water partition coefficient. The freely-dissolved concentrations of neonicotionoids calculated by this equation with K_{ow} values predicted by JChem deviated by more than two orders of magnitudes from the measured tissue concentrations (based on Fig. 3.4 and literature data) (Ashauer et al., 2010; Englert et al., 2017). Therefore, instead of using K_{ow}, we calculated the partitioning ratio as the ratio between the tissue and water equilibrium concentrations measured in the uptake experiments for imidacloprid (Fig. 3.4) and with data from another publication (thiacloprid) (Foucreau et al., 2013). Reference standard toxicity data (LC_{50}) were retrieved from the EPA ecotoxicology database (https://www.epa.gov/chemical-research/ecotoxicology-database). If LC_{50} data were not available for *G. pulex*, data for *Daphnia magna* were used. The TUs for each compound with available LC_{50} (Tab. SB5) were summed up in order to predict an additive effect of all compounds at each site (Warne & Hawker, 1995):

$$log \sum TU = log \sum \left(\frac{C_i^{fd}}{LC_{50,i}} \right)$$

3.3.4 DNA extraction, sequencing, and genotyping

Genomic DNA was extracted from 140 *G. pulex* individuals from differently polluted sites (H1, H3–H8) using the Qiagen DNeasy Blood & Tissue kit. To avoid contamination by endoparasites, common in the gut of freshwater amphipods, only appendages (pereopods) were used. After DNA quality check using gel electrophoresis and a nanodrop spectrophotometer, a fragment of the mitochondrial COI gene was amplified for

twenty samples per site. For details on PCR conditions and primer selection refer to the section SB2 and Tab. SB7.

For microsatellite analysis, 17 markers (Gergs et al., 2010; Švara et al., 2019; Westram et al., 2010) (Tab. SB8) were amplified from 80 DNA samples mainly belonging to polluted and non-polluted sites analyzed in the exposure experiments (H1, H3, H4, H6). The amplification was done according to the protocol described in Švara et al. (2019) and Schuelke (2000). Allele sizes were determined using an ABI Prism 3130XL Genetic Analyzer.

3.3.5 Genetic variation analysis

The genetic variation of *G. pulex* from the Holtemme River was investigated with two methods, comprising protein-coding mitochondrial COI sequence analysis and analysis of non-coding microsatellite nuclear loci. With the two methods, cryptic diversity at the species (COI) and population (microsatellites) levels can be examined. The sequenced COI fragments were assembled and aligned with sequences of *G. pulex* from other European rivers acquired from the National Center for Biotechnology Information (NCBI) and compared for their phylogenetic relation and genetic distances by the maximum likelihood analysis in MEGA7 (Kumar et al., 2008). Genetic differentiation was analyzed by pairwise fixation index (F_{st}) comparison in Arlequin 3.5. (Excoffier & Lischer, 2010). For microsatellite loci, diversity parameters and diversification between amphipods from different locations were estimated in Fstat 2.9.3.2 (Goudet, 1995) and Arlequin 3.5. The population genetic structure in the river was determined in Structure 2.3.4 (Raj et al., 2014) and the effective population sizes were estimated in NeEstimator 2.0.2 (Do et al., 2014). Analyses and visualization of the genetic data are described in detail in section SB3 in the SI.

3.3.6 Imidacloprid toxicity experiment

Gammarus pulex from three sampling locations (H1, H4, H6) were exposed to imidacloprid (≥98% Purity, CAS-No. 138261-41-3, Sigma-Aldrich) at 130 µg/L (0.025% DMSO) and 270 µg/L (0.05% DMSO), along with medium and solvent controls (0.05% DMSO) for 14 d. Exposures were set up in 1 L glass beakers in a volume of 500 mL Aachner Daphnien Medium (ADaM) (Klüttgen et al., 1994) as an exposure medium. For further details on the experimental set-up refer to section SB4 in the SI.

During the experiment, the beakers were checked for dead/immobile amphipods (lethal/sub-lethal effect) at least every twelve hours. Amphipods were classified as dead when no movement of extremities was observed and as immobile when repeated contacts with a glass rod did not stimulate movement although pleopod motion indicated that amphipods were alive. As a measure of sensitivity, the time until mortality reached 50% (LT_{50}) in each treatment was quantified with the non-linear Hill model (see S5) (Heidel & Maloney, 1999) and compared using the 95% confidence intervals. For comparison of immobility data from different treatments and samplings sites, the Kruskal-Wallis rank sum test was applied as normal distribution of data was not assumed. Data analysis was done in GraphPad Prism version 5.01 and in R (Team, 2016).

3.3.7 Imidacloprid uptake and depuration kinetics

To determine the kinetics of imidacloprid bioaccumulation and depuration in *G. pulex* tissue, *G. pulex* from the locations H2 (non-polluted) and H6 (polluted) were exposed to imidacloprid as described in sections 2.6 and SB4. Exposures were performed at 25 µg/L (≙ $1/10^{th}$ of LC_{50}) for seven days (uptake period) and subsequently in uncontaminated ADaM for four days (depuration period). Control amphipods were kept in ADaM with 0.05% DMSO for seven days and afterwards in uncontaminated ADaM for four days. Amphipods were sampled at 17 time points. Four to six amphipods with a total tissue mass of 150 mg were pooled and immediately frozen at -20°C. After QuEChERS (Inostroza et al., 2016b) extractions, imidacloprid concentrations in the tissue were measured using LC-HRMS (see section SB1).

Uptake data were fitted with the one phase association model, using the least squares method. Initial internal concentration C_0 was set to zero with the accumulation rate constant K, time t, and maximal saturation estimated with the model. Depuration data were fitted with the one phase decay model, using the least squares fitting method. To compare the accumulation and depuration efficiency, the models were compared using an extra sum-of-squares F-test. Modelling was performed with GraphPad Prism version 5.01.

3.4 Results

3.4.1 Organic micropollutants in the Holtemme River

3.4.1.1 Micropollutants in water samples

The number and amount of identified micropollutants was strongly related to the presence of WWTP effluent (Fig. 3.1a, Tab. SB4). Out of 60 screened organic compounds, four were found in the water samples from site H3 upstream of WWTP1 and 32 in water samples from sites H4–H8, downstream of WWTP1. The concentrations of the analyzed compounds were, in comparison to the upstream site, higher downstream of WWTP1 (Fig. 3.1a). From the analyzed compounds, 7-diethylamino-4-methylcoumarin showed the highest concentrations, between 873–1785 ng/L, at sites H4–H8. The effluent of WWTP1 is the source of this fluorescent dye (Muschket et al., 2018). The corrosion inhibitors 1H-benzotriazole and 5-methyl-1H-benzotriazole and the diuretic hydrochlorothiazide showed relatively high concentrations in the samples from locations downstream of WWTP1 (H4–H8) with 350–734 ng/L, 204–486 ng/L, and 268–511 ng/L, respectively.

3.4.1.2 Micropollutants in *Gammarus pulex* tissue samples

The WWTP effluents significantly contributed to the amount and abundance of micropollutants in the *G. pulex* tissue samples, as in total 10 compounds were detected in *G. pulex* samples from upstream (sites H1, H3) and 28 from downstream of WWTP1

(sites H4–H8) (Tab. SB3). The micropollutant concentrations detected in tissue samples collected downstream were up to 200 times higher than in the samples collected from site H1 (Fig. 3.1b). Among the detected compounds in the tissue extracts the industrial compound 7-diethylamino-4-methylcoumarin at 21–67 ng/g wet tissue in samples from downstream of WWTPs, was most abundant. It was followed by the transformation product 7-amino-4-methylcoumarin, the antidepressant citalopram at 4.2–9.6 ng/g and the rubber additive transformation product 2-benzothiazolesulfonic acid at 2.8–7.7 all at sites H4–H8.

With their high toxic potential for *G. pulex*, identified insecticides were of special interest. The neonicotinoid insecticide imidacloprid was detected in the amphipod tissue samples from the sites downstream of WWTPs (2.4–4.3 ng/g at sites H4–H8) (Fig. 3.1b). The second detected neonicotinoid, thiacloprid, was found also upstream of WWTP1 (0.21–0.35 ng/g at sites H1 and H3), but the concentrations were higher downstream of WWTP1, reaching 1.2 ng/g at the site H8 (0.64–1.2 ng/g at sites H4–H8). Fipronil was detected downstream of WWTP2 at sites H6 and H7 (0.64 and 0.12 ng/g, respectively). Pesticide tissue concentrations were the highest in the samples from H8, the last location before the confluence with the Bode River.

3.4.1.3 Toxic unit values

The amounts of the detected compounds at each site are reflected by TUs. For 14 compounds detected in *G. pulex* tissue, toxicity data were available in the EPA ecotoxicology database (Tab. SB5). The sum of TUs in samples from all locations downstream of WWTP1 exceeded 10^{-2}, while at locations H1 and H3 TUs were below 10^{-3} (Fig. 3.1c, Tab. SB6). In the samples from sites H4–H8, cumulated TUs amounted to > 10^{-3} with imidacloprid as the major contributor to these TUs (> 10^{-2} TUs). Additionally, the corrosion inhibitors 1H-benzotriazol and 5-methyl-1H-benzotriazole, the neonicotinoid insecticide thiacloprid, and the pharmaceuticals verapamil, metoprolol, and propranolol, each with up to 10^{-4} TUs, contributed substantially to the sum of TUs.

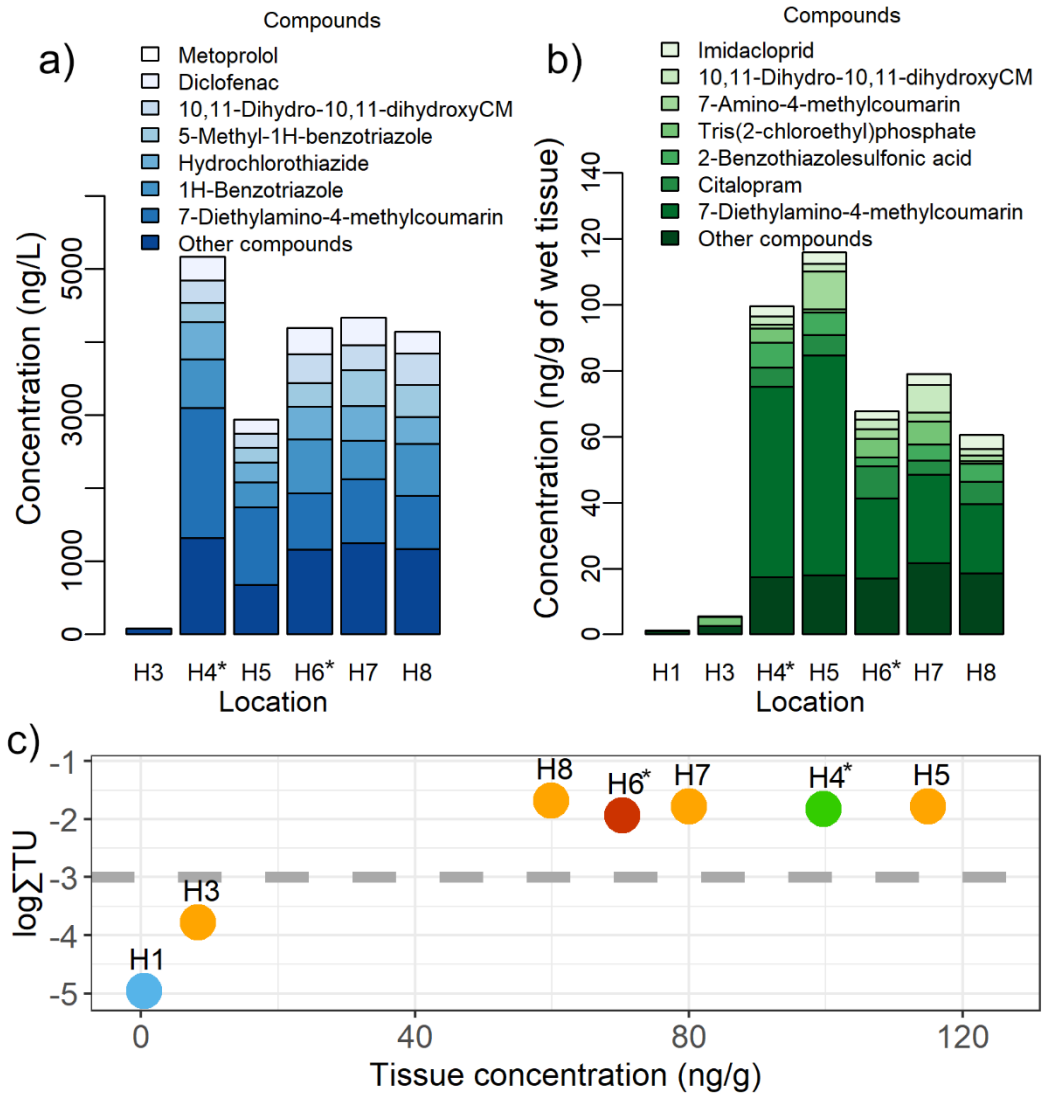

Figure 3.1 Organic micropollutant levels in the Holtemme River. (a) Detected concentrations of the seven most prevalent compounds in each water sample from the Holtemme River. (b) Detected concentrations of the seven most prevalent compounds in the *G. pulex* tissue extracts. (c) Sum of toxic units (TUs) for each sampling site based on the calculated TUs for all compounds detected in *G. pulex* tissue samples. The colors of the circles representing sites H1, H4, and H6 correspond to the colors in figures 3.3 and 3.4. The dashed line at 10^{-3} TUs marks the threshold for expected adverse effects; at TUs > 10^{-3} adverse effects are expected to occur. Asterisks denote the locations directly downstream of WWTPs.

3.4.2 Population genetic analysis

3.4.2.1 COI sequences comparison

Comparisons of 658 base pair COI sequences of 127 *G. pulex* amphipods from seven locations in the Holtemme River and of twelve *G. pulex* amphipods from the reference river Parthe revealed a significant variation across sequences. Fifteen variable nucleotide sites were identified in the sequences of amphipods from the Holtemme River and an additional variable site in the amphipods from the reference group. The sequences from the Holtemme River comprised 16 distinct haplotypes, of which nine were represented by more than a single specimen. The three most common haplotypes gpcoi1, gpcoi2, and gpcoi3, were found among 39.4%, 19.7%, and 16.5% of the amphipods, respectively. Sequences were most diverse at location H6 with eight and least diverse at location H1 with four different haplotypes. Site-specific haplotypes were identified at sites H4 and H6, while no site-specific haplotypes were found at H1 and H8.

The population genetics structure of *G. pulex* from the Holtemme River based on the COI analysis was not pollution-related. All of the most common haplotypes are present in the samples from polluted as well as non-polluted locations, with only a few location-specific haplotypes (Fig. 3.2). *Gammarus pulex* from the Parthe River belonged to one distinct haplotype characterized by a single different base, and a reference sequence for *G. pulex* E from the Brandenburg region by the difference of two bases. The fixation index for COI sequences across all nucleotides within the Holtemme River was 0.012, suggesting low genetic structuring. Pairwise F_{st} values were mostly lower than 0.05 and not significant (Tab. SB10). Two significant values between locations H3:H6 and H4:H6 were detected with fixation indices 0.10 and 0.07, respectively, explaining the low diversification. On the phylogenetic tree (Fig. SB2), a cluster of samples belonging to *G. pulex* lineage E *sensu* Grabner et al. (Grabner et al., 2015; Švara et al., 2019) from the Holtemme River, Parthe River, and from the Brandenburg region (G_pulex_E) can be recognized consistently, without supported structure within this cluster. Phylogenetic comparison also showed small genetic distances of less than 0.003 among all *G. pulex* samples from the Holtemme River (Tab. SB9). The distances to the samples from the Parthe River and Brandenburg

reference sequences, which are also spatially closest to the Holtemme River, were all below 0.003. Genetic distances to other *G. pulex* lineage C and D were 29 and 40 times higher, respectively.

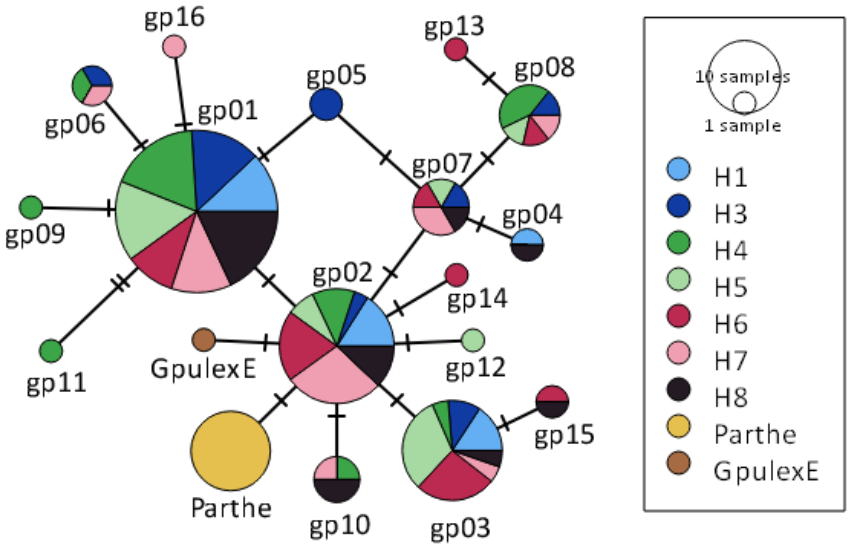

Figure 3.2 Minimum spanning network of the analyzed COI sequences of *Gammarus pulex* belonging to the clade E from seven sampling locations at the Holtemme River (H1 and H3–H8) and two reference locations Parthe and Brandenburg (GpulexE) in different colors. Each pie chart represents a different haplotype. Their sizes represent the number of samples detected for each haplotype. Hatch marks between the pie charts represent a single nucleotide change.

3.4.2.2 Microsatellite analysis

Similar to COI sequence analysis, large microsatellite variability with no pollution-related structure was detected among the Holtemme River samples. In total, 75 alleles were found with allele variability of 54–59 alleles across 17 microsatellite loci in amphipods from each of the four analyzed locations (Tab. 3.1). From one to nine alleles per microsatellite locus were found in total (Tab. SB8) with nine alleles detected for loci gp10 and gp28, eight for gp37, and only a single allele for locus Gapu-9 as all pairs of loci were unlinked. A higher

number of private alleles was observed in amphipods at sites with higher allelic richness, with no significant differences in expected and observed heterozygosities across all loci. Null alleles were detected for four loci, namely g8, g9, gp11, gp37, at frequency rates of 0.06, 0.02, 0.08, and 0.36, respectively. The highest effective population size (∞) was detected at site H6 and the lowest (87.3) at site H1. No structural divergence within the sampled amphipods was detected as the likelihood values estimated in Structure Harvester suggest a single population based on the K value (Fig. SB3). Pairwise F_{st} comparison of different locations did not confirm significant COI structuring results, but showed a weak (F_{st} = 0.017), yet significant difference between H1 and H3 (Fig. SB11). A slightly increased inbreeding rate was detected at H6 (Tab. 3.1).

Table 3.1 Microsatellite diversity indices including the total number of detected alleles (N), allelic richness per all loci (AR), detected number of private alleles per all loci (N_{pa}), observed (H_o) and expected (H_e) heterozygosity, inbreeding coefficient (F_{is}), and effective population size (N_e).

Location	N	AR	N_{pa}	H_o	H_e	F_{is}	N_e
H1	59	2.83	0.26	0.38	0.41	0.03 (-0.05–0.10)	87.3 (27.4–∞)
H3	54	2.58	0.19	0.40	0.42	0.00 (-0.09–0.10)	∞ (55.3–∞)
H4	57	2.65	0.23	0.40	0.43	0.03 (-0.01–0.14)	∞ (45.2–∞)
H6	59	2.86	0.37	0.42	0.49	0.08 (-0.03–0.18)	∞ (149.7–∞)

3.4.3 Imidacloprid toxicity experiments

The laboratory exposure experiments with different imidacloprid concentrations indicated site-specific differences in sensitivities across *G. pulex* from the Holtemme River. The initial mortalities occurred simultaneously at 4 h in amphipods from sites H1, H4, and H6 in both the 130 µg/L and 270 µg/L treatments. The mortality rates at the end of the experiment reached 46% (H1) and 56% (H4, H6) in the 130 µg/L imidacloprid treatment and 66% (H1), 78% (H4), and 68% (H6) in the 270 µg/L imidacloprid treatment. In the

treatment with 130 µg/L imidacloprid, LT_{50} values were reached at 184 (164.5–205.8) h (H6), 269.1 (234.9–308.2) h (H4), and 501.7 (304.1–824.8) h (H1), while LT_{50} values in the 270 µg/L imidacloprid treatment were reached earlier, i.e., after 102.2 (92.2–113.3) h (H6), 146.9 (130.3–165.5) h (H4) and 187.3 (169.4–207.1) h (H1) (Fig. 3.3a). The confidence intervals of LT_{50} values did not overlap between H1–H6 and H4–H6 in the low concentration treatments and between H1–H4, H1–H6, and H4–H6 in the high concentration treatments. The LT_{50} differences between polluted and non-polluted sites were at 41.4 and 85.1 h (22%–45%) in the high concentration treatments and at more than 232 h (54%) in the low concentration treatments. In controls/solvent controls, mortalities first occurred after 82 h/92 h (H1), 56 h/32 h (H4) and 68 h/82 h (H6). They reached 9%/8% (H1) and 12%/14% (H4, H6) by the end of the experiment (Fig. SB4).

For immobility rates, indicating sub-lethal effects of imidacloprid that amphipods can recover from (Nyman et al., 2013), significant differences were observed in *G. pulex* from polluted (H4, H6) and non-polluted locations (H1) (Tab. SB12). In contrast to controls, in which all amphipods were mobile throughout the experiment (Fig. SB4), increased immobility was observed in all treatments at the first observation time point (4 h) (Fig. 3.3b). On average, 35–60% and 77–96% of amphipods were immobile in 130 µg/L and in 270 µg/L imidacloprid treatments, respectively. Twice as many amphipods were immobile in treatments from polluted locations (H4, H6) compared to the non-polluted site H1. By the end of the experiment the percentages of immobile amphipods decreased to 43% in H4 and to 20% in H1 and H6 in the lower concentration treatments and to 77% in H4, 48% in H6, and 32% in H1 in the higher concentration treatments.

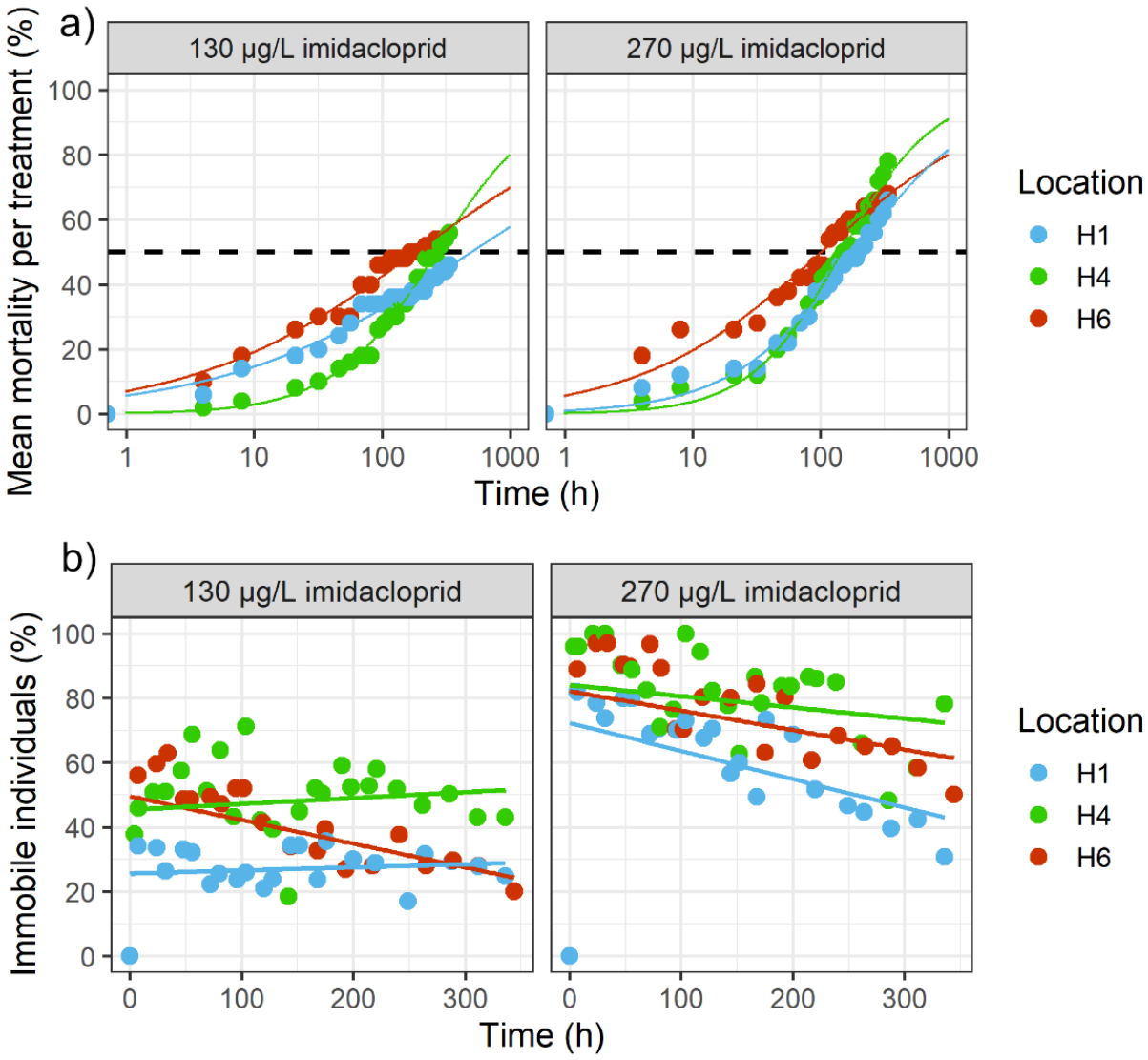

Figure 3.3 Toxic effects of imidacloprid on *Gammarus pulex* from different sampling locations. (a) Mean mortalities of amphipods from locations H1, H4 and H6 in 130 µg/L and 270 µg/L imidacloprid treatments in exposures over 14 d (336 h). Each dot marks the number of dead amphipods per beaker in %. 50 individuals (10 individuals in each of 5 replicates) correspond to 100%. Regressions were calculated with the Hill equation (SB5). The dotted line marks 50% mortality. (b) Percentages of immobile amphipods in 130 µg/L and 270 µg/L imidacloprid treatments over 14 d (336 h) of exposure. Lines were fitted to the data for each sampling location using linear regression.

3.4.4 Uptake and depuration

Upon exposure to imidacloprid, the tissue concentrations of imidacloprid in *G. pulex* from polluted (H6) and non-polluted (H2) locations indicated similar uptake kinetics. After 48 h of exposure, the mean tissue concentration in amphipods from sites H2 and H6 reached equilibrium at 225 ng/g and 228 ng/g wet weight tissue, respectively (Fig. 3.4). Afterwards, tissue concentrations varied between 200.5 ng/g and 261.9 ng/g, and between 182.6 ng/g and 258.7 ng/g in amphipods from H2 and H6, respectively. The imidacloprid uptake rates of amphipods from different locations were similar (0.125 and 0.091 in *G. pulex* from H2 and H6, respectively; $p = 0.605$).

Parameter estimates from the depuration models for *G. pulex* from polluted and non-polluted locations differed significantly ($p = 0.016$), with depuration rate constants of 0.166 (H2) and 0.046 (H6). Imidacloprid tissue concentrations reached equilibrium in the amphipods from location H6 already after 34 h at 126 ng/g; thereafter, no further changes in tissue concentrations were seen (Fig. 3.4). In contrast, imidacloprid tissue concentrations in amphipods from H2 did not reach equilibrium by the end of the experiment with imidacloprid tissue concentrations at 79 ng/g. The amphipods from the controls showed constant concentrations from the start of the experiment, with 8.2 ng/g and 0 ng/g imidacloprid detected in the samples from H6 and H2, respectively.

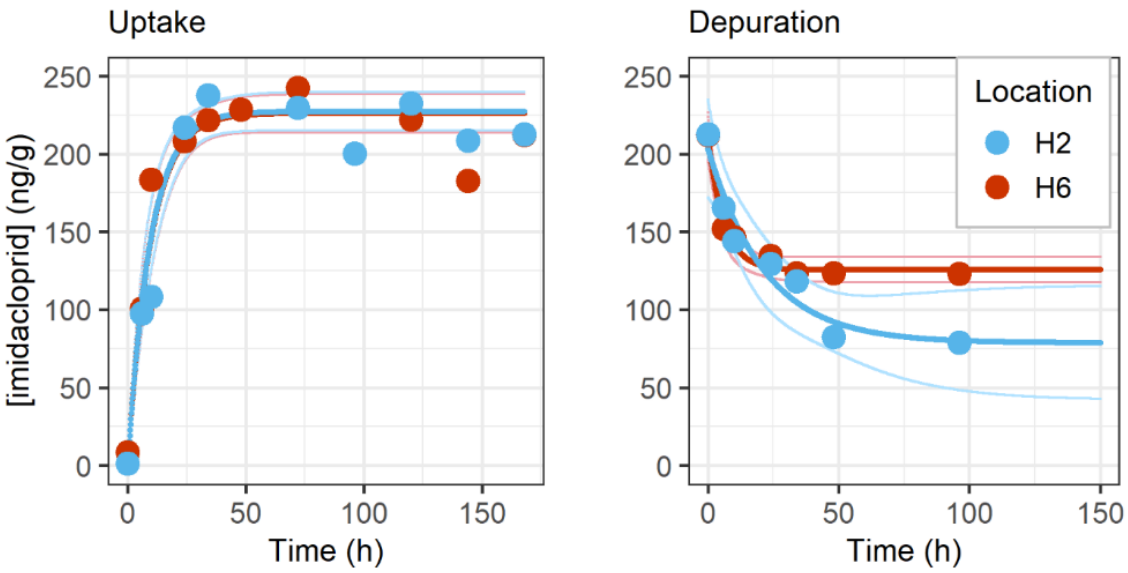

Figure 3.4 Uptake and depuration kinetics of imidacloprid in tissue of amphipods sampled at locations with low (H2) and high (H6) levels of organic pollutants. Regressions were modeled with a one phase association (uptake) and a one phase decay (depuration) model. The lighter blue and red lines denote confidence intervals of the models (95%).

3.5 Discussion

We addressed the question whether sensitivities of *G. pulex* to pollution stress in a river with different levels of pollution differ due to acclimation or adaptation or due to an impaired organisms' condition as a consequence of chronic exposure to toxicants. Our data indicate that differences in sensitivities of *G. pulex* to imidacloprid exposure along the Holtemme River rather originate from local exposure to toxic anthropogenic micropollutants than from adaptive adjustment at differently polluted sites, as the *G. pulex* population in the Holtemme River was found to be genetically homogenous.

3.5.1 Toxic potential of anthropogenic micropollutants in the Holtemme River

Detected organic micropollutant concentration levels in the Holtemme River samples are in a range similar to the levels reported for various European rivers (Beckers et al., 2018; Inostroza et al., 2016a; Inostroza et al., 2016b; Miller et al., 2015; Munz et al., 2018).

Micropollutant analysis from up- and downstream of WWTP1 indicated that this WWTP is a significant source of pesticides, pharmaceuticals, and other organic micropollutants. Of the detected compounds, insecticides with their comparatively larger TU values show a particularly high adverse potential for *G. pulex*. Tissue concentrations of imidacloprid in amphipods sampled downstream of WWTP1 were above 4 ng/g (Tab. SB3). Based on equilibrium partitioning, this concentration corresponds to a water concentration of 0.4 ng/mL. This is in the range of imidacloprid concentrations measured in other European rivers (Iancu et al., 2019; Masiá et al., 2013) that were found to affect the feeding behavior of *G. pulex* (0.81 ng/mL) (Agatz et al., 2014). Thus, imidacloprid in the Holtemme River water, in the presence of other adverse factors (Liess et al., 2001), may be a major contributor to sub-lethal effects (i.e., reduced feeding rates). *Gammarus pulex* individuals sampled downstream of WWTP1 were therefore predisposed by exposure to neonicotinoids and potential sub-lethal effects, which may already exert a selective pressure at these sites in the Holtemme river (Shahid et al., 2018b).

Notably, toxicity data for only a few compounds were available for *G. pulex* and therefore toxicity data for *D. magna* were used. Although toxicities to *G. pulex* and *D. magna* correlate for most compounds (Ashauer et al., 2011), toxicity estimations for further compounds for this species would be extremely valuable for more precise assessments of the impacts of chemicals in the environment of this species. Likewise, we want to emphasize the importance of examining the micropollutant levels in the tissue of riverine organisms in addition to water grab samples, as certain toxic compounds, such as imidacloprid, were found in tissue only but not in water samples. Thus, comprehensive information on the present micropollutants can only be obtained by looking at both matrixes, as it enables a more precise toxicity assessment (De Lange et al., 2006).

3.5.2 River pollution patterns and *Gammarus pulex* population structure are not linked

Although there is evidence for the presence of a selective pressure in the river, our genetic data on population diversity and structure indicate the absence of genetic differentiation

of *G. pulex* populations in relation to pollution. This is consistent with preceding studies on *G. pulex* population structure, which suggest that amphipods from one river mostly belong to one genetically homogeneous population within a clade, but at a regional scale, i.e. between different rivers, a complex population structure with distinct populations often exists (Lagrue et al., 2014; Švara et al., 2019; Weiss & Leese, 2016). Surprisingly, in contrast to our observations and the aforementioned studies, two populations and increased rates of private alleles for *G. pulex* in the Holtemme river due to anthropogenic pollution of the river were demonstrated in a previous study (Inostroza et al., 2016a). As pollution conditions in the river were comparable between the two studies, pollution seems not to be the cause for the observed differences. Different sets of microsatellites used in the two studies are a likely explanation for differing results. For this study we selected a robust microsatellite set and avoided primers with many stuttering peaks used in the previous study (e.g. Gam 2, Gam 4), as suggested by Weiss and Leese (2016) (see also Švara et al., 2019).

The homogenous genetic structure of *G. pulex* in the Holtemme River is shaped by different factors. Firstly, migration from the upstream sites with low pollution pressure to sites with higher pollution pressure (Inostroza et al., 2016a) most likely prevents major shift of allele frequencies in the polluted river section. Although slightly inbred, amphipods living downstream of the WWTP effluents did not show drastic reduction of the effective population size and allelic richness, the two parameters are often observed in populations under selection due to toxic exposure (Hoffmann & Willi, 2008). Secondly, in comparison to a low *G. pulex* abundance and effective population size found at upstream sites H1 and H2, high abundances in the polluted river sections and large effective population sizes directly after WWTPs can be maintained due to abundant food supply (fungi, biofilms) resulting from the input of anthropogenic nitrate and organic carbon, that enter the river through WWTP effluents and agricultural field drainage (Brase et al., 2018; Karrasch et al., 2019). Additionally, the number of private alleles does not show any dramatic increase downstream of the WWTPs in our study. Thus, slightly increased allelic richness values downstream of WWTPs are probably due to a larger allele pool in lower reaches because of migration to the river. Within rivers comparable to the Holtemme River, connectivity, migration, and historic colonization have been argued to often determine population

genetic composition of *Gammarus* amphipods, rather than pollution (Weiss & Leese, 2016).

3.5.3 *In situ* exposure to anthropogenic pollution results in an increased sensitivity of *G. pulex*

As *G. pulex* from the Holtemme River form a single population, differences in molecular targets originating from adaptation in amphipods from the different sites are an unlikely reason for differential sensitivities of amphipods from different sites against exposure to imidacloprid. The highest detected difference in survival time between amphipods from two sites in the Holtemme River was 54%, which is partially in line with the findings of Weston et al. (2013) who found differences in sensitivities of amphipods within the same clade and location to be smaller than one-fold (Weston et al., 2013). Larger sensitivity differences between genetically divergent populations are associated with respective mutations or shifted allele frequencies, which could also be expected in *G. pulex*, but only on a regional scale, where several populations or even cryptic species are present (Feckler et al., 2012; Lagrue et al., 2014; Švara et al., 2019).

Given the genetic homogeneity of *G. pulex* across the Holtemme River, we can assume that the physiological states of amphipods were different between upstream and downstream sampled individuals. The amphipods used for the experiments here were lab-acclimated for seven days, which is a period commonly used in comparable studies (1–7 d) (Feckler et al., 2012; Russo et al., 2018; Weston et al., 2013). It proves sufficient to harmonize in situ physiological state differences of the amphipods from the different sites due to factors such as temperature (refer to Tab. SB1), food availability, and competition. Yet, this time period may not have been sufficient for recovery of amphipods from toxic micropollutants accumulated in the tissue, as many compounds persist in *G. pulex* tissue for weeks (e.g. imidacloprid) (Ashauer et al., 2010). In the elimination experiment, imidacloprid tissue levels decreased by about 50% within two to three days, however, tissue levels then remained stable and did not show any significant decrease until the end of the experiment (Fig. 3.4). It is conceivable that imidacloprid, together with other

micropollutants (e.g. thiacloprid) taken up by the amphipods at sites H4 and H6, enhance such chronic toxic burden, that could in the exposure experiment be reflected in higher immobility or mortality rates in the initial phase of the exposure to imidacloprid. Thus, the reduced capacity to eliminate imidacloprid accumulated in the tissue by amphipods from polluted sites, in addition to the effects of sequential exposure (Ashauer et al., 2007) and differences in damage recovery of closely related amphipods (Ashauer et al., 2015), may explain the finding of higher sensitivity of amphipods from polluted sites against imidacloprid exposure.

3.5.4 Ecological implications

Our data show that within a genetically homogeneous *G. pulex* population site-specific differences in sensitivities to anthropogenic micropollutant exposure can occur. These sensitivities are related to the site-specific pollution conditions. Sensitivity of amphipods to micropollutants is enhanced when amphipods are chronically exposed to toxic compounds in their natural habitat, as these compounds accumulate in the tissue. However, although more vulnerable from exposure to anthropogenic micropollutants, *G. pulex* exposed to toxic micropollutants benefit from the high abundance of food in the polluted but nutrient-rich habitats in the Holtemme River. Together with higher food abundance, other factors, such as habitat availability, higher temperatures, and favorable oxygen and pH conditions (Crane, 1994; Meijering, 1991), can contribute to higher growth rates (Sutcliffe et al., 1981) and increased abundance of *G. pulex* in these reaches compared to the more oligotrophic upstream habitats. In addition to favorable environmental parameters, large effective population size and high abundance of *G. pulex* can be facilitated by migration of genetically diverse *G. pulex* from non-polluted parts of the river. By contrast, predisposition of *G. pulex* in polluted river sections through exposure to micropollutants may lead to temporal phases of increased sensitivity due to seasonal pollution peaks. Such peak events may result in severe consequences for a *G. pulex* population in a stream, such as large fluctuations of population size (Schulz & Liess, 1999) and a reduced trophic transfer. After all, G. pulex has a key role as a shredder of organic debris and as food source for fish (Altermatt et al., 2019) Therefore, we would like to

emphasize the importance of information on the population genetic composition of the studied organisms in toxicological studies with organisms originating from habitats with different levels of pollution. As our study shows, toxic organic micropollutants did not select for a *G. pulex* genotype adapted to thrive in polluted habitats in the river, but lead to higher sensitivity against compound exposure in amphipods.

Supplementary information

LC-HRMS sample preparation and analysis; PCR conditions, sequencing and genotyping information; sequence and microsatellite data analyses; exposure experiments conditions; non-linear Hill model; haplotypes of COI sequences; map of the sampling sites; likelihood values from Structure Harvester analysis; maximum likelihood phylogenetic tree; graphs with control data; mortality data with standard errors; list of sampling sites; list of analyzed compounds; compounds detected in *G. pulex* tissue samples; compounds detected in water samples; standard toxicity test median acute effect concentration data; toxic units for compounds found in the *G. pulex*; primers used for COI sequencing; primers used for microsatellite amplification; pairwise genetic distances between COI sequences; pairwise F_{st} values for COI sequences; pairwise F_{st} values for microsatellite data; Kruskal-Wallis one-way test of mobility data.

Acknowledgements

We would like to thank Dr. Matthias Liess and Oliver Kaske from UFZ and Vera Dackermann, Lara Hermsen, and Jona Frederik Schulze, who took part at the ecotoxicology course at the UFZ in Leipzig in 2017, for their support of field sampling and laboratory experiments. Dr. Eberhard Küster, Anže Švara, and Elena Motivans are acknowledged for fruitful discussions during the study and comments on the manuscript. We also thank three anonymous reviewers for their constructive comments on the manuscript. An academic license for Marvin, JChem for Excel and InstantJChem was

provided by ChemAxon (Budapest, Hungary). The first author was partially supported by a fellowship by Deutsche Bundesstiftung Umwelt (DBU). The QExactive Plus LC-HRMS used is part of the infrastructure initiative CITEPro (Chemicals in the Terrestrial Environment Profiler) funded by the Helmholtz Association. This work is a part of the Integrated Project “Healthy Aquatic Ecosystems” at the Helmholtz Centre for Environmental Research - UFZ. The authors declare no conflicts of interest.

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Chapter 4

Reduced genetic diversity of freshwater amphipods in rivers with increased levels of anthropogenic organic micropollutants

Author's contribution statement 3

Declaration of author contributions to the publication:

Reduced genetic diversity of freshwater amphipods in rivers with increased levels of anthropogenic organic micropollutants

Status: Published in Evolutionary Applications

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The contributions of the doctoral candidate and co-authors:

(1) Concept and design
Doctoral candidate: 70%
Co-authors 2, 6 and 7: 30%

(2) Conducting laboratory analysis
Doctoral candidate: 60% (carried out field work, DNA extraction, analysis)
Co-authors 3, 5, and 7: 40% (helped with field work, sample preparation, and with DNA and LC-MS analysis)

(3) Compilation of data sets
Doctoral candidate: 65% (raw data evaluation, genotyping, LC-MS analysis, statistical modelling)
Co-authors 2, 3, 4, and 5: 35% (contribution to chemical data and genetic data evaluation, support of modelling)

(4) Data interpretation
Doctoral candidate: 70% (genotype data, LC-MS data, and toxicity data interpretation)
Other co-authors: 30% (genotype data, LC-MS data, and toxicity data synthesis)

(5) Drafting of manuscript
Doctoral candidate: 70%
Other co-authors: 30%

I hereby certify that the information above is correct.

_____

Date and place Signature doctoral candidate

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4.1 Abstract

Anthropogenic chemicals in freshwater environments contribute majorly to ecosystem degradation and biodiversity decline. In particular anthropogenic organic micropollutants (AOM), a diverse group of compounds, including pesticides, pharmaceuticals, and industrial chemicals, can significantly impact freshwater organisms. AOM were found to impact genetic diversity of freshwater species; however, to which degree AOM cause changes in population genetic structure and allelic richness of freshwater macroinvertebrates remains poorly understood. Here, the impact of AOM on genetic diversity of the common amphipod *Gammarus pulex* (Linnaeus, 1758) (clade E) was investigated on a regional scale. The site-specific AOM levels and their toxic potentials were determined in water and *G*. *pulex* tissue sample extracts for 34 sites along six rivers in central Germany impacted by wastewater effluents and agricultural run-off. Population genetic parameters were determined for *G*. *pulex* from the sampling sites by genotyping 16 microsatellite loci. Genetic differentiation among *G*. *pulex* from the studied rivers was found to be associated with geographic distance between sites and to differences in site-specific concentrations of AOM. The genetic diversity parameters of *G*. *pulex* were found to be related to the site-specific AOM levels. Allelic richness was significantly negatively correlated with levels of AOM in *G*. *pulex* tissue ($p < 0.003$) and was reduced by up to 22% at sites with increased levels of AOM, despite a positive relationship of allelic richness and the presence of waste-water effluent. In addition, the inbreeding coefficient of *G*. *pulex* from sites with toxic AOM levels was up to 2.5 times higher than that of *G*. *pulex* from more pristine sites. These results indicate that AOM levels commonly found in European rivers significantly contribute to changes in the genetic diversity of an ecologically relevant indicator species.

Running title: Micropollutants impact genetic diversity

Keywords: *Gammarus pulex*, anthropogenic pollution, population genetics, microsatellites, LC-HRMS, evolutionary toxicology

4.2 Introduction

Chemical pollution, river regulation, and the invasion of alien species degrade freshwater ecosystems worldwide (Grizzetti et al., 2017; Ormerod et al., 2010; Rohr et al., 2006; Vörösmarty et al., 2010). This degradation becomes evident by biodiversity declines on both local and global scales (Thieme et al., 2010), as a third of all freshwater species faces a high extinction risk (Collen et al., 2014). In particular, anthropogenic organic micropollutants (AOM) were recognized as one of the major drivers of the biodiversity declines in freshwaters (Liess & von der Ohe, 2005; Malaj et al., 2014; Münze et al., 2017). AOM include bioactive compounds, such as pesticides (Pimentel, 2009) and pharmaceuticals (Daughton & Ternes, 1999; Ginebreda et al., 2010) that mostly pass standard water treatment in wastewater treatment plants (WWTP) and are discharged into rivers and streams where they accumulate in the habitats of freshwater organisms and in their tissues (Stamm et al., 2016). AOM in the environment were shown to impact species distribution, freshwater community composition, and the species' ecological function (Burdon et al., 2019; Englert et al., 2017; Liess & von der Ohe, 2005; Peschke et al., 2014). The effects of AOM across different levels of the biological organization do not only affect species distribution and function but also drive the evolution of species by affecting intraspecific genetic diversity and traits in exposed populations (Bickham et al., 2000).

The effects of AOM on genetic diversity in exposed natural populations are diverse and often difficult to predict due to the immense diversity of AOM that enter freshwater ecosystems (Brown et al., 2009). Different AOM can modify the genetic diversity of species in direct and indirect ways. AOM directly affecting a species' gene pool comprise genotoxic and mutagenic chemicals with the potential to modify DNA integrity (Bickham, 2011). Such AOM, for instance antineoplastic agents and aromatic amines (Muz et al., 2017; Steger-Hartmann et al., 1997), can alter DNA replication and chromosome structure and can cause nucleotide substitutions, deletions, or duplications (Devaux et al., 2011; Lacaze et al., 2011; Theodorakis et al., 2001). The emergence of new genetic variants may increase the genetic diversity of species, but it can also lead to deleterious mutations causing reduced reproductive fitness (Bickham et al., 2000). In addition to direct genetic effects, AOM can cause changes in the genetic diversity of a species in an indirect way

by affecting species fitness. In exposed species, selective effects of AOM may promote specific genotypes by adverse short-term or sublethal long-term effects (Brown et al., 2009). Short-term effects, for example by pesticides, can result in high mortality rates that increase genetic drift (Coors et al., 2009; Coutellec et al., 2013) or select genotypes resistant to a direct specific toxic impact (Bell & Gonzalez, 2009). Long-term exposure to sublethal AOM levels can not only cause effects such as reduced mobility or feeding ability (Englert et al., 2017; Nyman et al., 2013), but also promote species traits associated with increased resistance to AOM.

Genetic shifts in species impacted by AOM can be associated with altered genetic diversity parameters, such as reduced allelic richness or an increased inbreeding rate (Brown et al., 2009). In populations adapted to exposure to toxic AOM, the overall allelic richness can be reduced with increased frequency of certain alleles associated with resistance to pesticides with specific modes of action (Bickham et al., 2000). For example, tolerance-related alleles were found to be prevalent in *Hyallela azteca* (Saussure, 1858) living in habitats contaminated with pyrethroid insecticides and rare in *H. azteca* from nonpolluted sites (Weston et al., 2013). In addition to adaptation-related alleles, genetic change of species living in environments with toxic contaminants is associated with reduced genetic diversity rates in genetic markers that are not necessarily related to specific adaptive change (Brown et al., 2009). For instance, the diversity of alleles was reduced in marine and terrestrial amphipods originating from sites with sediments polluted with polyaromatic hydrocarbons (PAHs) and heavy metals (Bach & Dahllöf, 2012; Ungherese et al., 2010).

Increased levels of AOM are not exclusively associated with the reduction of genetic diversity in natural environments. In very mobile species, changes in genetic diversity may be compensated by gene flow from sites without AOM (Lenormand, 2002). Furthermore, mutagenic AOM can significantly increase the genetic diversity of exposed populations (Eeva et al., 2006). For example, increased genetic diversity in redbreast sunfish, *Lepomis auritus* (Linnaeus, 1758), was associated with the presence of mutagenic chemicals from toxic paper mill effluents (Theodorakis et al., 2006). A species' genetic diversity can also be enhanced by the presence of AOM with different modes of action (Whitehead et al.,

2017). Thus, in contrast to, for example pesticides directly impairing physiological fitness of exposed species, endocrine disruptors can cause shifts in genetic inheritance, for example by altered gametogenesis (Alves da Silva et al., 2018; Coulaud et al., 2015; Xuereb et al., 2011).

Despite multiple evidences for changes in the genetic diversity of species exposed to environmental pollutants, such as mutagenic chemicals, heavy metals, or PAHs (Bach & Dahllöf, 2012; Theodorakis et al., 2006; Ungherese et al., 2010; Weigand et al., 2018), data on genetic changes in freshwater macroinvertebrates exposed to AOM in rivers are scarce. In most cases, studies on AOM's impact on the genetic diversity of freshwater macroinvertebrates investigated alterations at the local scale (Inostroza et al., 2016a; Inostroza et al., 2016b; Švara et al., 2021). In a recent study investigating the frequencies of specific alleles to reveal the genetic structure of *Gammarus pulex* (Linnaeus, 1758) sampled along a pollution gradient in a river, the physiological condition of the amphipods was found to depend on AOM contamination at the respective sampling site, while the genetic structure of *G. pulex* within the river did not show AOM-dependent changes (Švara et al., 2021). However, due to the limitations of the study performed in a single river with a few sampling sites, the relationship between the genetic diversity of *G. pulex* and different levels of AOM in rivers remains unclear.

To expand upon the previous results and comprehensively determine the association between the genetic diversity of species and different levels of AOM, genetic diversity parameters of the amphipod species *G*. *pulex* and site-specific AOM profiles were here investigated on a regional scale in central Germany. We compiled and examined a comprehensive data set comprising data on AOM concentrations in water and *G*. *pulex* tissue, AOM toxicity levels for *G*. *pulex*, and several *G*. *pulex* genetic diversity parameters for 34 sites across six rivers from three catchment areas. The study sites were selected based on criteria assumed to result in the diversity of study sites with regard to pollutant levels. We selected different locations along a stream course and considered the absence/presence of effluents from wastewater treatment plants (WWTP) and run-offs from agricultural and urban areas. In the previous study on the genetic divergence of *G*. *pulex* from polluted and nonpolluted sites within a river, *G*. *pulex* individuals from the

different sites were found to not be genetically different (Švara et al., 2021). Thus, we hypothesize that (1) divergence in population genetic structure within rivers and across rivers in a region corresponds to distances between sites rather than to different levels of toxic AOM in the rivers. In contrast to divergence in species genetic structure, increased levels of toxic AOM at the sites downstream of the main sources of pollution (e.g., WWTP, agriculture) likely exert selective pressure on amphipods and affect species genetic diversity parameters. Therefore, we expect (2) AOM-related reduction of allelic richness and effective population size along with an increase in the inbreeding rates of *G*. *pulex* at sites with high AOM concentrations and toxicity levels in the studied region.

4.3 Materials and Methods

4.3.1 Study sites and sampling

Samples for genetic and chemical analyses were taken at eight reference sites (upstream of settlements and WWTP) and 26 AOM-polluted sites along six rivers (Altenau River (A), Eine River (E), Holtemme River (H), Parthe River (P), Saale River (S), Wipper River (W)) belonging to three catchments in the states of Lower Saxony, Saxony, Saxony-Anhalt, and Thuringia in central Germany (Fig. 4.1). The rivers flow through forest, urban, and agricultural landscapes with run-offs and WWTP effluents as the main sources of anthropogenic water contaminants. The numbers of WWTP located at the analyzed river stretches were: one at the Altenau River, two at the Eine River, two at the Holtemme River, two at the Parthe River, two at the Saale River, and five at the Wipper River (Fig. 4.1). At each site, the common Palearctic amphipod species *G*. *pulex* was collected along with water samples. The chemical analysis of the two types of samples enabled detection of a broad spectrum of freely dissolved and tissue-bound, potentially ecotoxicologically relevant AOM. In parallel to the sample collection, water parameters comprising temperature, pH, O_2 concentration, and conductivity were measured (Tab. SC1). *Gammarus pulex* amphipods were caught by kick-net sampling (0.5 mm mesh size) across the whole width of a river with at least five locations per sampling site. Sampled amphipods were morphologically identified using a taxonomical key (Altermatt et al., 2019). The abundance of *G*. *pulex* at each site was estimated by the recorded number of

individuals per catch. Amphipods for chemical analysis were rinsed with distilled water at the sampling site, dried on a clean paper towel, and stored at −20°C until analysis. For DNA analysis, *G. pulex* specimens were stored in absolute ethanol. One milliliter of river water samples were collected and frozen at −20°C until chemical analysis. For detailed information on the sampling sites, refer to Tab. SC1.

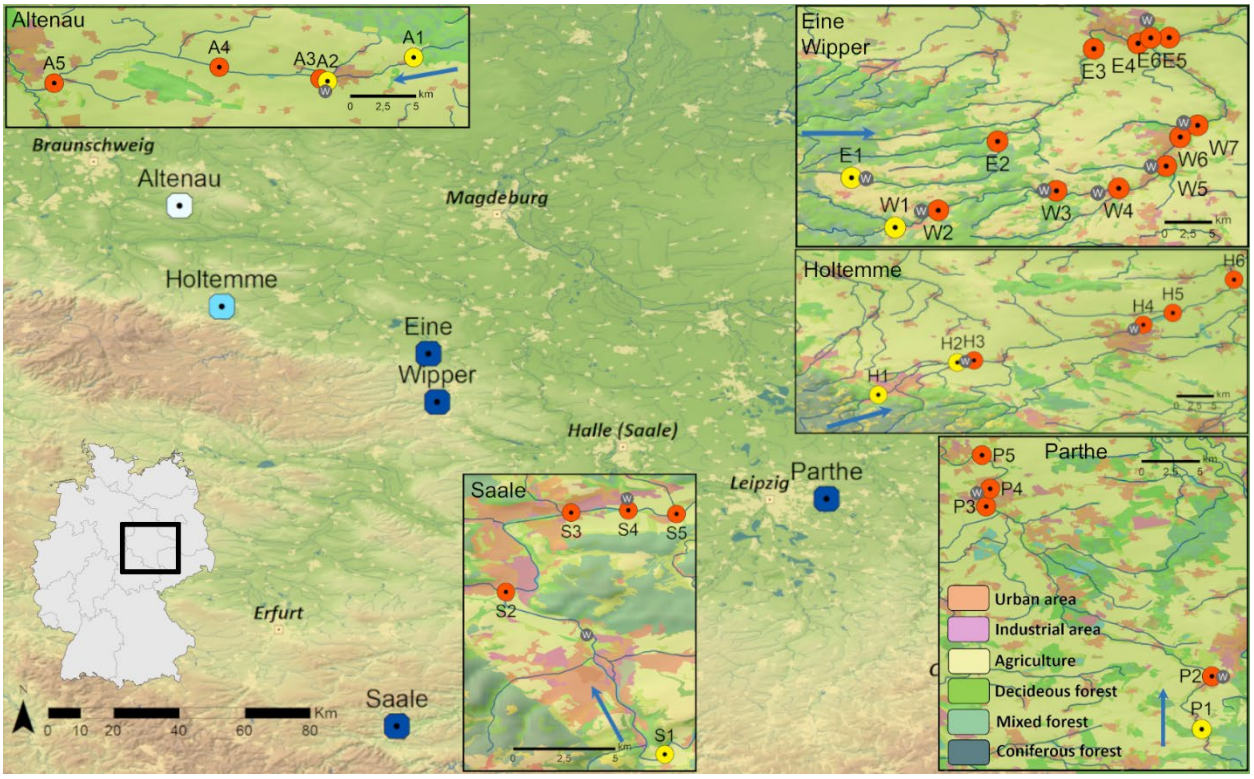

Figure 4.1 Map of the studied region with the six rivers and the sampling sites. The region is indicated by a square on the map of Germany (bottom left). The large map shows an overview of the region with the studied rivers and the major cities marked for orientation. Symbols colored in different shades of blue indicate different catchments: Oker River catchment—white, Bode River catchment—light blue, and Saale River catchment—dark blue. The studied sites are shown in the panels with each sampling site marked with colored circles (A—the Altenau River, E—the Eine River, H—the Holtemme River, P—the Parthe River, S—the Saale River, and W—the Wipper River). The colors of the circles in the detailed maps indicate whether a wastewater treatment plant (WWTP) is located upstream of the study site (yellow—no WWTP; red—WWTP). Grey circles with a white “W” indicate the locations of WWTP. Blue arrows indicate the direction of the waterflow. Land use in the areas of the detailed maps is indicated by the colors explained in the legend in the bottomright corner of the figure: green colors—different forest types, red—urban areas, purple—industrial areas, and yellow—agricultural areas. The length of the scale bars in the panels indicates 5 km.

4.3.2 AOM quantification and toxicity estimation

AOM concentrations in water and *G*. *pulex* tissue samples were analyzed by a Thermo Ultimate 3000 liquid chromatography (LC) system coupled with a quadrupole-orbitrap high-resolution mass spectrometer (HRMS; Thermo QExactive Plus) as described in Švara et al. (2021). For details on sample preparation and LC-HRMS measurement refer to Section SC1 in the Supplementary information.

To quantify AOM in the samples, raw data from the LC-HRMS analysis were converted into the.mzML format using 'ProteoWizard v3.0.18265'. The peak list for each batch was generated by MZmine v2.32 (Pluskal et al., 2010) with settings as suggested by Beckers et al. (2020). The list was annotated for 523 compounds from water samples and 497 compounds from *G*. *pulex* tissue samples and corrected for blanks. The analyzed compounds comprised AOM with a wide spectrum of hydrophobicity and application categories including pesticides, pharmaceuticals, and household and industrial known to occur in Central European rivers regularly (Beckers et al., 2018; Inostroza et al., 2016a; Inostroza et al., 2016b; Munz et al., 2017). Refer to SC1.4 in the Supplementary information for details on data evaluation and to Tab. SC2 for a list of analyzed compounds.

The toxic potential of the analyzed AOM was estimated by converting the measured *G*. *pulex* tissue concentrations into toxic units (TU). TUs were calculated based on the lethal concentrations for 50% of individuals in standard toxicity tests (LC_{50}) for the respective compounds given in the EPA ecotoxicology database (https://www.epa.gov/chemical-research/ecotoxicology-database). Data from the database were retrieved as a text file (exotox_ascii_15_09_2020) and the mean was calculated from all LC_{50} values obtained in 24 or 48 h exposure experiments for *G*. *pulex* for a respective compound. If no LC_{50} data for a compound were available for *G*. *pulex*, LC_{50} data for *Daphnia magna* Straus, 1820 were used. The freely dissolved fraction (C^{fd}) of each compound *i* was estimated based on the measured tissue concentrations according to the equilibrium partitioning theory using the following equation:

$$C_i^{\mathrm{fd}} = \frac{C_i^{\mathrm{tG}}}{f_{LIPID} D_{OW}}$$

where C^{tG} is the total measured concentration [ng/g of wet tissue] of a compound in *G*. *pulex*; f_{LIPID}, the lipid fraction value predicted for *G*. *pulex* by Ashauer et al. (2010; 1.34% of total body weight); and D_{ow}, the n-octanol-water distribution coefficient. The coefficient values were calculated using ACD Perfecta 2014. The potential toxicity of the individual AOM for *G*. *pulex* was determined by calculating TU values (Tab. SC5). The TUs for all compounds in an extract were summed to predict the potential for an additive adverse effect of those chemicals, as described in Švara et al. (2021):

$$\log\sum \mathrm{TU} = \log\sum\left(\frac{C_i^{\mathrm{fd}}}{\mathrm{LC}_{50,\mathrm{i}}}\right)$$

4.3.3 Assessment of *G. pulex* genetic diversity and structure

To assess the population genetic structure and genetic diversity parameters of *G*. *pulex*, 16 microsatellite loci were genotyped in a total of 931 individuals from the different sampling sites (10–30 individuals per site; Table SC8). Genomic DNA was extracted from pereopods of each individual using the DNeasy Blood & Tissue kit (Qiagen). DNA integrity was checked on an agarose gel, followed by DNA concentration quantification using a NanoDrop spectrophotometer (NanoDrop Technologies Inc.). A segment of the mitochondrial cytochrome oxidase I (COI) gene from randomly selected individuals (>5 per river) was sequenced to assess whether *Gammarus* cryptic lineages were present in the studied region. For details on polymerase chain reaction parameters, sequencing conditions, and primers refer to Švara et al. (2021) and Tab. SC6.

Microsatellite loci (Tab. SC7) were amplified and genotyped from 20 (Holtemme River sites) to up to 30 (all other sites) *G*. *pulex* DNA samples following the protocol described in Švara et al. (2019). Microsatellite genotype data for each individual and each locus with missing genotype information (>20%) were removed from the data set. Null alleles and deviations from Hardy-Weinberg equilibrium were assessed using the R package ‘popgenereport’ (Adamack & Gruber, 2014). Subsequently, rarefied allelic richness and

private allele rates were calculated in 'HP-Rare' (Kalinowski, 2005) for *G*. *pulex* from each sampling site, followed by the estimation of observed (H_o) and expected (H_e) heterozygosity and the inbreeding coefficient (F_{IS}) using the R package 'Hierfstat' (Goudet, 2005). Pairwise differentiation and its statistical significance among *G*. *pulex* from different sampling sites and rivers were estimated by fixation index (F_{st}) values, with the deviation from zero tested by applying 10,000 permutations of the analyzed loci. The effective population size at each site was estimated with the linkage disequilibrium model in 'NeEstimator 2.0.2' (Do et al., 2014) and an alpha value of less than 0.05. Hierarchical variance significance of the genetic differentiation among sites and rivers was calculated by analysis of molecular variance (AMOVA) in the R package 'Poppr' (Kamvar et al., 2014).

To assess the composition of genotypes in the rivers, a population structure analysis was performed in Structure 2.3.4. (Raj et al., 2014). An admixture model without any a priori information was run 10 times for clusters K from 1 to 10 using 1,200,000 MCMC steps and discarding the first 200,000 steps as a burn-in. The optimal number of clusters was determined in Structure Harvester (Earl & von Holdt, 2012) with the Evanno method (Evanno et al., 2005). Using CLUMPP 1.1.2. (Jakobsson & Rosenberg, 2007), the runs were merged into a single plot and visualized in DISTRUCT 1.1. (Rosenberg, 2004).

The association between distance and genetic differentiation among sites was tested using the Mantel test by comparing pairwise F_{st} values and waterway distances between all sites for regional comparison and sites within each river for local comparison. Distances between sites were estimated using the 'network analyst toolbox' in ArcGIS (ESRI). The impact of environmental pollution on genetic differentiation between sites was assessed by partial Mantel tests correlating pairwise F_{st} values against distances based either on total AOM levels or TUs, while accounting for the effect of waterway distances.

4.3.4 Analyses of AOM relation to genetic diversity parameters

Relations between AOM levels and amphipod genetic diversity indicators, including allelic richness, private allele rates, inbreeding coefficients (F_{IS}), effective population size (N_e), and *G*. *pulex* abundance, were analyzed by linear mixed-effect models (LME). Genetic diversity indicators were explained by linear fixed effects of distance from the spring, toxic units based on the AOM from *G*. *pulex* tissue samples, total concentration of detected AOM in amphipod tissue, presence of WWTPs before the sampled sites, conductivity, pH, and saturation of water with oxygen, allowing a random intercept for each river. The values of the total concentration of detected compounds and effective population sizes were log-transformed to avoid the effects of very low values close to 0. The sites, for which, due to limited numbers of *G*. *pulex*, AOM-tissue concentrations were not available, were excluded from the analysis (i.e., S2, W5, W6). Using the function lmer in the package 'lme4' (Bates et al., 2015), a global model including all environmental site characteristics was constructed. To select for the fixed effects contributing to differences in the analyzed indicators, the best fitting models were selected based on the lowest Akaike information criterion (AICc; delta AICc of less than 5 were considered) and the highest log-likelihood using the dredge function from the package 'MuMIn' in R (Burnham & Anderson, 2002).

Structural equation models (SEM) were used to fit allelic richness against total AOM concentration and distance from the source for all sites in each river. The models were fitted by generalized least squares by applying the sem function from the R package 'lavaan' (Rosseel, 2012). In the global model for all rivers, TUs and *G*. *pulex* abundance were included as intermediate explanatory variables, as differences in total concentrations of AOM could be reflected in TU values and in *G*. *pulex* abundances.

4.4 Results

4.4.1 AOM detected in water and *Gammarus pulex* tissue samples

Numbers of detected AOM and their concentrations in the water samples indicated different site-specific pollution patterns. In total, 236 compounds were detected in water

samples from 34 sites. Most compounds were found in water samples from sites P4, S5, P5, and W7 with 152, 131, 130, and 104 compounds, respectively. The highest total AOM concentrations were 107.8 and 96.4 µg/L in water from sites W5 (Wipper River) and P4 (Parthe River), respectively (Fig. 4.2a). Total AOM concentrations were lowest in water from sites H1 and H2 (0.2 and 0.4 µg/L, respectively; Holtemme River) and A2 (0.5 µg/L; Altenau River). From the analyzed AOM, the industrial chemical 1H-benzotriazole (47.2 µg/L at site W5), the pharmaceutical theophylline (41.1 µg/L at site W5 and 37.4 µg/L at site P1), and the metformin transformation product guanylurea (14.8 µg/L at site P4) showed the highest concentrations. The herbicide metazachlor, the sweetener acesulfame (both detected at all 34 sites), the industrial chemical melamine, and the sweetener cyclamate (both detected at 33 sites) were found at most sites. Several compounds were found in water samples from only a single site (refer to Tab. SC3 for an overview). Among the detected AOM, suspected carcinogens tris(2-chloroethyl)phosphate at sites S5, E2, E5, and P4 and melamine at 31 sites were detected in the studied rivers (Tab. SC3).

In *G. pulex* tissue samples, a total of 253 compounds were found at 31 sites. Most compounds were detected in tissue samples from sites E6 (155 compounds), P4 and P5 (109 compounds), E5 (103 compounds), and E3 and E4 (83 compounds). The highest (8.4 µg/g at H3) and the lowest (0.1 µg/g at H1) total AOM concentrations were detected in tissue samples from the Holtemme River (Fig. 4.2b). The biocides benzyldimethyldodecylammonium and didecyldimethylammonium, the herbicide pendimethalin, and the surfactant decylsulfate were detected in tissue samples from all 32 sites. 7-(ethylamino)-4-methylcoumarin (up to 7.6 µg/g at H3), tetrapropylammonium (up to 1.8 µg/g at E3), and pendimethalin (up to 1.5 µg/g at A1) were found at the highest concentrations among compounds detected in tissue extracts. The lowest number of compounds was detected in *G. pulex* tissue from sites H1, A1, and A2 (19, 43, and 49, respectively). Several compounds were detected in tissue samples from only one site; for detailed information see Tab. SC4. The total AOM concentration generally increased at sites downstream of a WWTP effluent entering the river, yet, it did not linearly increase with distance from the first site (e.g., in the Altenau or the Eine Rivers) and at the sites downstream of the WWTP effluents (e.g., in the Altenau River). Among the detected AOM,

a pharmaceutical with a genotoxic potential, tamoxifen, and its metabolite 4-hydroxytamoxifen were found at sites S2 and S4 and at sites P4, W2, and W3 (Saale, Parthe, and Wipper Rivers). The potential mutagen carboline was detected at sites E2, E6, and P1 (Eine and Parthe Rivers). Suspected carcinogens tris(1,3-dichlorisopropyl)phosphate (TDCPP; at sites E4, S4) and tris(2-chloroethyl)phosphate (at sites H2–H5, E1–E4, E6, P1–P4) were measured in *G*. *pulex* tissue extracts (Tab. SC4).

The differences among concentrations of AOM in water samples from the same river were larger than those measured in *G*. *pulex* tissue samples from the same river (Fig. 4.2a, b). While AOM concentrations in water samples were highest for pharmaceuticals, food ingredients, industrial chemicals, and biocides, AOM concentrations in *G*. *pulex* tissue were highest for industrial chemicals, herbicides, pharmaceuticals, and fungicides. The correlation of the AOM concentrations in water and *G*. *pulex* tissue samples was rather low, with a Pearson's correlation coefficient of 0.28 across all sites.

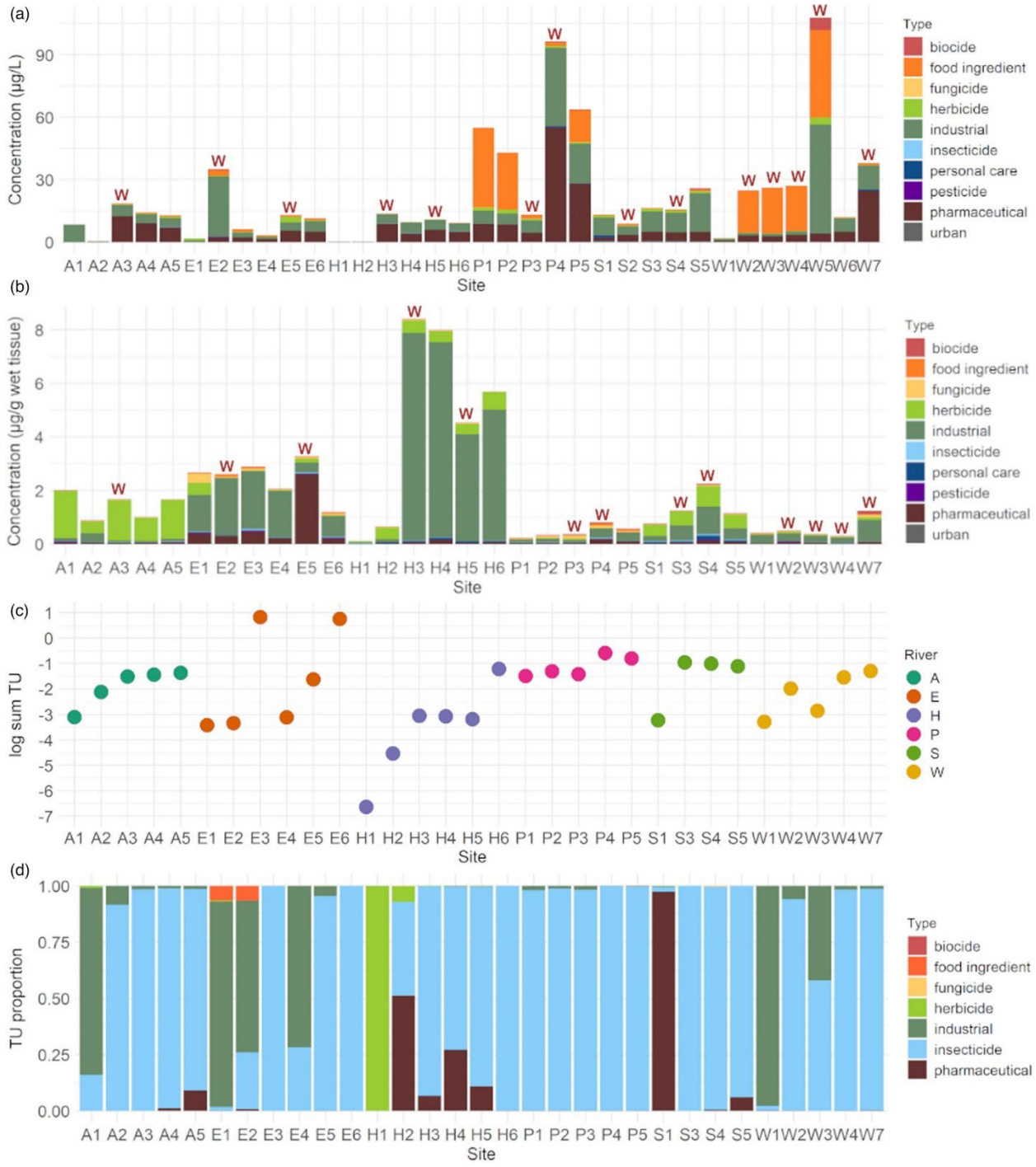

Figure 4.2 Levels of AOM and their toxicities for *Gammarus pulex*. (a) Total concentrations of AOM from different application types (types are marked by different colors) in water samples from each site. (b) Total concentrations of different AOM types measured in *G. pulex tissue* samples from each site. (c) Toxic units (TU) calculated for AOM found in *G. pulex* tissue samples from each site. (d) TU ratio of AOM with different application types based on AOM concentrations measured in *G. pulex* tissue levels from each site. The sites situated directly downstream of WWTP effluents are marked with a red "W".

4.4.2 Site-specific toxic potentials of AOM

The TU values calculated from *G. pulex* tissue concentrations indicated an increased toxic potential downstream of the major pollution sources in the studied rivers. The sites with the highest TU values were either the ones located the furthest downstream at the sampled river stretches or the ones located downstream of the WWTP effluents and include sites from the Eine River (E3 at 6.74, E6 at 5.75), the Parthe River (P4 at 0.26, P5 at 0.16), and the Saale River (S3 at 0.11, S4 at 0.10, SC5 at 0.08; Fig. 4.2c). The lowest potentials for adverse effects from AOM in amphipods (TU <0.001) were indicated at sites upstream of WWTP effluents and run-offs from agricultural areas (H1, H2, E1, E2, W1, and A1).

TU values were assessed for 44 AOM (Tab. SC5) and were highest for the organophosphate transformation product 3,5,6-trichloro-2-pyridinol, insecticides acetamiprid and imidacloprid, and the pharmaceutical acetaminophen. The insecticide 3,5,6-trichloro-2-pyridinol was detected in two samples (E3, E6); respective TUs were > 0. The neonicotinoid insecticide acetamiprid was detected at 17 sites. At sites P4, P5, S3, and S4, the TU attributed to acetamiprid exceeded 0.01, the value known to cause acute effects in crustaceans (Malaj et al., 2014). Several other AOM, including insecticides (e.g., imidacloprid) and a pharmaceutical (i.e., acetaminophen), exceeded 0.001 TU (Tab. SC5), and thus the threshold for chronic adverse effects for *G. pulex* (Malaj et al., 2014).

Acute and chronic TU levels were mostly attributed to insecticides (acetamiprid, imidacloprid, thiacloprid, clothianidin) and their transformation products, often contributing more than 95% of the total TUs (Fig. 4.2d, Tab. SC5). Another compound group that contributed significantly to the toxicity at the polluted sites included pharmaceuticals (e.g., acetaminophen and citalopram). Some AOM groups did not exceed the threshold for chronic toxicity, yet they significantly contributed to total TU. For example, at the sites with low TUs, industrial chemicals (A1, E1, E2, E4, W1, and W3), herbicides (H1 and H2), pharmaceuticals (H2, H4, and S1), and even food ingredients (E1 and E2) significantly contributed to the total TUs.

4.4.3 Genetic diversity and structure of *G. pulex*

Based on the analyzed COI segment sequences, *G*. *pulex* samples from the studied rivers belonged to a single genetic lineage. Similarities of all sequences were greatest with those from *G*. *pulex* samples from the Brandenburg region (Fig. SC1). Therefore, all sampled *G*. *pulex* individuals could be assigned to clade E (Fig. SC1), according to the classification of Grabner et al. (2015).

In total, 931 *G*. *pulex* individuals were genotyped using microsatellites; upon quality control, data from 928 individuals were further analyzed. Across all 16 analyzed microsatellites, 138 different alleles were detected. All loci were polymorphic in *G*. *pulex* from the six rivers (Tab. SC8). Averaged rarefied allelic richness per locus was highest in the Parthe River (2.98) and lowest in the Holtemme River (2.69; Tab. SC8); ranges were 2.74 to 2.89 in the Altenau River, 2.34 to 2.89 in the Eine River, 2.43 to 2.88 in the Holtemme River, 2.75 to 2.99 in the Parthe River, 2.76 to 2.94 in the Saale River, and 2.61 to 2.85 in the Wipper River. The numbers of river-specific private alleles per locus were highest in the Saale River (0.32) and lowest in the Wipper and the Parthe Rivers (0.18). No significant linkage between loci was detected when considering all sites. Null alleles were consistently detected for locus gp37, which was excluded from the structure analysis (Tab. SC9). Observed heterozygosity across sites varied from 0.23 at the site directly at the most downstream WWTP at the Wipper River (W6) to 0.40 at the furthest upstream site at the Saale River (SB1; Tab. SC8). Expected heterozygosity varied from 0.35 at W6 to 0.44 at the site downstream of the WWTP at the Saale River (S5) and at the Parthe River (P3). The F_{is} values were lowest at upstream sites of rivers, including sites H1 and H2 at the Holtemme River and site S1 at the Saale River with −0.017, 0.026, and 0.073, respectively. The F_{is} values were highest mostly at more downstream sites. For example, F_{is} = 0.439 and F_{is} = 0.282 at sites W4 and E6 in the Wipper and the Eine Rivers, respectively (Fig. SC3).

Cluster values K with the highest probability that resulted from structure analysis of *G*. *pulex* genotypes were K = 2, K = 3, and K = 6 (Figure SC4). For K=6, each cluster belonged to a specific river (Fig. 4.3d). Some individuals with a genotype membership of another than the predominant cluster in a respective river were found particularly at sites

E1, E6, H6, W1, and W2. The F_{st} values corresponded to the result of the genetic structure analysis with values significantly different from zero for all pairwise comparisons of rivers. Genetic differentiations were observed to be largest between *G*. *pulex* from the Saale River and the Holtemme River, from the Saale River and the Eine River, and from the Eine River and the Altenau River (Fig. 4.3a). These pairwise comparisons indicated a subpopulation structure between sites with lower F_{st} values (F_{st} <0.1 in Holtemme:Eine and Altenau:Parthe:Wipper), consistent with K = 3 from the structure analysis (Fig. 4.4).

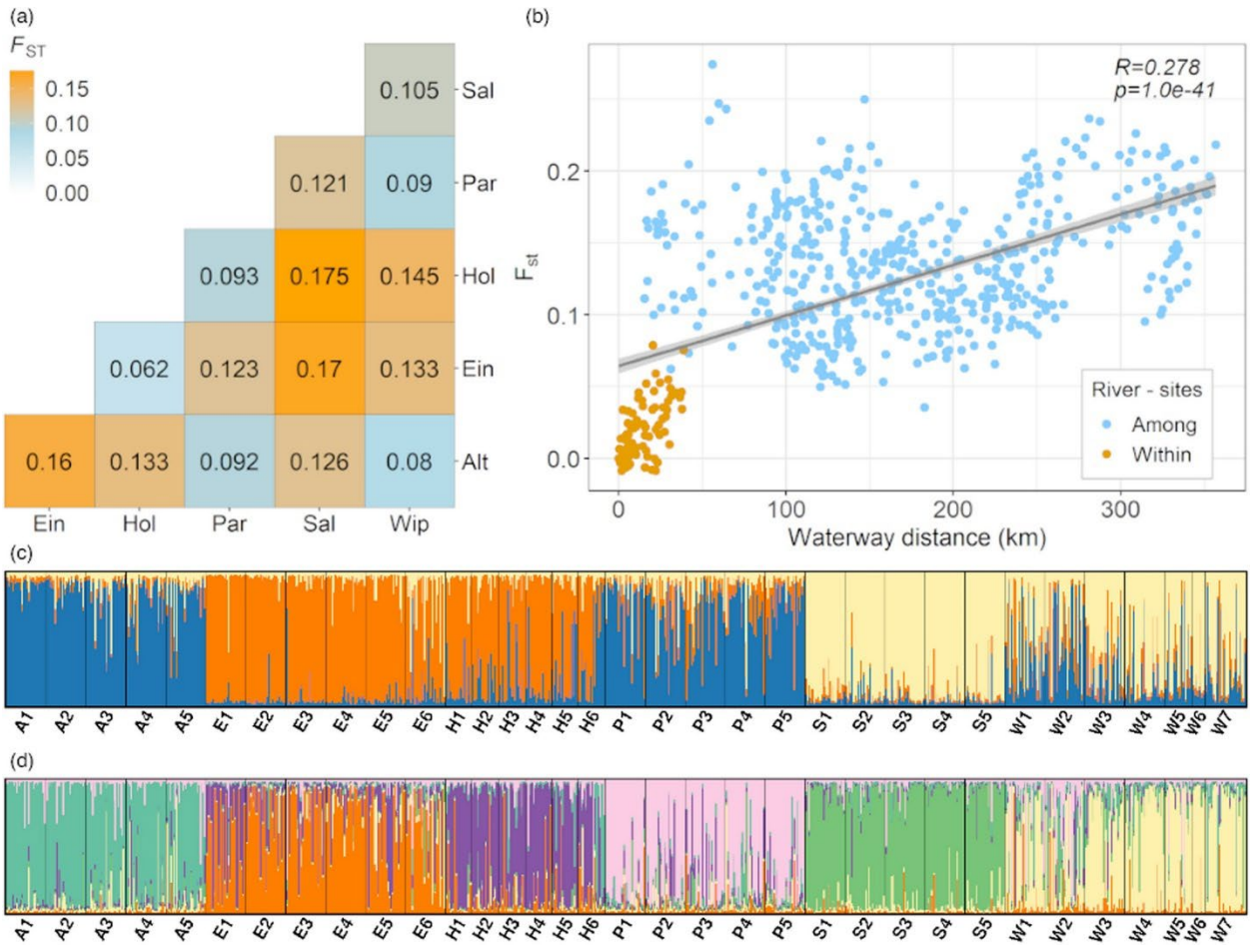

Figure 4.3 Genetic differentiation of *Gammarus pulex* in the studied rivers. (a) Pairwise F_{st} values estimated based on pooled *G*. *pulex* genotypes from different sites belonging to each river. All values are significantly different from zero. (b) Mantel test of pairwise F_{st} values between every pair of sites and respective waterway distances. Orange dots indicate pairwise comparison of sites within each river. Blue dots indicate pairwise comparison of sites among rivers. Structure analysis of *G*. *pulex* from 34 sites in six rivers with

memberships/ancestry proportion to different clusters (c) K = 3 and (d) K = 6. Each vertical line represents a genotype of a single amphipod.

The genetic differentiation of *G*. *pulex* within each river was lower than among rivers. F_{st} was highest with 0.079 for *G*. *pulex* from sites S1 and S4 (Saale River), followed by 0.075 for *G*. *pulex* from sites E1 and E6 (Eine River) and 0.055 for *G*. *pulex* from sites W1 and W6 (Wipper River; Tab. SC10). The highest F_{st} values were detected when comparing *G*. *pulex* genotypes from the most upstream and the most downstream sites in the rivers (Tab. SC10). Of 81 pairwise F_{st} values for comparisons within rivers, 35 comparisons showed significant differences from 0 (Tab. SC10). A significant positive relationship was detected between F_{st} values and waterway distances regionally (among all sites; $r = 0.527$, Mantel $p < 0.001$; Fig. 4.3b) and locally (within single rivers; Fig. SC2), except for the Holtemme River for which the F_{st} value–waterway distance relationship was nonsignificant ($p > 0.05$). Surprisingly high F_{st} values were detected for two sites located close to each other (i.e., P1 and P2; Fig. SC2c) and for two sites with similar pollution and toxicity patterns (i.e., H4 and H6; Fig. SC2d). At the regional scale and accounting for the effect of waterway distances, genetic differentiation increased with differences in total AOM levels ($r = 0.173$, Mantel $p < 0.01$). Within rivers, partial Mantel tests were not significant, except for the Altenau River. Genetic differentiation did not increase with differences in TUs neither regionally nor locally (Mantel $p > 0.3$).

4.4.4 Relationship between AOM and *Gammarus pulex* genetic diversity indices

The association between *G*. *pulex* genetic diversity parameters and AOM concentrations detected in *G*. *pulex* tissue was indicated by LMEs. A significant contribution of fixed effects to LME was indicated for 4 of the 5 analyzed model indicators, including allelic richness, F_{is}, abundance, and N_e (Tab. SC11). The distribution of private alleles could not be explained by any linear effect across analyzed rivers. According to the AICc and log-likelihood values, models without explanatory variables described genetic diversity parameters equally well as the models with defined fixed effects, suggesting that other

than the here considered fixed effects influenced the assessed genetic parameters (Tab. SC11).

Three of the analyzed fixed effects, total AOM concentration, TUs, and the presence of WWTP effluent, showed a significant correlation with the analyzed indicators ($p < 0.05$). The presence of WWTP effluent and total concentration of AOM best-described changes in allelic richness in *G. pulex* (Tab. SC11). The presence of WWTP effluent showed a positive relationship to the allelic richness across the six rivers, yet, the relationship was not confirmed by analysis for each river individually (Tab. 4.1, Fig. 4.4b). In contrast to the WWTP effluent, total AOM concentration was negatively correlated with the allelic richness at the studied sites (Tab. 4.1, Fig. 4.4a). According to the LME regression, allelic richness was 12% lower at the highest measured AOM concentrations in comparison with the lowest concentrations of AOM. It was also up to 22% reduced at sites with increased levels of AOM in comparison to sites with low AOM pollution. The distribution of F_{is} from the analyzed sites was associated with TUs calculated from AOM detected in *G. pulex* (Tab. 4.1). The calculated TUs were positively related to the F_{is} rates (Tab. 4.1, Fig. 4.4c), which, according to the model, spanned from F_{is} of 0.04 at low TU values to F_{is} of 0.36 at high TU values. The abundance of amphipods at sampling sites was best described by the WWTP effluent and total concentration of AOM (Tab. 4.1). The abundance of *G. pulex* was negatively correlated with WWTP effluents and positively correlated with the total concentration of AOM (Tab. 4.1). Abundances were lowest at sites with very high or very low TU of AOM in both river water and *G. pulex* tissue samples (e.g., at sites H1, W5, W6). Finally, the most informative fixed effect associated with the effective population size of *G. pulex* was the presence of WWTP effluent, with a positive but nonsignificant relationship (Tab. 4.1).

The relationship between the water concentrations of total AOM and allelic richness was confirmed by the global SEM. The model combining allelic richness, total AOM water concentration, and distance to the source with intermediate variables indicated the strongest negative relation between total AOM and allelic richness (−0.79, $p < 0.02$) and a positive relationship between distance to the river source and allelic richness (Fig. 4.4d). The negative relationship between total AOM and allelic richness was also detected in

models assessing parameters of single rivers. The effects of total AOM on allelic richness spanned from the value of −0.15 in the Wipper River to the value of −0.94 in the Saale River (Fig. SC5), yet ranged from significant (rivers Eine, Wipper) to nonsignificant (rivers Altenau, Holtemme, Parthe, Saale), indicating river-specific patterns (Tab. SC12).

Table 4.1 Parameter values from LMEs for the analyzed genetic diversity indicators allelic richness, inbreeding coefficient (F_{is}), abundance, effective population size (N_e) and respective fixed effects. The parameters intercept estimation (Estimate), standard error (SE), t-values, and p-values are indicated. Fixed effect indicating a wastewater treatment plant effluent source upstream of the sampling sites (WWTP +), log_{10} of the total concentration of the detected AOM in G. pulex tissues (log_{10} total AOM); toxic unit values calculated from AOM concentrations detected in *G. pulex* tissues (TU_{gam}).

Indicator	Fixed effect	Estimate	SE	t-value	p-value
Allelic richness	Intercept	3.189	0.160	19.875	< 0.001
	WWTP +	0.173	0.049	3.526	0.002
	log_{10} total AOM	-0.184	0.056	-3.308	0.003
F_{is}	Intercept	0.313	0.062	5.089	< 0.001
	TU_{gam}	0.066	0.029	2.282	0.032
Abundance	Intercept	-0.409	0.562	-0.728	0.474
	WWTP +	-0.462	0.166	-2.772	0.012
	log_{10} total AOM	0.775	0.194	3.985	0.001
$log_{10} N_e$	Intercept	1.722	0.141	12.240	< 0.001
	WWTP +	0.293	0.163	1.794	0.0854

Note: The parameters intercept estimation (Estimate), standard error (SE), *t*-values, and *p*-values are indicated. Fixed effects indicate a wastewater treatment plant effluent source upstream of the sampling sites (WWTP +), log_{10} of the total concentration of the detected AOM in *G. pulex* tissues (log_{10} total AOM); toxic unit values calculated from AOM concentrations detected in *G. pulex* tissues (TU_{gam}).

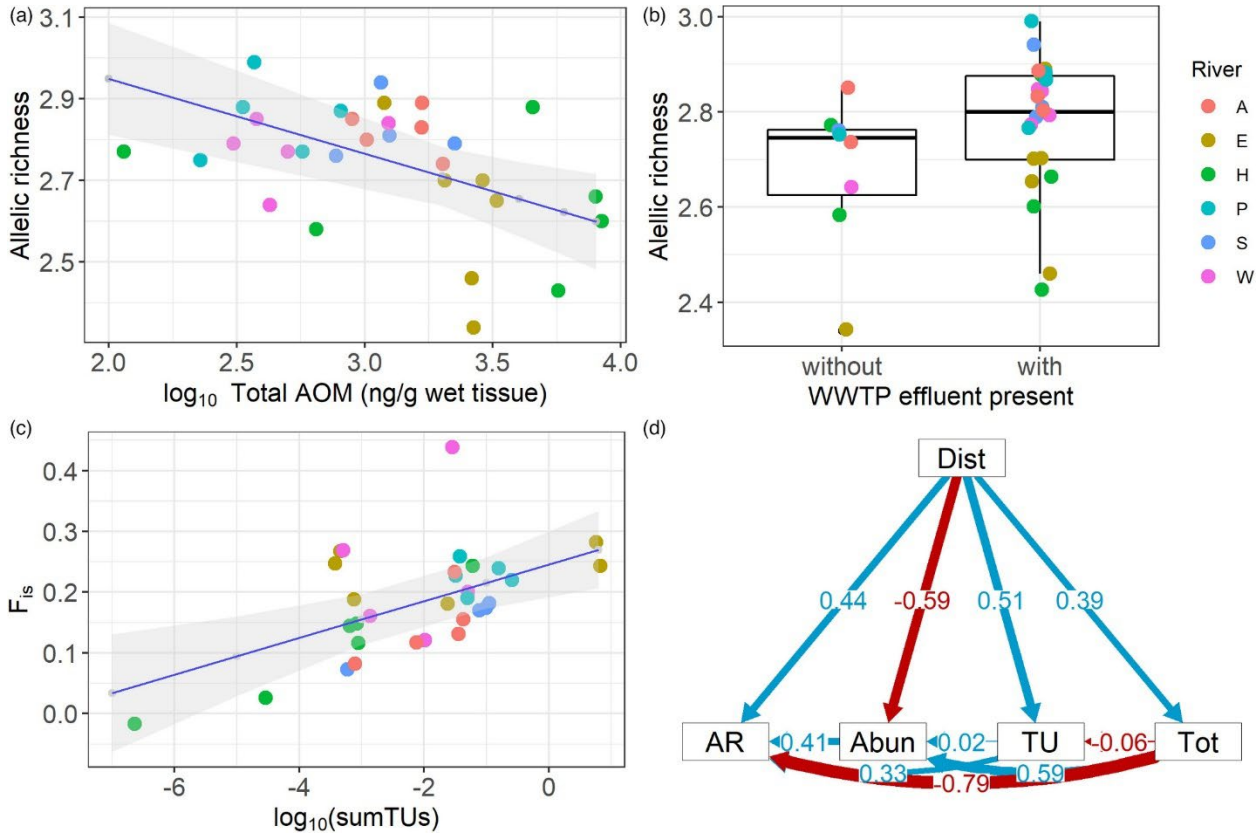

Figure 4.4 Relationship of *Gammarus pulex* genetic diversity parameters and AOM indices from the sampling sites at the investigated rivers. (a) Scatterplot based on a linear mixed-effects model (regression line with 95% confidence interval shaded) of allelic richness values for *G. pulex* and the total AOM tissue concentration at the respective site. Circles represent the sampling sites, colors represent different rivers (A—the Altenau River, E—the Eine River, H—the Holtemme River, P—the Parthe River, S—the Saale River, and W—the Wipper River). (b) Box plots of allelic richness values of *G. pulex* from sampling sites upstream (without) or downstream (with) of a WWTP effluent. (c) Scatterplot based on a linear mixed-effects model (regression line with 95% confidence interval shaded) of inbreeding coefficient (F_{is}) values and toxic units ($\log_{10}$(sumTUs)). Circles represent the sampling sites, colors represent different rivers (refer to "A"). (d) Structural equation model graphical output indicating the relationship of site-specific parameters allelic richness (AR), total concentration of AOM in *G. pulex* tissue (Tot), distance from the source (Dist), and intermediate parameters of TUs (TU) and *G. pulex* abundance (Abun). Blue arrows represent a positive relationship and red arrows a negative relationship of two parameters. The width of the arrows, together with the indicated values, represents the magnitude of the indicated relationship.

4.5 Discussion

In this study, the association of genetic diversity of the freshwater amphipod species *G*. *pulex*, common in likewise polluted and pristine sections of rivers in central Europe, and AOM levels in the habitats of *G*. *pulex* was investigated. The population genetic structure of *G*. *pulex* in the studied region corresponded to river catchments and showed a weak correlation with the respective AOM contamination across rivers. However, genetic diversity parameters indicated a significant trend of reduced allelic richness and enhanced inbreeding rates of *G*. *pulex* from sites with increased levels of AOM.

4.5.1 The genetic structure of *G. pulex* relates to the connectivity among sites

According to the genetic structure analysis performed here, populations of *G*. *pulex* in the studied region are strongly defined by the within-river connectivity and the geographic distance between studied sites. The river-related genetic structure of the examined species is in line with previous studies demonstrating the importance of riverine network and species colonization history for the genetic structure of amphipods in rivers (Weiss & Leese, 2016; Westram et al., 2013). Indeed, populations with a specific genetic structure of the amphipod *G*. *fossarum* were found to be confined by different river catchments based on neutral loci (Westram et al., 2013). Such river-related genetic structure can be maintained by a high migration rate within a river that can compensate for selective drivers including local environmental stressors, such as increased pollution, large temperature oscillations, food scarcity, or increased competition (Lenormand, 2002).

In addition to river-related *G*. *pulex* population structure, sites within rivers with significant differentiation levels were detected. The within-river differentiation largely followed the isolation by distance pattern, yet it was high among some sites within rivers. For instance, reference site E1 and the other sites in the Eine River showed significant differentiation. Such differentiation is, due to reduced allelic richness at the reference site, likely not the result of the increased AOM input downstream of this site. Another factor contributing to isolation and reduced genetic diversity of *G*. *pulex* at the site E1 could be drought, as

some parts of the stream dry out in summer. Finally, organic compounds released from the WWTP downstream of the site E1 could contribute to the absence of *G*. *pulex* directly downstream of the WWTP and to the spatial and genetic isolation of *G*. *pulex* at site E1. The other sites with significant differentiation were, in contrast to the sites in the Eine River, spatially distant from each other, or did not indicate the difference in pollution patterns.

Genetic differentiation has been found to be comparatively large not only among *G*. *pulex* from distant rivers but also in some *G*. *pulex* populations from spatially proximate rivers (Fig. 4.3a). As shown by previous studies, genetic differentiation within *Gammarus* populations living at sites close to each other can be significant (Weiss & Leese, 2016; Zickovich & Bohonak, 2007). Some of this differentiation may be associated with increased levels of AOM as shown by the partial Mantel test (see Result Section 3.3). However, large genetic differentiation rates between populations were shown to be associated with migration barriers, local and seasonal gene flow bottlenecks, and drift (Reid et al., 2016; Weston et al., 2013). In *G*. *pulex* studied here, historic migration events could, on the one hand, explain the genetic similarity of *G*. *pulex* in geographically more distant rivers (e.g., Parthe and Altenau) and, on the other hand, the significant genetic divergence among *G*. *pulex* in geographically proximate rivers (e.g., rivers Eine and Wipper; Alp et al., 2012; Weiss & Leese, 2016). Moreover, the dispersal of amphipods by birds may contribute to gene flow to remote, hydrologically little connected sites, leading to low genetic differentiation among sites (Figuerola & Green, 2002; Rachalewski et al., 2013). Such events could promote the introduction of novel genotypes to the established populations in the river that could be reflected by differences in genetic membership of some individuals within a river.

4.5.2 Genetic diversity of *Gammarus pulex* at sites with AOM contamination

The negative relationship between allelic richness and total concentration of AOM (Fig. 4.4a) and a positive relationship between inbreeding rates of *G*. *pulex* and TUs (Fig. 4.4c) determined for the studied sites confirms the hypothesis that *G*. *pulex* from AOM-polluted

habitats exhibits reduced genetic diversity. The reduced genetic diversity in *G*. *pulex* at sites with comparatively high levels of AOM can be attributed to an increased probability of genetic drift and loss of rare alleles (Hoffmann & Willi, 2008). The effects of genetic drift on populations exposed to AOM are even more likely when considering many other environmental and biological stressors, such as high summer temperatures that facilitate the susceptibility of organisms to toxicants (Brans et al., 2021). Similar to the findings presented here, reduced genetic diversity was found in populations of *Daphnia magna* in ponds with increased levels of AOM, suggesting their selective pressure (Coors et al., 2009). In exposed *G*. *pulex*, the selective pressure of AOM with toxic potential might promote genotypes beneficial in toxic environments. However, selective effects of AOM might be masked by gene flow between sites or the examined microsatellites may not be associated with genes under selection, resulting in no significant genetic change in the studied populations. Still, migration does apparently not proceed at a rate that would compensate for low allelic richness at sites with increased AOM levels. In addition, increased inbreeding rates in *G*. *pulex* strongly relate to estimated toxic levels of AOM. Increased inbreeding can enhance the effects of AOM in *G*. *pulex*, as it was shown to affect survival, reproduction, resistance to disease and predation, and susceptibility to environmental stress (Keller & Waller, 2002). These effects, resulting in reduced population fitness, are especially likely to occur in populations with a strong competition between males (Meagher et al., 2000). To prevent negative effects and retain a low inbreeding rate, inbreeding avoidance mechanisms exist (Pusey & Wolf, 1996), yet these appear to be ineffective in *G*. *pulex* living in the more polluted river sections.

In addition to the findings of decreased allelic richness and high inbreeding at sites with increased levels of AOM, allelic richness was increased at the sites downstream of WWTP effluent discharges (Fig. 4.4b). This contrasts with the finding of reduced allelic richness downstream of the main pollution sources and is mainly due to comparatively low AOM levels and high allelic richness detected at multiple sites downstream of the WWTP effluents (e.g., A4, E4, E6, P3, W3, W4). These results might reflect the complexity of the environmental conditions co-affecting the genetic diversity of *G*. *pulex* at the sites downstream of the WWTP. The effects of AOM may be altered by nutrients entering the river *via* the WWTP effluents. In fact, the abundance of *G*. *pulex* was particularly high at

some downstream sites (e.g., A3–A5, H3–H6), which could be related to high nutrient levels from WWTP effluents and thus the high abundance of food for *G*. *pulex*. The higher genetic diversity of *G*. *pulex* at sites WWTP effluents may thus be due to the abundance of *G*. *pulex* at these sites that is comparatively high because of the increased availability of food. Furthermore, comparatively high genetic diversity at downstream sampling sites may be due to the comparatively long distance to the river source. According to the LME, distance from the source was not an important parameter for increased allelic richness within a river (see Tab. SC11). Yet, the allelic richness and private allele values were highest at the most downstream sampling sites, which may be due to their proximity to the river confluence and therefore enhanced gene flow from other *G*. *pulex* populations (Alther et al., 2021).

4.5.3 AOM compounds with the potential to alter the genetic diversity of *G. pulex*

AOM can alter the genetic diversity of exposed species by causing mutagenic effects by exerting direct selective pressure or by affecting a species' gene pool through nonselective effects (Bickham, 2011). Mutagenic effects may lead to an increased genetic diversity at sites with mutagenic and genotoxic compounds present (Theodorakis et al., 2001). At sites E6 (Eine River) and W2 (Wipper River), where mutagenic or genotoxic compounds were found, allelic richness in *G*. *pulex* was highest; contrariwise, at site P1, where one compound with mutagenic potential was found, allelic richness was lowest in *G*. *pulex* across sites in the Parthe River. Thus, additional research would be necessary to reveal whether mutagenic and genotoxic AOM significantly contribute to changes in allelic richness in a multiple-stressors context.

From the detected AOM, insecticides can be expected to exert selective pressure because of their high toxic potential. Particularly, the detected insecticides fipronil, imidacloprid, thiacloprid, clothianidin, and acetamiprid were shown to cause adverse effects in *G*. *pulex* by hindering mobility and feeding (Englert et al., 2017) and, thus, select for tolerant individuals. Concentrations of insecticides in the same toxicity range as detected at sites with high toxic potentials (such as at e.g., E3, E6, P4, P5) were shown to reduce genetic

diversity and promote specific genotypes adapted to the particular insecticide exposure, for example, for pyrethroid exposure in *H. azteca* (Weston et al., 2013). An increased tolerance of *G*. *pulex* to these AOM may improve *G*. *pulex* performance; however, selection for tolerant individuals likely results in the observed inbreeding and a decrease in allelic richness at these sites. In addition, *G*. *pulex* at sites with high AOM levels are exposed to other pesticides possibly exerting indirect selective pressures. These include herbicides, fungicides, and biocides, which affect freshwater communities by reducing the quantity and diversity of periphyton, freshwater plants (e.g., MCPA, DEET, and pendimethalin), or fungi (carbendazim) that *G*. *pulex* feeds upon. It may be assumed that these indirect effects lead to an increase in intraspecific competition and genetic drift; however, there is no experimental evidence yet for the consequences of these indirect effects. Furthermore, effects caused by freely dissolved AOM in water and by AOM in *G*. *pulex* tissue should be compared in future studies, as the detected compound concentrations and exposure duration often largely differ. For instance, AOM may persist over long time periods in the tissue of *G*. *pulex*, while the exposure of *G*. *pulex* to some freely dissolved compounds would only occur at the time of events such as the release of wastewater from the WWTP.

For many AOMs detected at sites with reduced genetic diversity of *G*. *pulex*, no information on acute toxicity for *G*. *pulex* is available (see Tab. SC2). These AOMs that were detected at exceptionally high concentrations, often exceeding 100 µg/L, included the pharmaceuticals diclofenac, theophylline, valsartan, hydrochlorothiazide, 4-aminoantipyrine, the industrial chemicals 1H-benzotriazole, melamine, guanylurea, 2-benzothiazolesulfonic acid, 7-diethylamino-4-methylcoumarin, and tris(1-chloro-2-propyl)phosphate and the food additive triethylcitrate. Some of these AOMs can contribute to alterations of a species' genetic diversity by increasing selective pressure or altering the inheritance of alleles by affecting species reproduction or behavior. For example, diclofenac may exert selective pressure on and increase genetic drift of *G*. *pulex,* as it was shown to cause reduced survival of macroinvertebrates, including amphipods, when in a mixture with other AOM (Miller et al., 2015). In addition to the acute toxic effects of AOM, endocrine disruptors found in *Gammarus* amphipods were shown to alter the male-female ratio, influence reproductive success, and alter population size and allele

frequencies in the exposed populations (Gross et al., 2001; Watts et al., 2002). In the current study, 7-diethylamino-4-methylcoumarin, a driver of antiandrogenic effects in fish, was for the first time detected at high concentrations in the Eine River and confirmed for the Holtemme river (Muschket et al., 2018; Švara et al., 2021). The effects of this coumarin in amphipods remain unknown. However, it could importantly contribute to a complex pattern of allele frequency change, as antiandrogenic effects were indicated to contribute to genetic diversity change by altering sexual behavior in fish (Alves da Silva et al., 2018).

4.5.4 Ecological relevance of AOM effects in *Gammarus pulex*

Altered genetic diversity of a species can have significant consequences for the species' ecological performance. Reduced genetic diversity within populations can impact the species' abundance and thus its ecological function, interspecific competition, and the species' ability to recover from disturbance (Randall Hughes et al., 2008). In the current study, such low abundance accompanied by a small effective population size of *G*. *pulex* was detected at sites with extremely high, potentially acutely toxic levels of AOM (e.g., W5, W6, see Tab. SC8). *Gammarus pulex* at these sites may, due to reduced genetic diversity, show disrupted ecological performance, which was found to be associated with decreases in survival, body size, and reproduction (Aguirre-Gutierrez et al., 2015).

Reduced abundance and genetic diversity of a species may cause changes in interspecific competition and species community structure. In the Eine River, *G*. *pulex* was found to co-occur with *Gammarus roeselii* Gervais, 1835. *Gammarus pulex* can survive in sympatry with other amphipod species (Altermatt et al., 2019); however, the abundance of *G*. *pulex* was reduced at sites E2–E6 inhabited by both species. The allelic richness within *G*. *pulex* was not reduced at these sites. In contrast, comparatively low genetic richness but high abundance of *G*. *pulex* was seen at site E1 (Eine River), where no *G*. *roeselii* were found, indicating a genetic bottleneck for *G*. *pulex*. Comparable abundance patterns were shown for amphipod species that occupy similar ecological niches in streams, suggesting that colonizing history majorly influences species composition (Little & Altermatt, 2018).

In addition to interspecific competition, low genetic diversity at sites with comparably high AOM levels can affect the ability of *G*. *pulex* to respond to environmental changes in the long run. In populations with critically reduced genetic diversity the effects of environmental stress factors may be more pronounced, increasing species mortality and threatening its survival (Pearman & Garner, 2005). Moreover, chronic exposure to toxic AOM was shown to increase the susceptibility of *G*. *pulex* to additional acute stress (Švara et al., 2021). Thus, a combination of multiple environmental stressors, such as temperature extremes or increased parasitism, in parallel with increased levels of AOM, poses an increased adverse risk for *G*. *pulex* populations exhibiting low genetic diversity.

In conclusion, our results indicate that AOM contamination of rivers and streams can significantly shape the population genetic diversity of *G*. *pulex*. A decline in the genetic diversity of the species may lead to decreased species robustness to environmental stress that, in the long run, can affect the survival of this keystone species and enhance the risk of the loss of its ecosystem function.

Acknowledgments

We would like to thank Saskia Mertins, Sophie Oster, and Sergej Grebenyuk who took part in the ecotoxicology course at the UFZ in Leipzig in 2018 and Ute Lohse and Jörg Alheim for their support of the field- and laboratory work. Helge Norf, Anže Švara, and Elena Motivans Švara are acknowledged for fruitful discussions during the study and comments on the manuscript. An academic license for Marvin, JChem for Excel, and InstantJChem was provided by ChemAxon (Budapest, Hungary). The ArcMap waterway data were provided by the German Federal Agency for Cartography and Geodesy. The first author was partially supported by a fellowship of the Deutsche Bundesstiftung Umwelt (DBU) and the Helmholtz-RSF Joint Research Groups program from the Helmholtz Association and the Russian Science Foundation (RSF) (LaBeglo2/HRSF-0048). The QExactive Plus LC-HRMS used is part of the infrastructure initiative CITEPro (Chemicals in the Terrestrial Environment Profiler) funded by the Helmholtz Association. This work was a part of the Integrated Project “Healthy Aquatic Ecosystems” at the Helmholtz Centre

for Environmental Research—UFZ. Open Access funding enabled and organized by Projekt DEAL.

Conflict of interest

The authors declare no conflict of interest

Data availability statement

Data on measured AOM concentrations and microsatellite genotypes are available on Dryad (https://doi.org/10.5061/dryad.zw3r2288p). Sequencing data are available in GenBank.

4.6 Literature cited in Chapter 4

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Chapter 5

General conclusions and outlook on the use of population genetics for the assessment of the effects of AOM

General conclusions and remarks

In this chapter, general insights and conclusions from the results obtained in the previous chapters are presented. The main conclusions of the thesis are: 1) Applied analytical methods allow a robust and precise characterization of AOM with potential to affect the genetic diversity of *G. pulex;* 2) Population genetics approaches coupled with high-resolution chemical analytics enable to detect AOM-associated genetic changes in populations of an indicator species; 3) The population genetic pattern of *G. pulex* and its relationship to AOM was clear when comparing local and regional scales. Locally, *G. pulex* survives and forms viable populations without a significant relationship between species' genetic structure and AOM pollution patterns. However, regionally, spatially distinct populations can be identified; 4) To maintain high genetic diversity, *G. pulex* needs pristine river sections and sufficient connectivity among sites; 5) Finally, this research shows that there are effects from AOM pollution on the genetic diversity of amphipods and confirm that a reduced environmental pollution needs to be the goal for the future.

5.1 Applied analytical methods enabled characterization of toxic potentials of AOM for the analyzed species

Using LC-HRMS, over 200 AOM were determined in the assessed water and *G. pulex* tissue samples. The detected AOM prove that the method is successful in the detection of diverse, potentially environmentally harmful chemicals from water as well as from tissue of *G. pulex.* Thus, the method can robustly support the assessment of factors influencing the genetic diversity of the studied amphipod species. In comparison to previous studies assessing AOM from tissues, the applied target screening proved extremely successful; in some of the recent studies assessing AOM from amphipod tissue, fewer than 70 chemicals were detected (e.g., Inostroza et al., 2016; Munz et al., 2018; Shahid et al., 2018). The number of detected compounds depends on the available and selected targets in the target screening method, which was higher in our studies in contrast to the previous ones. Due to the successful detection of diverse AOM with a broad spectrum of application

types, the target screening methodology applied here proved suitable for further use in the assessment of AOM burden for freshwater arthropods. To be able to produce comparable data across different organism-groups, ecosystems, and geographic regions, it would be beneficial to apply an equivalent method and chemical targets in different studies. Moreover, a set of key compounds that pose risk to survival of freshwater organism and comprise as broad spectrum of AOM as possible, could be proposed in the future.

In the assessment of AOM, the main advantages of the target screening approach using water and *G. pulex* tissue samples were the efficient sample processing time and the precision of AOM determination with detection levels as precise as a few nanograms per millilitre of sample. The detection of chemicals was time efficient as up to several dozens of samples were processed in parallel by the application of the QuEChERS method. The inclusion of different sample types also enabled the screening of a chemically and temporally broad spectrum of AOM present at sites with *G. pulex*. The water samples consist of AOM containing mostly polar chemicals that are transported downstream in the water column. In contrast, AOM in *G. pulex* comprise polar and non-polar compounds that accumulate in tissues and may not all be transported further downstream. On top of the efficient AOM detection, the toxicity assessment for certain AOM using TUs was possible if reference toxicity data was available. Thus, the approach enabled to determine the selective potential of AOM from each site on the assessed *G. pulex* populations.

However, target screening of AOM in water and *G. pulex* tissue also indicated some shortcomings. The potential of AOM to affect amphipods may differ based on the size of the amphipods and lipid contents; larger amphipods may have higher lipid content than the smaller ones. Thus, the accumulation of certain hydrophobic AOM may be increased in bigger amphipods (Viganò et al., 2007). This could be considered in the future studies by measuring amphipod dry weight and lipid contents. In addition to the influence of different lipid contents on the accumulation of potentially harmful AOM in amphipods, some compounds that could potentially affect amphipods survival and genetic diversity may not be detected at all with the applied methods. For instance, extremely hydrophobic compounds, such as aromatic amines, which could be mutagenic and could promote new

variants in the exposed *G. pulex*, can only be detected by the application of passive samples and the analysis using GC-MS method (Muz et al., 2017). To improve the comprehensiveness of the assessment and estimation of the potential impact of some hydrophobic compounds, application of such an approach would be recommended.

From the detected AOM, TUs approach was used to characterize the selective potential of AOM on *G. pulex*. Based on this information, site-specific AOM toxicity was associated to differences in species' genetic diversity. However, to go a step further from potential toxicity estimation for *G. pulex*, actual effects of toxic AOM could be validated in laboratory experiments. The toxicity of selected AOM can be tested with the effect-based approaches in amphipods originating from populations from polluted and non-polluted river section. Such an approach would have the potential to empirically indicate selective effects of the environmentally-representative AOM mixtures on species phenotype and genetic diversity of amphipods. As shown here in chapter three, this could be valuable as the toxic effects at a specific concentration of a compound may significantly differ among populations. In addition to empirical testing of the selective potential of each compound in different populations, empirical tests of mutagenicity and sub-lethal effects could be performed. For instance, an *in situ* test of mutagenic compounds on *G. pulex* model populations could be performed to assess mutagenic effects. Effects of AOM on feeding of *G. pulex* can be tested with measuring shredding performance of different populations. Finally, reproduction rates in different populations can be assessed using different parameters, such as counting of copulas, eggs in females, or neonates. Such data could offer further insight in the potential effects of detected AOM on genetic diversity of *G. pulex*.

5.2 The main groups of AOM driving genetic impairment were identified

The AOM groups, which were investigated at multiple sites for the potential to cause adverse effects and selective pressure in *G. pulex* comprised insecticides, pharmaceuticals, industrial chemicals, and herbicides. Among the AOM with especially

high potential toxicity were neonicotinoid insecticides including acetaminophen, imidacloprid, and thiacloprid. The effects of these insecticides that can cause selective pressure on freshwater invertebrates are, in general, well understood and comprise lethal and sub-lethal effects (Englert et al., 2017; Gibbons et al., 2015; Münze et al., 2017; Nyman et al., 2013; Reiber et al., 2021). Among the most obvious effects are changes in amphipod survival, mobility, and feeding that prevent affected individuals to be active and compete for mates in the exposed populations (Nyman et al., 2013). Although some effects of the chemicals on amphipods are well-understood, studies on evolutionary change in populations exposed to such lethal and sub-lethal effects of insecticides are lacking and should be promoted. In-vitro and in-situ tests coupled with precise genetic characterization of exposed individuals and populations would be especially beneficial. Such studies should be conducted to understand the effects associated with the impairment of the gene pool in natural populations of organisms. In Chapter 3, it was shown that toxic exposure, mostly due to the presence of insecticides, increases the sensitivity of *G. pulex* in polluted river sections. Although the increase of sensitivity in these amphipods was clearly indicated, the physiological mechanisms leading to the increased sensitivity and the contribution of environmental factors, such as differences in river temperatures and potential eutrophication, were out of the scope of the study. To better understand the physiological mechanisms caused by AOM and their interaction with the most relevant environmental factors, experiments focusing on physiological condition, metabolic rates, expression of selected genes, and energy budget of exposed animals should be performed.

The other two groups of AOM with the potential for causing genetic alterations in *G. pulex*, include pharmaceuticals and industrial AOM, which consist of extremely diverse compounds. Pollutants from these groups mainly enter rivers through WWTP and can occasionally be found in *G. pulex* tissue at concentrations that are acutely toxic to amphipods (see Chapter 4). Still, most of the compounds belonging to these groups did not indicate high toxicity, despite accumulation at high concentrations. However, the effects of these compounds, when present in the tissue of amphipods, are in general not very well understood. This is mostly due to a lack of studies on the specific effects and fate of compounds in invertebrate metabolism pathways. The effects of industrial

chemicals and pharmaceuticals in *G. pulex* may result in endocrine disruption or changed behavior that can be reflected by altered activity, behavior, and reproduction success including different mating behavior and fertility rates (Geffard et al., 2010; Gross et al., 2001; Peschke et al., 2014; Xuereb et al., 2011). However, no studies showing a direct connection between altered behaviour, mating activity in affected individuals, and the genetic diversity in affected populations of *Gammarus* amphipods have been performed thus far. Therefore, the acquisition of such data on AOM affecting amphipod behavior would be especially beneficial in the future.

5.3 Application of genetic markers in the assessment of toxic effects in populations of *G. pulex*

Population genetics was applied to compare the effect of AOM on the genetic diversity parameters of *G. pulex* within and between the studied rivers. According to the findings presented here, the application of the novel set of microsatellite markers enabled the estimation of several population genetics indices of the freshwater indicator macroinvertebrate species *G. pulex.* As shown in chapter 2, applied microsatellite loci clearly indicated genetic differentiation of spatially separated populations of *G. pulex* living in three different rivers. Moreover, using 16 microsatellites, locally specific genetic patterns and differentiation within spatially proximate populations were detected in chapter 4. Thus, in chapter 4, the genetic structure of different populations living only several kilometers from each other, i.e., near the confluence of the Eine River and Wipper River, was retrieved. In addition to significant differences among populations living in different rivers, a sub-population structure with significant genetic differentiation was successfully detected for sites from the Eine River. These results show that the applied set of microsatellite loci is suitable for application in future studies on fine-scale genetic structure in *Gammarus* species.

In chapters 3 and 4, the microsatellites were applied in a genetic diversity parameter analysis, estimating allelic richness, private allele rates, effective population size,

observed and expected heterozygosity, and inbreeding rates of *G. pulex*. Among the analyzed parameters, the increased levels of AOM were significantly associated with reduced allelic richness and increased inbreeding in *G. pulex*. Thus, the application of microsatellites proved suitable for fine scale genetic diversity analysis of populations of freshwater macroinvertebrates in relationship to AOM. This conclusion is in line with studies that applied microsatellites in the assessment of population genetic patterns in *G. fossarum*, e.g., in the assessment of allelic richness and population structure in relation to river morphology change (dams), river networks, and amphipods' migration ability (Alther et al., 2021; Seymour et al., 2016; Weiss & Leese, 2016). In addition to the listed environmental factors, AOM are likely an important factor contributing to altered genetic diversity of species, as shown here. Thus, in environmental studies, the application of microsatellites to detect significant negative changes in species' genetic diversity can supplement chemical assessment and toxicity estimation in the identification of areas where contaminants act selectively and threaten long-term species survival. On the one hand, a reduction in the genetic diversity of a common and relatively AOM-resistant species, such as *G. pulex,* can indicate the potential for selective effects of toxic AOM in a specific ecosystem. On the other hand, a detection of a reduced genetic diversity in a rare and endangered species can enable to predict potential bottlenecks that could threaten species survival in an area of interest (e.g., Hamill et al., 2007). Therefore, microsatellites or other comparable population genetic methods should be applied in the future in the analysis of genetic diversity parameters as an effective and cost-efficient method in the assessment of the effects of anthropogenic disturbances in natural populations.

5.4 Genetic indices corresponding to AOM in freshwater habitats

In chapter 4, the allelic richness of *G. pulex* was, based on LME and SEM, significantly negatively correlated to the total concentration of detected AOM. The trend of reduced allelic richness was observed across the studied rivers; however, the relationship was not significant for each single river. A larger sample set of further analyzed sites may enable

the determination of the significance of this relationship for each single river. At sites indicating a significant negative relationship between AOM and allelic richness, the most likely driver of changes in the genetic diversity of amphipod populations exposed to AOM appear to be pesticides. Pesticides were significantly correlated to increasead inbreeding rates and were detected at levels that translated into the highest toxic potential for *G. pulex.* They drive adverse effects in the species, as shown previous studies (Agatz et al., 2014; Ashauer et al., 2011; Nyman et al., 2013; Shahid et al., 2018b). However, as the results presented here do not indicate causality of the negative relationship between reduced genetic diversity and increased levels of AOM, further *in vitro* and *in situ* investigations would be necessary to completely understand how insecticides select for specific genotypes in a single stressor scenario, mixture of different AOM scenario, and, finally, in an exposure to AOM and multiple environmental stressors. In addition, it would be extremely beneficial to test whether environmentally relevant mixtures of AOM compounds, which are not necessarily acutely toxic but are detected at high concentrations, cause sub-lethal chronic effects (e.g., endocrine disruption). From the population genetics perspective, a test of the impacts of such AOM on *Gammarus* population genetic structure and diversity could be performed.

In contrast to the association between AOM and the genetic diversity of *G. pulex,* the results in this thesis indicated that changes in the genetic structure and differentiation among *G. pulex* from different sampling sites were, in general, not related to differences in the amount of AOM or toxicity of AOM in rivers. As suggested by other studies, at least three conditions need to be met for a natural population to genetically shift towards a different optimum, e.g. for tolerance to a toxic contaminant in an exposed population, and a different genetic pattern in relation to other, non-exposed populations (Hoffmann & Willi, 2008). First, the decline in population size in the focal area needs to be large enough to affect genetic variation. Second, to preserve site-specific variation, a certain degree of isolation is necessary to prevent an input of common alleles from nearby populations. Third, the contaminants need to exert a sufficiently strong effect to select for specific genotypes in the given population. Within the studied rivers, there is a high chance that not all of these criteria were met, especially as the studied sites within rivers proved to be well connected to each other. On the other hand, toxicity of the detected AOM was often

in a chronic or even acute range, which contributes to the low genetic diversity at these sites. Despite reduced genetic diversity of *G. pulex*, it is likely that the dynamic spatial and temporal patterns of AOM pollution in combination with migration of individuals from non-contaminated sites prevent the adaptation to a single driver of selection and local genetic separation of populations. This was also confirmed by the fact that there was no exclusive genotype detected for any of the studied microsatellite loci in *G. pulex* from sites with high AOM levels and toxicity potential. The low abundance of *G. pulex* at some of the studied sites might indicate that the toxicity of AOM and other environmental stressors could lead to site-specific conditions unfavorable for the survival of amphipods regardless. To investigate to which degree amphipods are adapted to local, river-related contamination with AOM, a comparison of *G. pulex* sensitivities among rivers with separated populations should be conducted and compared to controls from pristine sites from the upstream reaches of the studied rivers.

5.5 Applicability of microsatellites in comparison to other population genetics methods

In addition to *G. pulex* microsatellite genotyping, a COI sequence comparison method was applied in the study described in chapter 3 to assess the genetic structure of *G. pulex* in the Holtemme River. In contrast to the microsatellite nuclear loci-based method that can be applied in the assessment of fine-scale spatial genetic patterns, the COI genetic region is located in the mitochondrial genome and is therefore usually better conserved than microsatellites and inherited only through females (Waugh, 2007). The COI barcoding method is likely to identify genetic differentiation patterns at the species level rather than at the population level within a single species (e.g. Delić et al., 2017; Gurkov et al., 2019). The COI haplotype analysis used in chapter 3 indicated similar results to microsatellite genotyping; a single within-river population was detected in the Holtemme River as a result of COI barcode analysis and microsatellite analysis. However, in contrast to the microsatellite results, the genetic differentiation among populations from other rivers was not successfully identified based on the barcode sequences. Nevertheless, the method

still enabled the retrieval of information on genetic diversity patterns in the river, i.e., by the assessment of haplotype diversity. According to these results, the application of COI barcode analysis in relating genetic diversity indices to AOM patterns might be especially applicable when comparing different species living in AOM-polluted environment at a larger geographical scale. In such cases a single set of microsatellites cannot be applied due to large interspecific genetic differences. Thus, the main advantage of this method is the applicability and estimation of genetic diversity and population structure across genetic lineages, species and cryptic species, and even different taxa living in comparable environments polluted with AOM. For instance, genetic diversity parameters of insect and crustacean species living in different environments can be directly compared with this method.

Currently, in addition to microsatellite analysis and barcoding, the whole genomes are becoming available for more and more species and are starting to be implemented in ecotoxicological research (Osterberg et al., 2018; Poynton et al., 2018). With novel sequencing techniques and their widespread application, the utilization of such genome-wide analysis is becoming affordable and widely applicable (Pool et al., 2010). Techniques such as double digested reduced-representation genome sequencing (ddRADseq) already enable the screening of nucleotide diversity across the whole genome and predict changes in populations with higher precision. This genome-screening approach provides genetic diversity information based on thousands of polymorphisms and can be associated with specific genomic regions and functional genes. Therefore, future studies on organisms' genetic diversity and adjustment to pollution could be based on this approach. Such studies should be performed when the more cost- and time-effective microsatellite method would not be expected to produce significant results. Furthermore, the attention of studies investigating the evolutionary response of *G. pulex* to AOM could be focused on specific genomic regions relevant for toxicity response, including coding and non-coding DNA. This approach proved valuable in the assessment of population genetic response to freshwater pollution of organisms, such as fish and amphipod (Oziolor et al., 2019; Weston et al., 2013).

5.6 Importance of genetically diverse populations for species survival

The genetic diversity is one of the three key elements of biodiversity (besides ecosystem, and species diversity) and is especially important because it enables species to adjust to changes in its habitat. Based on the fact that species will have to adjust to ongoing global temperature increase, including higher temperatures in freshwater ecosystems, the diverse genetic pool of species will be increasingly important (Geffroy & Wedekind, 2020). Higher temperatures in rivers and more frequent droughts will affect indigenous amphipod species and amplify the effects of multiple stressors, including AOM, on freshwater biota. The effects of a combination of different stressors acting simultaneously is, however, difficult to predict. Dependent on the species and the magnitude of different stressors, a specific genotype may be beneficial in one scenario, but would disappear from the population in another (Roger et al., 2012). On the other hand, as shown in chapter 3 and 4, multiple stressors were not necessary the most important factor for the selection of a specific genotype at the studied site, as connectivity played a larger role. This has also been shown for riverine fish species, suggesting a 1.8 times larger impact of the riverine network on the genetic structure of a species in comparison to multiple stressors (Prunier et al., 2018). Due to this complex impact of different environmental factors influencing the genetic pool of a species in a river, the preservation of diverse communities and species is, thus, key for the continued survival of impacted species. In fact, it has already been shown that increased temperatures also increase the susceptibility of organisms for adverse effects of AOM (Macaulay et al., 2020). Consequently, even stronger negative effects of AOM on biodiversity should be expected in scenarios where the application of toxic contaminants will not be reduced and prevented from entering freshwater habitats. As a consequence, further loss of ecosystem functions and services can be expected. The most recent goals on biodiversity conservation worldwide aim for the opposite – slowing down the loss of biodiversity or even reverting the negative trend (Perino et al., 2021). These goals will be difficult to meet, however, by screening functional genetic diversity, novel genetic methods offer a way to assess the magnitude of effects on species genetic diversity, identify key units of species that should be a conservation priority, and support implementation of conservation measures in freshwater environments. Thus, the

application of these methods can also importantly contribute to the conservation of species under the pressure of environmentally relevant pollutants.

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Supplementary information A

Isolation and characterization of eleven novel microsatellite markers for fine-scale population genetic analyses of *Gammarus pulex* (Crustacea: Amphipoda)

Supplementary Tables A

Table SA1 DNA sequences of the COI gene region presented at the phylogenetic tree (Fig. 2.1a) with respective Genbank accession numbers.

	COI Sequence
***G._pulex*_Saale_1** **Genbank accession number:** **MN400975**	TACTTTATACTTTATCTTAGGGGCTTGGGCTAGTGTTGTTGGCACTTCCATGAGAGTG ATTATTCGCTCAGAACTAAGTACCCCTGGTAATTTAATCGATGATGACCAATTATATAA CGTCATAGTTACCGCTCACGCTTTTGTTATAATTTTTTTTATAGTCATACCTATTATAAT CGGTGGTTTCGGGAATTGACTGGTGCCATTAATGCTAGGTAGACCTGATATAGCTTTT CCGCGTATAAACAATATAAGATTTTGACTTTTACCTCCTTCTCTCACCCTTCTGCTTAT AAGTAGTATAGTAGAAAGAGGTGTAGGAACGGGTTGAACGGTATACCCTCCGTTGGC AGGTATCTCAGCTCATGGGGGTGGAGCGGTAGATCTAGCCATTTTTTCACTCCATTTA GCAGGGGCCTCCTCTATCCTAGGCGCTATTAATTTTATCTCTACTGTAATCAATATAC GTAGACCTGGTATATCTATAGACCAAACGCCTCTTTTTGTTTGGTCTGTCTTTATTACA GCTATCTTACTCCTATTATCCTTACCTGTTTTAGCCGGCGCTATCACTATGCTCCTGA CTGACCGAAACTTAAATACTTCTTTCTTCGACCCTAGCGGTGGAGGAGATCCTATTTT GTACCAACACTTATTC
***G._pulex*_Holtemme_1** **Genbank accession number:** **MN400976**	TACTTTATACTTTATCTTAGGGGCTTGGGCTAGTGTTGTTGGCACTTCCATGAGAGTG ATTATTCGCTCAGAACTAAGTACCCCTGGTAATTTAATCGATGATGACCAATTATATAA CGTCATAGTTACCGCTCACGCTTTTGTTATAATTTTTTTTATAGTCATACCTATTATAAT TGGTGGTTTCGGGAATTGACTGGTGCCATTAATGCTAGGTAGACCTGATATAGCTTTT CCGCGTATAAACAATATAAGATTTTGACTTTTACCTCCTTCTCTCACCCTTCTGCTTAT AAGTAGTATAGTAGAAAGAGGTGTAGGAACGGGTTGAACGGTATACCCTCCGTTGGC AGGTATCTCAGCTCATGGGGGTGGAGCGGTAGATCTAGCCATTTTTTCACTCCATTTA GCAGGGGCCTCCTCTATCCTAGGCGCTATTAATTTTATCTCTACTGTAATCAATATAC GTAGACCTGGTATATCTATAGACCAAACGCCTCTTTTTGTTTGGTCTGTCTTTATTACA GCTATCTTACTCCTATTATCCTTACCTGTTTTAGCCGGCGCTATCACTATGCTCCTGA CTGACCGAAACTTAAATACTTCTTTCTTCGACCCTAGCGGTGGAGGAGATCCTATTTT GTACCAACACTTATTC

***G._pulex*_Parthe_1** **Genbank accession number:** **MN400977**	TACTTTATACTTTATCTTAGGGGCTTGGGCTAGTGTTGTTGGCACTTCCATGAGAGTG ATTATTCGCTCAGAACTAAGTACCCCTGGTAATTTAATCGATGATGACCAATTATATAA CGTCATAGTTACCGCTCACGCTTTTGTTATAATTTTTTTTATAGTTATACCTATTATAAT TGGTGGTTTCGGGAATTGACTGGTGCCATTAATGCTAGGTAGACCTGATATAGCTTTT CCGCGTATAAACAATATAAGATTTTGACTTTTACCTCCTTCTCTCACCCTTCTGCTTAT AAGTAGTATAGTAGAAAGAGGTGTAGGAACGGGTTGAACGGTATACCCTCCGTTGGC AGGTATCTCAGCTCATGGGGGTGGAGCGGTAGATCTAGCCATTTTTTCACTCCATTTA GCAGGGGCCTCCTCTATCCTAGGCGCTATTAATTTTATCTCTACTGTAATCAATATAC GTAGACCTGGTATATCTATAGACCAAACGCCTCTTTTTGTTTGGTCTGTCTTTATTACA GCTATCTTACTCCTATTATCCTTACCTGTTTTAGCCGGCGCTATCACTATGCTCCTGA CTGACCGAAACTTAAATACTTCTTTCTTCGACCCTAGCGGTGGAGGAGATCCTATTTT GTACCAACACTTATTC

Table SA2 Distance matrix between COI sequences of samples from the Saale catchment and the reference samples from the Ruhr region. The genetic distances are listed on the bottom-left side of the table and the standard errors are on the top-right side of the table.

	Sal	**Hol**	**Par**	**Gp_E**	**Gp_C**	**Gp_D**	**Gf**
***G._pulex*_Saale_1**		0.001	0.002	0.002	0.014	0.014	0.024
***G._pulex*_Holtemme_1**	0.002		0.002	0.001	0.014	0.014	0.024
***G._pulex*_Parthe_1**	0.003	0.002		0.002	0.013	0.014	0.024
KT075230.1_*G._pulex*_E	0.003	0.002	0.003		0.013	0.014	0.024
KT075256.1_*G._pulex*_C	0.111	0.109	0.107	0.107		0.007	0.024
KT075232.1_*G._pulex*_D	0.116	0.118	0.116	0.116	0.039		0.024
KF521835.1_*G._fossarum*	0.278	0.28	0.28	0.278	0.277	0.268	

Supplementary information B

Chemical Pollution Levels in a River Explain Site-specific Sensitivities to Micropollutants within a Genetically Homogenous Population of Freshwater Amphipods

Supplementary Text B

Material and Methods B

SB1 LC-HRMS sample preparation and analysis

SB1.1. Water sample analysis

The pH of 1 mL water sample aliquots was adjusted to 3.5 by adding 10 µL of 2 M ammonium formate buffer to roughly match that of the LC eluent (0.1% formic acid, pH 2.6); 25 µL of an internal standard mixture containing 40 isotope-labelled compounds (40 ng/mL of each compound) and 25 µL of methanol were added. Matrix-matched calibration standards were prepared in the same way by spiking 1 mL water sample aliquots from a pristine stream (Wormsgraben, upper Harz Mountains, Germany) with the target analytes at concentrations from 1 to 5000 ng/L.

SB1.2. Body burden analysis

Gammarus pulex tissue samples were extracted using the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method according to Inostroza et al. (2016).

Accordingly, 900 mg of amphipod tissue was homogenized in a mixture comprising 2 mL acetonitrile, 2 mL water and 1 mL hexane using an Ultra-Turrax T-25 (IKA) for ca. 60 s and then the homogenate was vortexed for 60 s. Subsequently, 800 mg anhydrous $MgSO_4$ and 200 mg NaCl were added, the samples were vortexed again and centrifuged at 4000× *g* for 5 min. The supernatant was transferred to centrifugation tubes containing 50 mg primary-secondary amine and 400 mg anhydrous $MgSO_4$ for clean-up. The suspension was vortexed and centrifuged. The supernatant was moved into an evaporation vial and dried under the nitrogen stream at room temperature. Finally, 450 µL methanol and 50 µL of internal standard solution (final level 100 ng/mL) were added. Method-matched calibration standards were prepared by spiking target analyte solutions into 2 mL LC-MS grade water, which were processed the same way as the samples, corresponding to final levels of 0.1-200 ng/g in vial.

SB1.3. LC-HRMS analysis

Water samples and *Gammarus* extracts were analyzed 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 ionization source. LC separation was done on a Kinetex C18 EVO 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 min of 5% B, the fraction B was linearly increased to 100% within 12 min and 100% B were kept for 11 min. The eluent flow was diverted to waste and the column was rinsed

for 2 min 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 min. The injection volume was 5 µL for *Gammarus* extracts and 100 µL for water samples 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 kV (positive mode) or 3.5 kV (neg. mode), the sheath gas flow rate was 45 a.u. and the auxiliary gas flow rate 1 a.u.. Separate runs were conducted in positive and negative ion mode combining a full scan experiment (100-1000 m/z) at a nominal resolving power of 70,000 (referenced to m/z 200) and data-independent MS/MS experiments at a nominal resolving power of 35,000. For the latter, we acquired the data using broad isolation windows of about 50 Th (i.e., m/z ranges 97-147, 144-194, 191-241, 238-288, 285-335, 332-382, 379-429, 426-476) and 280 Th (i.e., m/z ranges 460-740, 730-1010), respectively.

SB2 Polymerase chain reactions, sequencing and genotyping information

COI PCR and sequencing reactions

50 µL PCR reactions contained 2.5 µL of 10 mM dNTPs, 10 µL 5X Green GoTaq Flexi Buffer (Promega), 4 µL of 25 mM $MgCl_2$, 1 µL of GoTaq DNA polymerase and 22.5 µL of deionized water. Each primer (Supp. 2) was diluted from the stock solution to 10 µM and 2.5 µL were added to the PCR reaction. Finally, 5 µL of DNA template with concentration between 40-80 ng/µL was added to the mix. The polymerase chain reaction (PCR) cycling setting was set to initial activation step of 2 min at 95°C, following by 34 cycles of 1 min denaturation at 95°C, 45 s annealing at 51°C and 1 min elongation at 72°C. The reaction was terminated after the final elongation of 5 min at 72°C. The PCR products were checked on the agarose gel and cleaned using GeneJET PCR Purification Kit (ThermoFisher Scientific) following the kit instructions. The 10 µL sequencing reaction of 150-250 ng of DNA with 1µL of the sequencing primer, Big dye mix (Thermo Fisher Scientific), and 5x sequencing buffer was prepared for each PCR product using the following program: 1 min at 96°C, 30 cycles of 10 s at 96°C, 5 s at 50°C and 4 min at 60°C. The products were purified by ethanol/EDTA (ethylenediaminetetraacetic acid) precipitation protocol (Applied Biosystems, 2010), and diluted in 10 µL HiDi formamide (Thermo Fisher Scientific). The samples were separated on an ABI Prism 3130XL Genetic Analyzer (Applied Biosystems).

SB3 COI sequence and microsatellite data analyses and visualization

COI Sequence Data

COI sequence reads were assembled and edited in Sequencher 5.4.5, with gaps coded as (-). Assembled sequences were aligned with *G. pulex* sequences from European rivers acquired from the National Center for Biotechnology Information (NCBI) (EU146924.1-G_pulex_E, KT075232.1-G_pulex_D, KT075256.1-G_pulex_C, KF521835.1-G_fossarum) using ClustalW in MEGA7 (Kumar et al., 2008) with default settings for alignment. Based on the alignment, a maximum likelihood tree with best fitting Tamura 3-parameter model and a nearest-neighbor-interchange tree inference method (Tamura et al., 2011) was constructed. The percentage concordance was calculated with 1000 bootstrap iterations. The phylogenetic tree was visualized with FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). The genetic distances between *G. pulex* samples were calculated using the Tamura 3-parameter model and a bootstrap variance estimation with 1000 replications. In order to test for population differentiation, pairwise fixation index (F_{st}) values were calculated in Arlequin 3.5 (Excoffier & Lischer, 2010) with 3024 permutations. The genetic structure network of *G. pulex* haplotypes from the Holtemme River and closely related samples was visualized using PopART (Leigh & Bryant, 2015).

Microsatellite Data

Microsatellite fragments were checked for stuttering and null alleles with Microchecker (Van Oosterhout et al., 2004). To analyze site-specific population patterns, allelic diversity, observed (Ho) and expected (He) heterozygosity, linkage disequilibrium, and Hardy-Weinberg equilibrium in each population were estimated using Fstat 2.9.3.2 (Goudet et al., 1995) and Arlequin 3.5.1.2. (Excoffier & Lischer, 2010). The population differentiation was determined in FreeNA (Chapuis & Estoup, 2007) by global and pairwise F_{st} comparison applying a null allele correction and 10000 permutations. The population structure was determined in Structure 2.3.4.(Raj et al., 2014). An admixture model without any *a priori* information was run 10 times for clusters K from 1 to 6 using 100000 MCMC steps and discarding the first 20000 steps as a burn-in. The optimal number of clusters was determined in Structure Harvester (Earl & von Holdt, 2012) with the Evanno method (Evanno et al., 2005). The effective population sizes for each location were estimated based on linkage disequilibrium approach and alleles with frequencies higher than 0.01 in NeEstimator 2.0.2 (Do et al., 2014).

SB4 Experimental conditions in exposure experiments

Each treatment/control included five replicates with ten amphipods per beaker. The beakers were kept in a chamber with a temperature of 15°C, air humidity of 70%, and a 16h:8h light:dark photoperiod. The exposure medium was aerated using air pumps (RENA Air 50) with the air being pumped through glass a pipette tip attached to the pump tube and inserted into the medium to avoid adsorption of chemicals.

The amphipods were first placed in the beakers containing ADaM medium and acclimated seven days prior to the start of the experiment with ten 1 cm^2 *Fagus sylvatica* leaf discs that were conditioned in stream water for two weeks. Eaten leaf disks were replaced by new ones. At the start of the exposure experiment, new beakers were filled with 0.5 L ADaM medium and spiked with imidacloprid stock solution for exposure treatments and with DMSO for solvent controls. The solutions in the beakers were stirred with a glass rod and the beakers were covered with glass lids to prevent water evaporation. Subsequently, fresh leaf discs and acclimated amphipods were added to the exposure beakers. Experimental solutions were exchanged after seven days to ensure stable imidacloprid concentrations throughout the experiment. New beakers with ADaM medium and exposure solutions were prepared, followed by a move of amphipods and leafs to the new beakers. For the verification of consistency of imidacloprid concentration in the media, a 1 mL water sample of all treatment/control solutions was taken for LC-HRMS quantification directly after spiking, directly before and after the medium exchange, and at the end of the exposure experiment.

SB5 Non-linear Hill model used to determine LT_{50} values in the exposure experiments

$$E(t_i) = \min + \frac{(\max - \min)}{1 + (\frac{LT_{50}}{t_i})^p}$$

where

$E(t_i)$ is the increase of mortality in time t_i

min is the observed value of dead animals at the time 0

max is the maximum number of dead animals (10)

t_i is the time of exposure to imidacloprid

p is the Hill number

LT_{50} is the value describing the time of the 50% mortality of all individuals

LT_{50} values were used as a measure of the sensitivity of animals from polluted and non-polluted locations.

SB6 COI Haplotype Sequences with GenBank Accession Codes

gp1 (MW221949)
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gp2 (MN400976.1)
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gp5 (MW221952)
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gp8 (MW221955)
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gp15 (MW221962)
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gp16 (MW221963)
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Supplementary Figures B

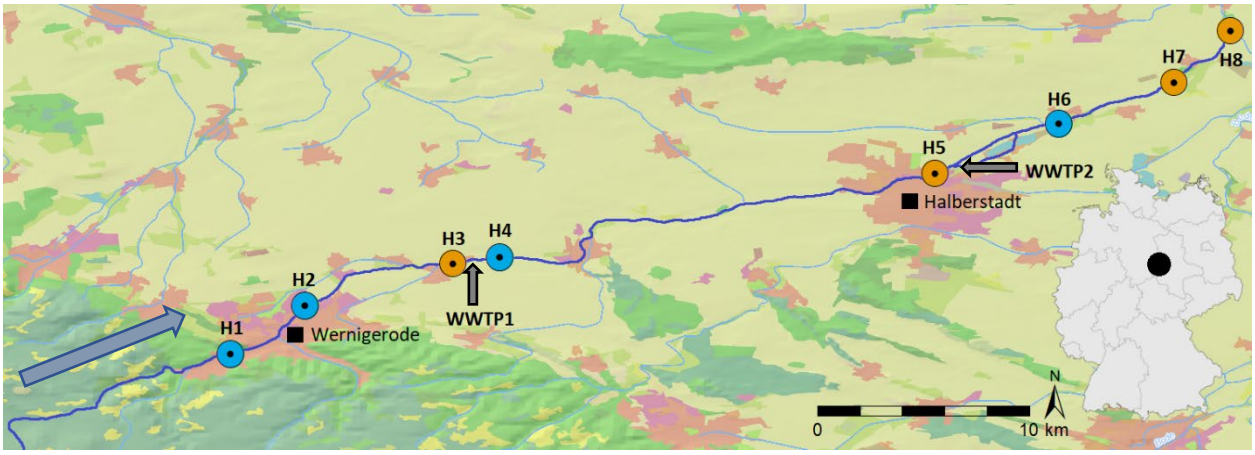

Figure SB1 Map of the studied section of the Holtemme River, Germany. Circles mark the sampling sites (H1-H8; orange and blue circles - locations of chemical (water, *G. pulex*) and genetic (*G. pulex*) analyses; blue circles - locations of additional *G. pulex* samplings for exposure experiments). Grey arrows indicate wastewater treatment plants (WWTP). The blue arrow indicates the flow direction of the river.

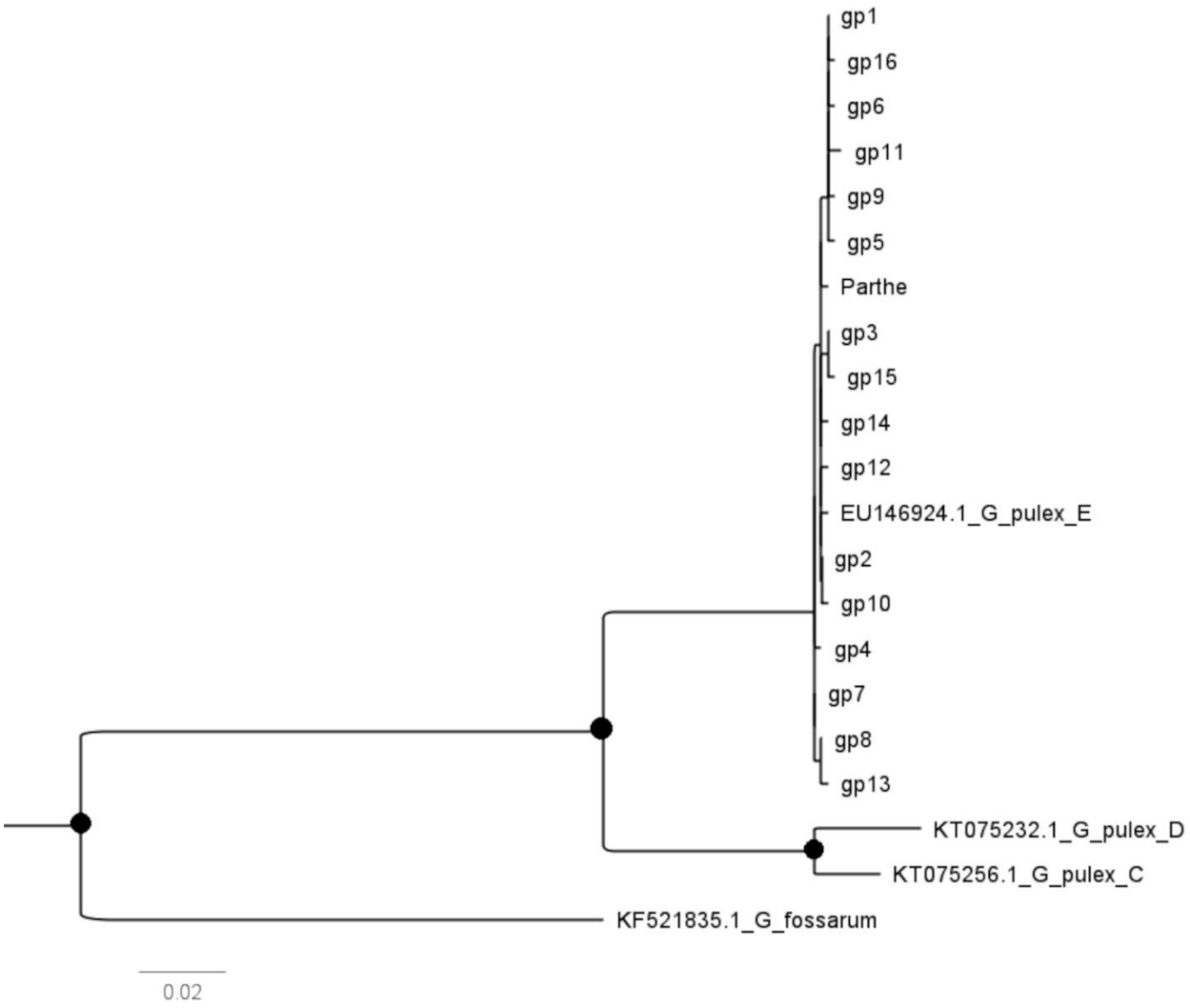

Figure SB2 Maximum likelihood phylogenetic tree of partial COI sequences from the Holtemme River, Parthe River and reference sequences from Genebank. Bootstrap values of > 99 are shown with black dots.

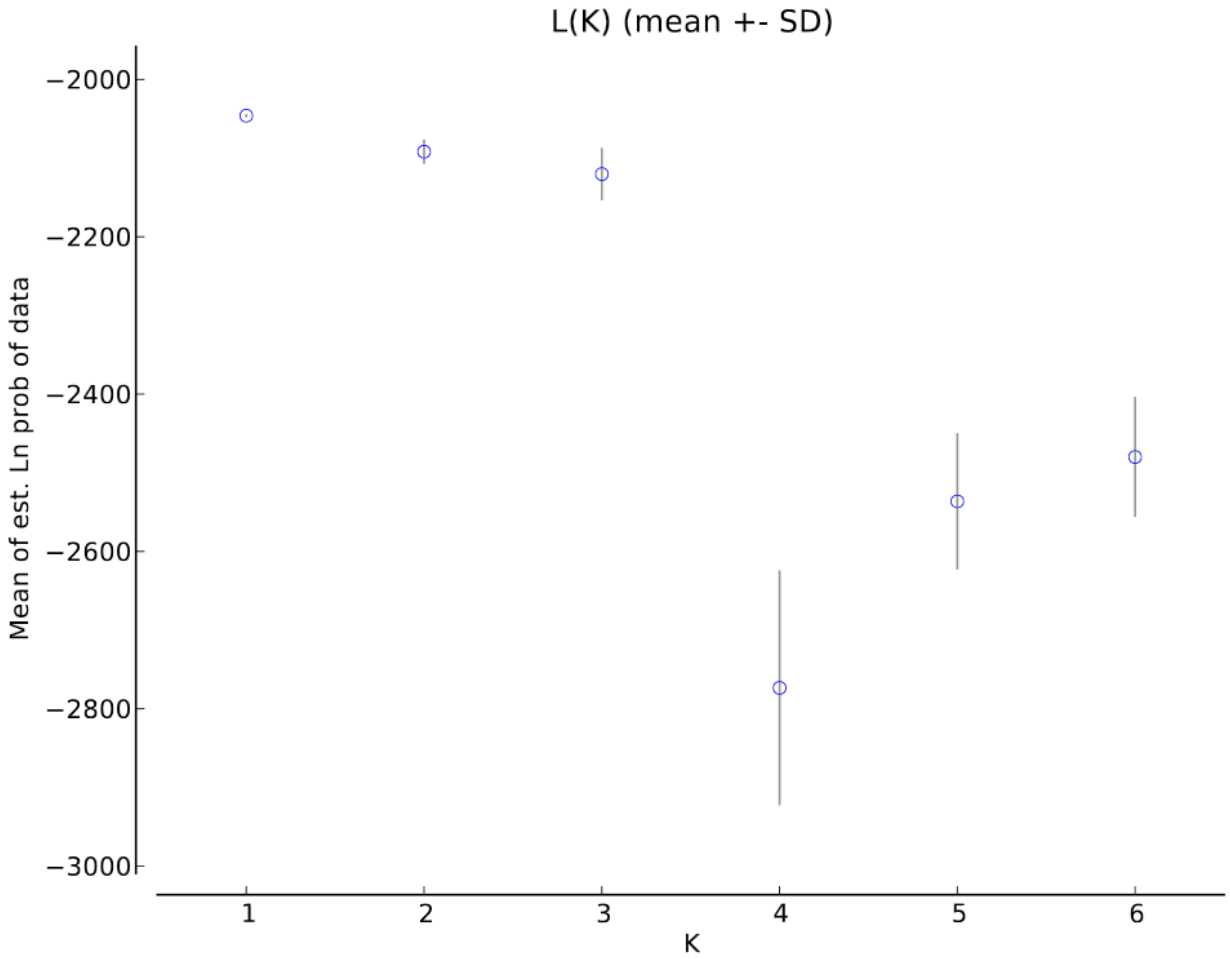

Figure SB3 Likelihood values for Structure analysis acquired from Structure Harvester for the given microsatellite samples with K selected from 1 to 6.

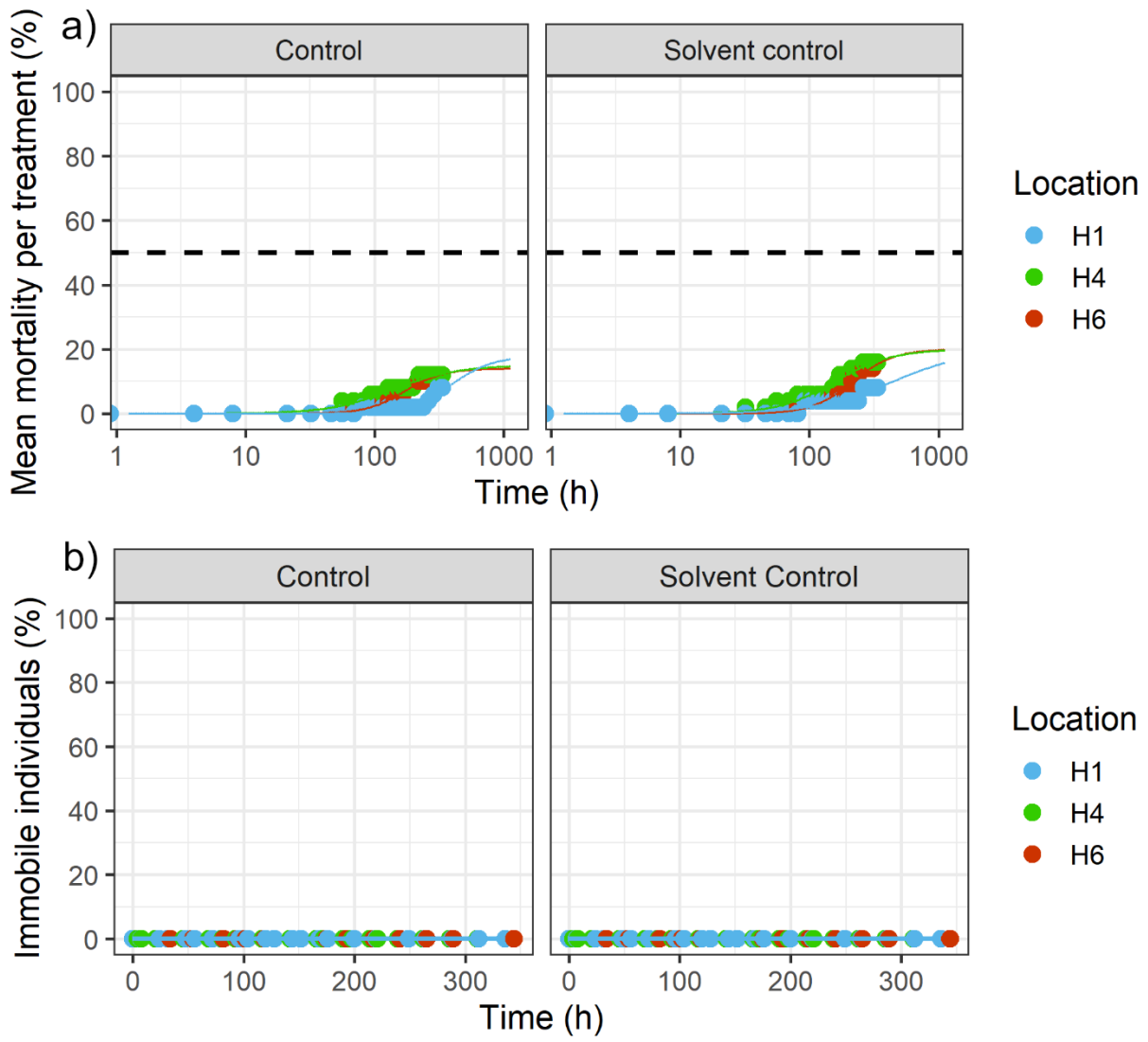

Figure SB4 Control treatments for exposure experiments with *Gammarus pulex* from different sampling locations. (a) Mean mortality per treatment for each location (mean value of dead amphipods per beaker in % of the total of 50 amphipods in 5 replicates) in control and solvent control treatments in a 14 d (336 h) exposure with amphipods from three sampling locations. The dotted line marks a mortality rate of 50%. (b) Percentage of immobile amphipods in controls and solvent controls across 14 d (336 h) exposure. 100% represents 50 individuals (10 individuals in each of 5 replicates). Lines were fitted to the data for each sampling location using linear regression.

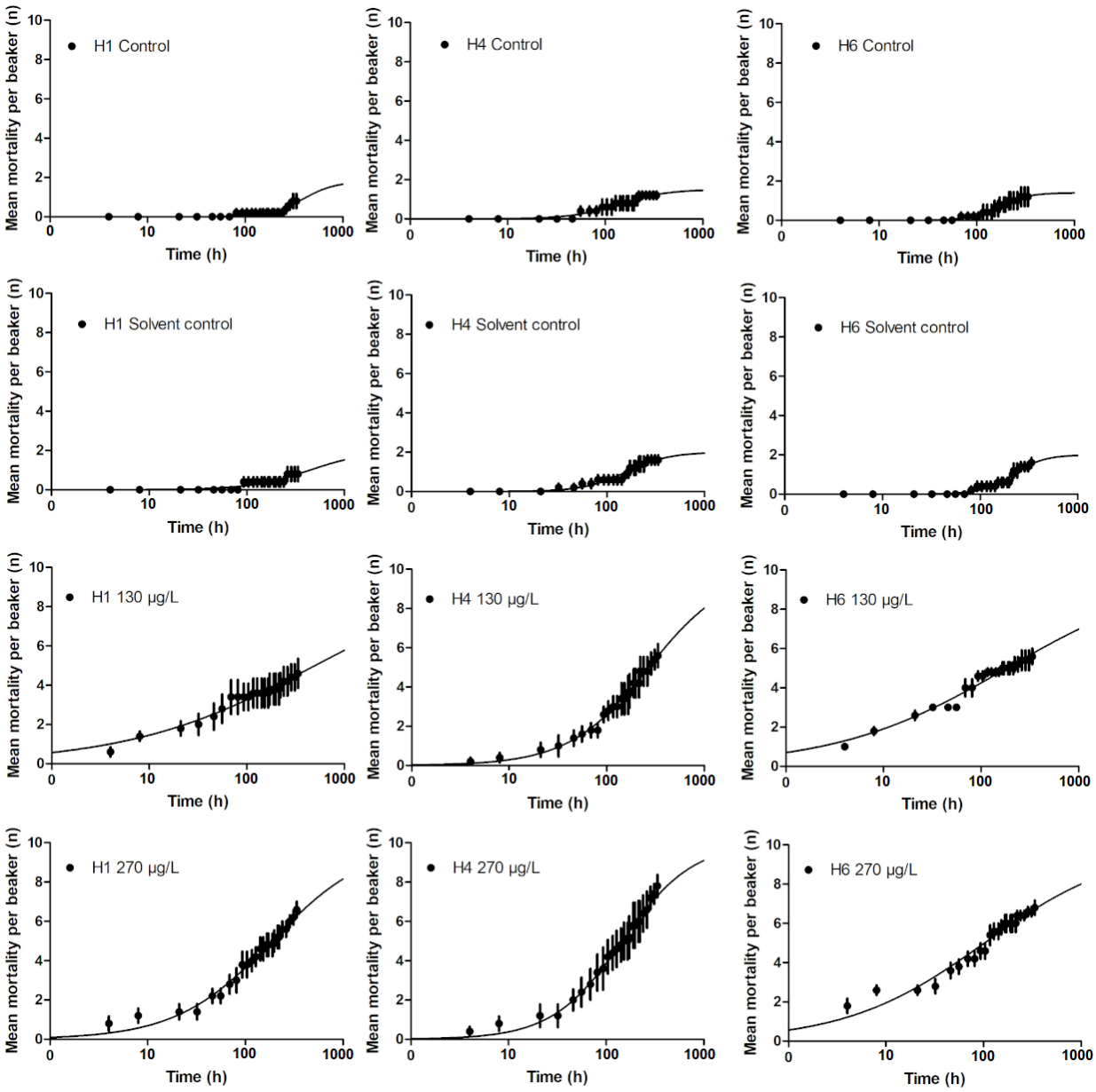

Figure SB5 Mean mortalities (± SEM) of *Gammarus pulex* in controls, solvent controls and imidacloprid treatments in the exposure experiment with amphipods from sampling sites H1, H4 and H6. N = 5.

Supplementary Tables B

Table SB1 List of sampling sites in the Holtemme River and in the Parthe River with information on type of samples, sampling date, *Gammarus pulex* abundance, pH value, temperature, water oxygen concentration, and water oxygen saturation.

Code	Location name	Sampling for	Sampling date (DD/MM/YYYY)	Coordinates (Lat., Long.)	*G. pulex* abundance (n per catch)	pH	T (°C)	Oxygen concentr-ation (mg/L)	Oxygen saturati-on (%)
H1	Wernigerode Hochschule	Genetics, analytics Exposure test	20/07/2017 20/07/2017	10.873714, 51.867732	<10	7.21	9.8	11.66	99.7
H2	Wernigerode town	Toxicokinetic	15/10/2017	10.791327, 51.847054	<10	7.33	10.6	12.05	100.2
H3	Silstedt upstream of WWTP1	Genetics, analytics	15/03/2017	10.853865, 51.865251	10–100	7.62	7.5	11.89	99.4
H4	Streuobstwiese, downstream of WWTP1	Genetics, analytics Exposure test	15/03/2017 24/05/2017	10.873714, 51.867732	100–200	7.55	8.3	11.91	101.8
H5	Halberstadt, upstream of WWTP2	Genetics, analytics	16/03/2017	11.057809, 51.903227	>500	8.53	5.2	12.52	99.1
H6	Groß Quenstedt, downstream of WWTP2	Genetics, analytics Exposure test Toxicokinetic	16/03/2017 11/06/2017 15/10/2017	11.110006, 51.923896	100–200	8.26	9.1	12.16	106.0
H7	Mobicos Nienhagen	Genetics, Analytics	15/03/2017	11.158756, 51.941673	10–100	8.34	8.4	12.92	108.0
H8	Holtemme–Bode confluence	Genetics, Analytics	16/03/2017	11.182576, 51.963426	<10	8.31	9.6	12.37	108.5
P1	Parthe, Glasten, Bad Lausick	Genetics	16/03/2017	51.153025, 12.701842	<10	7.11	10.3	10.26	98.3

Table SB2 List of detected compounds with additional information on the type of the compound, LogD, and $LogK_{OW}$ values, with calculated K_{gw} (*Gammarus*-water) for neonicotinoids.

Chemical compound	Group	LogD	$LogK_{OW}$ (K_{gw})
10,11-Dihydro-10,11-dihydroxycarbamazepine	Pharmaceutical TP	0.8	0.8
10,11-Dihydro-10-hydroxycarbamazepine	Pharmaceutical TP	1.7	1.7
1H-Benzotriazole	Corrosion inhibitor	1.3	1.3
2-Benzothiazolesulfonic acid	Industrial chemical	-0.4	-0.3
2-Hydroxycarbamazepine	Pharmaceutical TP	1.4	2.8
4+5-Methyl-1H-benzotriazole	Corrosion inhibitor	1.8	1.8
7-Amino-4-methylcoumarin	Industrial chemical	1.3	1.3
7-Diethylamino-4-methylcoumarin	Industrial chemical	2.9	2.9
Acesulfame	Sweetener	-1.5	-0.6
Acetamiprid	Pesticide	1.1	1.1
Acetyl-sulfamethoxazole	Pharmaceutical TP	0.1	0.9
Azithromycin	Pharmaceutical	-2.0	2.4
Benzophenone-3	UV filter	3.7	3.8
Carbamazepine	Pharmaceutical	2.8	2.8
Carbendazim	Pesticide	1.8	1.8
Citalopram	Pharmaceutical	1.3	3.8
Clarithromycin	Pharmaceutical	1.8	3.2
Clothianidin	Pesticide	0.5	0.5

Cyclamate	Sweetener	-1.8	0.6
DEET	Repellent	2.5	2.5
Denatonium	Bittern	1.9	1.1
Diclofenac	Pharmaceutical	1.4	4.3
Fipronil	Pesticide	4.5	4.5
Hexa(methoxymethyl)melamine	Industrial chemical	2.6	2.6
Hydrochlorothiazide	Pharmaceutical	-0.6	-0.6
Imidacloprid	Pesticide	-3.7	-0.6 (2.9)
Imidacloprid-guanidine	Pesticide TP	-1.7	0.7
Melperon	Pharmaceutical	1.3	3.2
Metoprolol	Pharmaceutical	-0.8	1.8
Metoprolol acid	Pharmaceutical TP	-1.2	-1.2
Phenylbenzimidazole sulfonic acid	UV filter	0.1	-0.1
Propiconazole	Pesticide	4.3	4.3
Propranolol	Pharmaceutical	0.0	2.6
Roxithromycin	Pharmaceutical	0.9	3.0
Sulfamethoxazole	Pharmaceutical	0.1	0.8
Tebuconazole	Pesticide	3.7	3.7
Terbutryn	Pesticide/biocide	2.9	2.9
Thiacloprid	Pesticide	2.1	2.1 (3.6)
Tri(butoxyethyl)phosphate	Flame retardant	5.0	5.0
Triphenylphosphate	Flame retardant	4.1	4.6
Tris(2-chloroethyl)phosphate	Flame retardant	1.4	1.8
Verapamil	Pharmaceutical	2.4	2.2

Table SB3 Concentrations of micropollutants in *Gammarus pulex* tissue in ng/g of wet tissue.

Compound	H1	H3	H4	H5	H6	H7	H8
1H-Benzotriazole	0	0	2.9	3.3	3.4	2.8	2.6
2-Benzothiazolesulfonic acid	0	0	7.6	6.7	2.8	5.0	5.5
2-Hydroxycarbamazepine	0	0	0.63	0.80	0	0.71	0.68
5-Methyl-1H-benzotriazole	0	0	1.83	1.98	1.24	1.47	1.29
7-Amino-4-methylcoumarin	0	0.21	1.26	11	3.1	2.7	1.9
7-Diethylamino-4-methylcoumarin	0	0	57	67	24	27	21
10,11-Dihydro-10-hydroxycarbamazepine	0	0	0.14	0.22	1.66	0.20	0.19
10,11-Dihydro-10,11-dihydroxycarbamazepine	0	0	0.63	0.80	0	0.71	0.68
Benzophenone-3	0	0.24	2.3	1.9	1.0	2.1	0.67
Carbamazepine	0	0	0.84	1.3	1.2	1.2	1.3
Citalopram	0	0	5.9	6.3	9.6	4.2	6.8
Clarithromycin	0.02	0.09	1.1	1.7	1.2	0.98	1.1
DEET	0.03	0.52	0.72	0.64	0.70	1.7	0.54
Denatonium	0	0	1.2	1.2	0.54	0.55	0.43
Fipronil	0	0	0	0	0.64	0.12	0
Imidacloprid	0	0	3.1	3.4	2.4	3.4	4.3
Melperon	0	0	0	0	0	1.8	0.8
Methyl-1H-benzotriazole	0	0.13	0	0	0	0	0
Metoprolol	0	0	2.8	0	0	3.1	3.7
Propiconazole	0	0	0.83	1.3	0.54	0.83	0.76
Propranolol	0	0	0.45	0.54	0.89	0.51	0.31
Roxithromycin	0	0	0	0	1.0	0.78	0.87
Tebuconazole	0	0.69	1.1	1.2	0.45	0.87	0.88
Terbutryn	0	0	0.11	0.14	0.17	0.25	0.16
Thiacloprid	0.21	0.35	0.97	0.80	0.64	0.80	1.2

Tri(butoxyethyl)phosphate	0	0	0	0.37	0.22	0	0
Triphenylphosphate	0	0.38	1.3	1.5	1.6	1.5	1.0
Tris(2-chloroethyl)phosphate	0.20	2.8	4.2	1.1	5.5	6.9	0.63
Verapamil	0	0.16	0.94	1.3	1.8	1.1	1.2

Table SB4 Concentrations of micropollutants in the Holtemme River water samples in ng/L.

Compound	H3	H4	H5	H6	H7	H8
1H-Benzotriazole	0	662	350	734	533	712
2-Benzothiazolesulfonic acid	6.4	142	37	86	72	181
2-Hydroxycarbamazepine	0	16	13	17	18	19
5-Methyl-1H-benzotriazole	0	265	204	323	486	436
7-Amino-4-methylcoumarin	0	212	161	122	252	152
7-Diethylamino-4-methylcoumarin	0	1785	1055	774	873	728
10,11-Dihydro-10-hydroxycarbamazepine	0	52	32	76	74	78
10,11-Dihydro-10,11-dihydroxycarbamazepine	0	307	191	391	344	428
Acesulfam	0	119	128	121	152	138
Acetyl-sulfamethoxazole	0	9.0	6.0	5.7	11	0
Azithromycin	0	186	0	0	0	0
Carbamazepine	0.31	144	93	174	155	171
Carbendazim	3.3	2.5	3.3	0	0	0
Clarithromycin	0	31	19	34	32	32
Cyclamate	71	70	50	57	75	0
DEET	0	5.1	0.77	0	0	1.8
Denatonium	0	19	6.4	31	21	41
Diclofenac	0	324	196	362	381	298
Hexa(methoxymethyl)melamine	0	0	0	94	0	0
Hydrochlorothiazide	0	511	268	455	473	370
Imidacloprid-guanidine	0	0	18	0	0	0
Melperon	0	17	21	21	26	23
Metoprolol acid	0	36	56	46	88	44
Metoprolol	0	251	135	253	198	228

Phenylbenzimidazole sulfonic acid	0	199	133	203	215	217
Propiconazole	0	8.7	7.0	3.1	6.0	6.3
Propranolol	0	8.4	6.2	7.9	6.6	8.8
Roxithromycin	0	22	15	23	17	26
Sulfamethoxazole	0	16	0	30	22	29
Tebuconazole	0	4.7	4.1	3.3	3.3	2.5
Terbutryn	0	0	0	2.2	0	0
Tri(butoxyethyl)phosphate	0	0	0	2.1	5.2	0

Table SB5 The standard toxicity test median acute effect concentration data (µg/L) in 48 h exposure experiments for *G. pulex* and for *D. magna* for compounds without available data for *G. pulex*.

Compound	EC_{50} *G. pulex*	EC_{50} *D. magna*
1H-Benzotriazole		107,000 (Seeland et al., 2012)
5-Methyl-1H-Benzotriazole		8,580 (Seeland et al., 2012)
Carbamazepine		111,000 (Han et al., 2006)
Clarithromycin		25,720 (Isidori et al., 2005)
DEET		75,000 (Forbis & Burgess, 1985)
Fipronil	88.3 (Hayasaka et al., 2012)	
Imidacloprid	21 (Agatz et al., 2014)	
Metoprolol		63,900 (Huggett et al., 2002)
Propranolol		1,600 (Huggett et al., 2002)
Tebuconazole	1,643 (Adam et al., 2009)	
Terbutryn		7,100 (Marchini et al., 1988)
Thiacloprid	350 (Beketov & Liess, 2008)	
Triphenylphosphate		530 (Scanlan et al., 2015)
Verapamil		15,210 (Jordão et al., 2016)

Table SB6 Calculated toxic units of compounds found in the *G. pulex* tissue samples from the Holtemme River from sites H1–H8 with the sum of logarithmic values of toxic units at each location in the final line.

	H1	H3	H4	H5	H6	H7	H8
1H-Benzotriazole	-	-	-4.1	-4.1	-4.1	-4.1	-4.2
5-Methyl-1H-benzotriazole	-	-	-4.5	-3.5	-3.6	-3.6	-3.7
Carbamazepine	-	-	-6.0	-5.8	-5.9	-5.9	-5.8
Clarithromycin	-7.4	-6.8	-5.7	-5.5	-5.7	-5.8	-5.7
DEET	-6.6	-5.3	-5.2	-5.2	-5.2	-4.8	-5.3
Fipronil	-	-	-	-	-4.8	-5.5	-
Imidacloprid	-	-	-1.8	-1.8	-1.9	-1.8	-1.7
Metoprolol	-	-4.2	-	-	-4.2	-4.1	-
Propranolol	-	-4.3	-4.2	-4.0	-4.2	-4.4	-
Tebuconazole	-	-5.2	-5.0	-5.0	-5.4	-5.1	-5.1
Terbutryn	-	-	-6.7	-6.6	-6.4	-6.3	-6.5
Thiacloprid	-5.0	-4.7	-4.3	-4.4	-4.5	-4.4	-4.2
Triphenylphosph-ate	-	-5.9	-5.3	-5.3	-5.3	-5.3	-5.4
Verapamil	-	-5.2	-4.5	-4.3	-4.2	-4.4	-4.4
Cumulative	-5.0	-3.8	-1.8	-1.8	-1.9	-1.8	-1.7

Table SB7 Primers used for the COI amplification.

Primer	Concentration	Sequence
LCO1490 (Folmer et al., 1994)	1.0 M	GGTCAACAAATCATAAAGATATTGG
HC02198 (Folmer et al., 1994)	1.0 M	TAAACTTCAGGGTGACCAAAAAATCA

Table SB8 Primers used for microsatellite loci amplification.

Code	Sequence	Repeat	Tag	Multi-plex	Range	N_a	Ref.
gapu-8	F:GAGCGTCATCATTTCCATCC R:GCCAATCAGGGAACTGAGAA	(AT)^8	No	1	244–252	4	[1]
Gapu-9	F:CTATGCCCAAGCTGGTTGTT R:TTCGCGTCATTCACTCGTAG	(ATT)^9	No	1	188	1	[1]
Gapu-23	F:CAGCAAGTGGTGCAGCTAAA R:CAGCCACATCGAAGCTGTAA	(GCA)^11	No	1	180–192	4	[1]
Gapu-29	F:CCTGCTCAGTAACAGCCTCA R:TCAAATCGAGAAGGCTACAACA	(TTAA)^4/(AT)^4	No	2	223–259	4	[1]
Gapu-30	F:AAGTCGTTGCCATTGCTCTC R:TCTTGGAGAGGGTGAGGTTG	(GT)^5/(ACA)5/(CAA)4+5	No	2	232–238	2	[1]
Gamm-fos28	F:ACCTCTCCATCCCTGATGC R:CATCGACCCGTCAGTATGTG	(AC)13	No	2	208–210	2	[2]
Gp10	F:TGAAATCGCACCCACTTCG R:AGCTTCCAACAAGATTCCACC	(AC)^18	M13	1	162–182	9	[3]
Gp11	F:CATGCGCGACTAACCAGAC R:GGATGACTGCCATGTGTACC	(ACT)^14	M13	1	325–337	5	[3]
Gp13	F:GGGAATTTGGCCTAGCGTATG R:TGCAGTGGAGATGGTAGTCG	(TA)^22	M13	1	91–95	3	[3]
Gp18	F:GCACCATGGAGTCGATTTAGG R:AAGTCATTGCTTGACGACGG	(ATT)^9	M13	1	111–126	4	[3]
Gp28	F:TTGTAGACCCGGCACATCC R:TTCCCACGGATCTTGCACC	(AC)^12	M13	2	269–361	9	[3]
Gp30	F:AAACGACACAGTCTTGACTTC R:CCCTTCTTTATACCAAATAACATTGCG	(AT)^22	CAG	1	100–106	4	[3]
Gp31	F:CCTAACTAGGGGGAATCGGC R:TGTCACACGAGACCCTGATG	(ATAC)^7	M13	3	128–148	5	[3]
Gp37	F:TGGGTATGTTTCGAATGATGTCTAC R:TCCCTGCTCTAAGAAATTTGCG	(AT)^14	M13	3	231–247	8	[3]
Gp42	F:GTAAGCTCAACTCCACGGC R:TCATGGTTGTAATGTTTGGATCAG	(AAT)^8	CAG	3	373–379	3	[3]

Gp55	F:CCACATCTGGTCTACACTGGG R:TGCGGACGCAAAGATGAAC	(AAC)^11	M13	2	288– 330	3	[3]
Gp68	F:TAACCTTGGGTGAGTGGCAG R:CCACCAGCGATTGTATGCAC	(ACGG)^ 8	CAG	2	92– 118	5	[3]

[1]Gergs, Rothhaupt, & Behrmann-Godel, 2010

[2]Westram et al., 2010

[3]Švara, Norf, Luckenbach, Brack, & Michalski, 2019

Table SB9 Pairwise genetic distances between COI sequences from amphipods belonging to different sites in the Holtemme river and the reference locations (Parthe, Brandenburg (G_pulex_E), Rhur (G_pulex_C, G_pulex_D)) situated in the bottom left part of the table. At the top right part of the table are standard error values of genetic distances.

	H01	H03	H04	H05	H06	H07	H08	Par.	G_p_E	G_p_C	G_p_D
H01	-	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.002	0.011	0.015
H03	0.002	-	0.001	0.001	0.001	0.001	0.001	0.002	0.002	0.011	0.015
H04	0.002	0.002	-	0.001	0.001	0.001	0.001	0.002	0.002	0.011	0.015
H05	0.002	0.002	0.002	-	0.001	0.001	0.001	0.002	0.002	0.011	0.015
H06	0.002	0.003	0.003	0.002	-	0.001	0.001	0.002	0.002	0.011	0.015
H07	0.002	0.002	0.002	0.002	0.002	-	0.001	0.002	0.002	0.011	0.015
H08	0.002	0.002	0.002	0.002	0.002	0.002	-	0.002	0.002	0.011	0.015
Parthe	0.003	0.003	0.003	0.003	0.003	0.003	0.003	-	0.002	0.011	0.015
G_pulex_E	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	-	0.011	0.015
G_pulex_C	0.088	0.088	0.088	0.088	0.088	0.088	0.088	0.086	0.089	-	0.005
G_pulex_D	0.120	0.119	0.120	0.119	0.120	0.120	0.121	0.118	0.120	0.039	-

Table SB10 Fixation index values for COI sequence comparison. Values in the lower left part of the table represent the pairwise COI F_{st} values between respective sites. The values in the top right part of the table represent the p-values of significance for the test between each two respective sites ($p < 0.05$).

	H1	**H3**	**H4**	**H5**	**H6**	**H7**	**H8**
H1	-	0.17+-0.00	0.35+-0.01	0.92+-0.00	0.52+-0.01	0.69+-0.01	0.83+-0.01
H3	0.038	-	0.64+-0.01	0.12+-0.01	0.02+-0.00	0.15+-0.01	0.32+-0.01
H4	0.004	-0.019	-	0.15+-0.01	0.04+-0.00	0.50+-0.01	0.66+-0.01
H5	-0.047	0.053	0.032	-	0.67+-0.01	0.28+-0.01	0.40+-0.01
H6	-0.013	0.105*	0.071*	-0.022	-	0.21+-0.01	0.13+-0.01
H7	-0.025	0.035	-0.009	0.013	0.022	-	0.92+-0.01
H8	-0.039	0.009	-0.018	-0.001	0.033	-0.036	-

Table SB11 F_{st} values for microsatellite samples from different sites. Asterisk denotes significant results ($p < 0.05$).

	H1	**H3**	**H4**
H3	0.017*		
H4	0.002	0.000	
H6	0.012	0.000	0.000

Table SB12 Results of the Kruskal-Wallis one-way comparison of mobility data in a 14-day exposure experiment across two treatments and three sampling locations.

Kruskal-Wallis rank sum test				
Locations	**Imidacloprid Concentration (µg/L)**	**Chi-squared**	**Df**	**p-value**
H1–H4	130	48.493	1	0.000
H1–H6	130	30.984	1	0.000
H4–H6	130	0.315	1	0.574
H1–H4	270	46.802	1	0.000
H1–H6	270	63.244	1	0.000
H4–H6	270	4.443	1	0.035

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Supplementary information C

Reduced genetic diversity of freshwater amphipods in rivers with increased levels of anthropogenic organic micropollutants

Supplementary Text C

Material and Methods C

SC1 LC-HRMS sample preparation and analysis

SC1.1. Water sample analysis

The pH of 1 mL water sample aliquots was adjusted to 3.5 by adding 10 µL of 2 M ammonium formate buffer to roughly match that of the LC eluent (0.1% formic acid, pH 2.6); 25 µL of an internal standard mixture containing 40 isotope-labelled compounds (40 ng/mL of each compound) and 25 µL of methanol were added. Matrix-matched calibration standards were prepared in the same way by spiking 1 mL water sample aliquots from a pristine stream (Wormsgraben, upper Harz Mountains, Germany) with the target analytes at concentrations from 1 to 5000 ng/L.

SC1.2. Body burden analysis

Gammarus pulex tissue samples were extracted using the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method according to Inostroza et al. (2016) Accordingly, 900 mg of amphipod tissue was homogenized in a mixture with 2 mL water,

2 mL acetonitrile, and 1 mL hexane using an Ultra-Turrax T-25 (IKA) for ca. 60 s followed by vortexing of the suspension for 60 s. Subsequently, 800 mg anhydrous $MgSO_4$ and 200 mg NaCl were added, the samples were vortexed again and centrifuged (4000× *g*) for 5 minutes. The supernatant was transferred to centrifugation tubes containing 400 mg anhydrous $MgSO_4$ and 50 mg primary-secondary amine. The suspension was vortexed and centrifuged. The supernatant was moved into an evaporation vial and dried under the nitrogen stream at 23 °C. Finally, 50 µL of internal standard solution and 450 µL methanol (final level 100 ng/mL) were added. Method-matched calibration standards were prepared by spiking target analyte solutions into 2 mL LC-MS grade water, which were processed the same way as the samples, corresponding to final levels of 0.1–200 ng/g in vial.

SC1.3. LC-HRMS analysis

Water samples and *Gammarus* extracts were analyzed 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 ionization source. LC separation was done on a Kinetex C18 EVO 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 min of 5% B, the fraction of B was linearly increased to 100% within 12 min and 100% B were kept for 11 min. The eluent flow was diverted to waste and the column was rinsed for 2 min 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 min. The injection volume was 5 µL for *Gammarus* extracts and 100 µL for water samples 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 kV (positive mode) or 3.5 kV (neg. mode), the sheath gas flow rate was 45 a.u. and the auxiliary gas flow rate 1 a.u. Separate runs were conducted in positive and negative ion mode combining a full scan experiment (100–1000 m/z) at a nominal resolving power of 70,000 (referenced to m/z 200) and data-independent MS/MS experiments at a nominal resolving power of 35,000. For the latter, we acquired the data using broad isolation windows of about 50 mass units (i.e., m/z ranges 97–147, 144–194, 191–241, 238–288, 285–335, 332–382, 379-429, 426–476) and 280 Th (i.e., m/z ranges 460–740, 730–1010), respectively.

SC1.4 Compounds quantification and estimation of toxic effects in *G. pulex*

Raw data from the LC-HRMS analysis were converted into .mzML format using ProteoWizard v3.0.18265. The peak list for each batch was generated by MZmine v2.32 (Pluskal et al., 2010), with settings set as suggested in Beckers et al. (2020) and annotated from a list of 534 target compounds. The extracted list was corrected for blanks according to equation 1 (see below). Signals below the threshold in the samples were removed, following by an intensity cut-off of peaks with intensity of 5,000 for negative mode and under 50,000 for positive mode batches, in order to remove the noise created by gap filling. The data containing compounds from negative and positive mode batches was assembled in a table.

Equation 1:

$$I_{thres} = \mu(I_{Blk}) + 2 * \sigma(I_{Blk})$$

I_{thres} = intensity threshold

$\mu(I_{Blk})$ = mean of peak intensities in blanks;

$\sigma(I_{Blk})$ = standard deviation of peak intensities in blanks

SC2 Polymerase chain reactions, sequencing and genotyping information

COI PCR and sequencing reactions

50 µL PCR reactions contained 2.5 µL of 10 mM dNTPs, 10 µL 5X Green GoTaq Flexi Buffer (Promega), 4 µL of 25 mM $MgCl_2$, 1 µL of GoTaq DNA polymerase and 22.5 µL of deionized Water. Each primer (Supp. 2) was diluted from the stock solution to 10 µM and 2.5 µL were added to the PCR reaction. Finally, 5 µL of DNA template with concentration between 40-80 ng/µL was added to the mix. The polymerase chain reaction (PCR) cycling setting was set to initial activation step of 2 min at 95°C, following by 34 cycles of 1 min denaturation at 95°C, 45 s annealing at 51°C and 1 min elongation at 72°C. The reaction was terminated after the final elongation of 5 min at 72°C. The PCR products were checked on the agarose gel and cleaned using GeneJET PCR Purification Kit (ThermoFisher Scientific) following the kit instructions. The 10 µL sequencing reaction of 150-250 ng of DNA with 1µL of the sequencing primer, Big dye mix (Thermo Fisher Scientific) and 5x sequencing buffer respectively was prepared for each PCR product. With the program as following: 1 min at 96°C, 30 cycles of 10 s at 96°C, 5 s at 50°C and 4 min at 60°C. The products were purified by ethanol/EDTA (ethylenediaminetetraacetic acid) precipitation protocol (Applied Biosystems, 2010), and diluted in 10 µL HiDi formamide (Thermo Fisher Scientific). The samples were separated on an ABI Prism 3130XL Genetic Analyzer (Applied Biosystems).

SC3 COI sequence analyses and visualization

COI sequence reads were assembled and edited in Sequencher 5.4.5, with gaps coded as (-). Sequence contigs and, as reference, sequences of *G. pulex* from European rivers acquired from National Center for Biotechnology Information (NCBI) (KT075230.1_G_pulex_E, KT075232.1_G_pulex_D, KT075256.1_G_pulex_C, KF521835.1_G_fossarum were aligned using ClustalW in MEGA7 (Kumar et al., 2008) with default settings for alignment. Based on the alignment, maximum likelihood tree with best fitting Tamura 3-parameter model and nearest-neighbor-interchange tree Inference method (Tamura et al., 2011) were constructed. Confidence in the obtained topology was assessed by bootstrapping the dataset 1000 times. The phylogenetic tree was visualized with FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

Supplementary Figures C

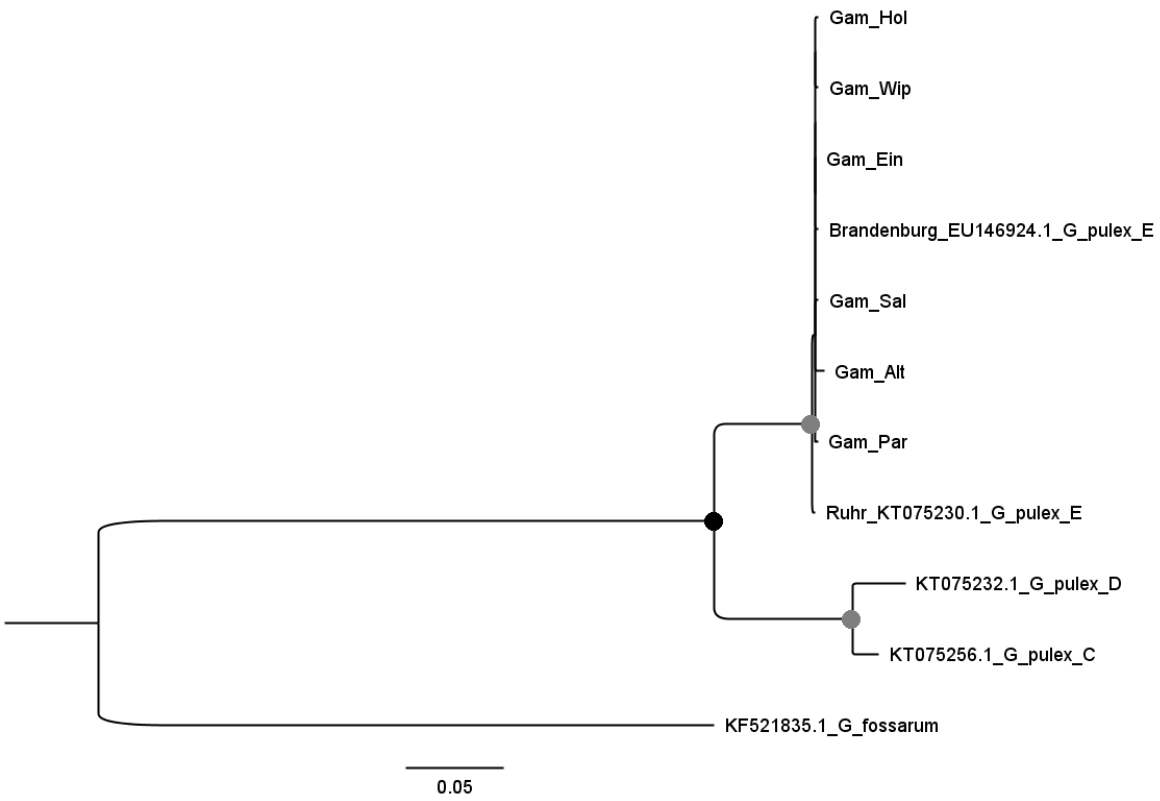

Figure SC1 Phylogenetic position of amphipods studied from six rivers in central Germany. The tree was constructed based on COI maximum likelihood comparison of the obtained *G. pulex* sequences indicating genotypes from six rivers. Black dot denotes the clade with bootstrap values of > 99, while grey dots denote bootstrap values of > 95. The sequences from the analysed rivers can be accessed in GenBank by the codes MN400976 (Gam_Hol), OL441362 (Gam_Wip), OL441361 (Gam_Ein), MN400975 (Gam_Sal), OL441360 (Gam_Alt), MN400977 (Gam_Par).

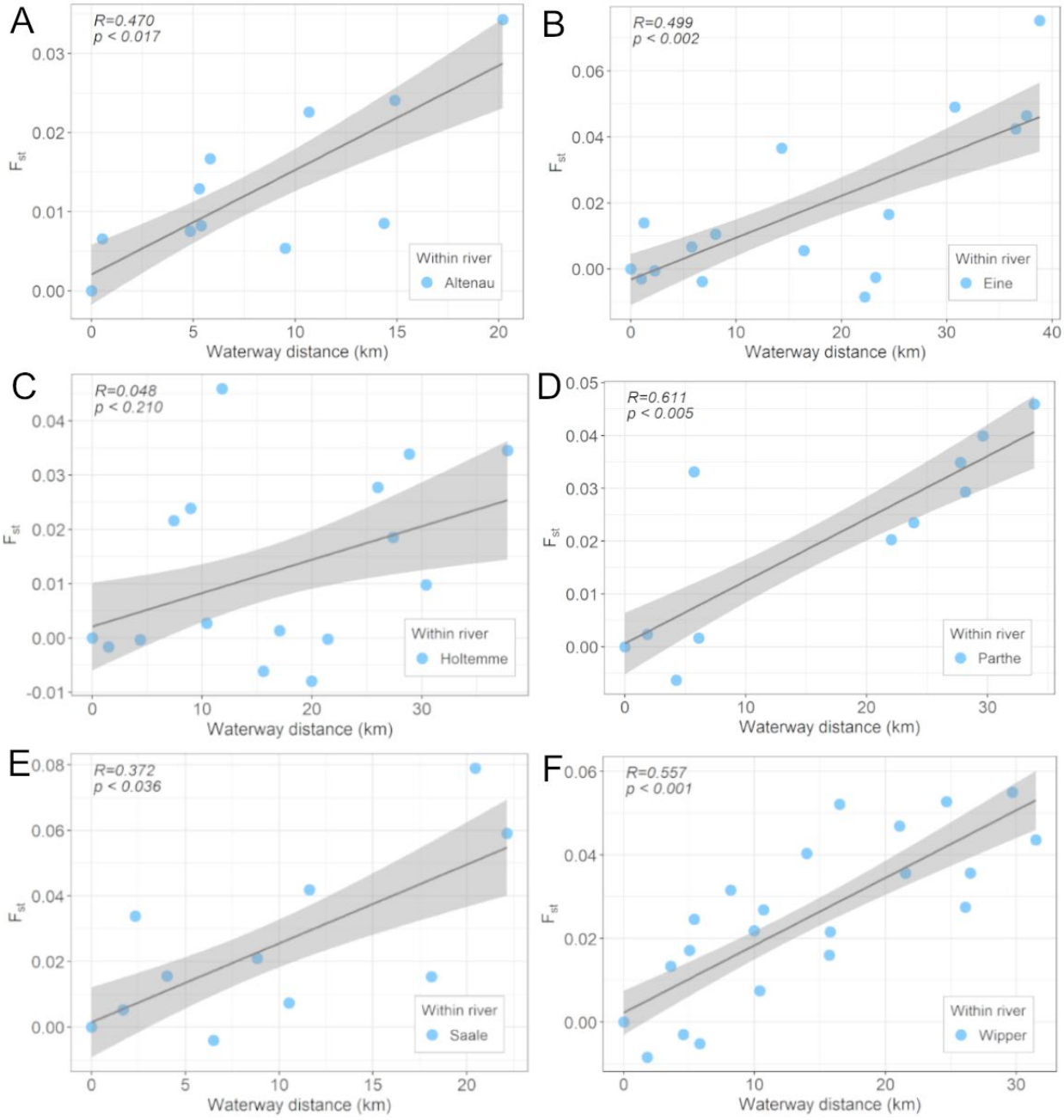

Figure SC2 Mantel test of pairwise F_{st} values between every pair of sites within each river (A–F) with respective waterway distances (km). Grey line indicates linear regression of the Mantel test with a 95% confidence interval (shaded area).

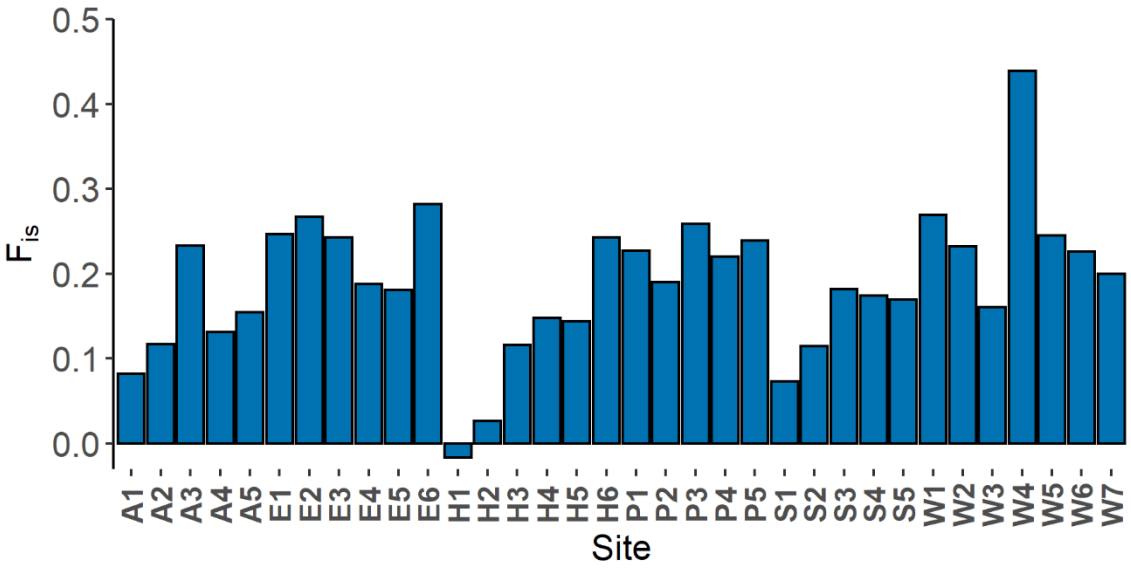

Figure SC3 Inbreeding coefficient rates (F_{is}) of amphipods samples from 34 sites along the Rivers in central Germany.

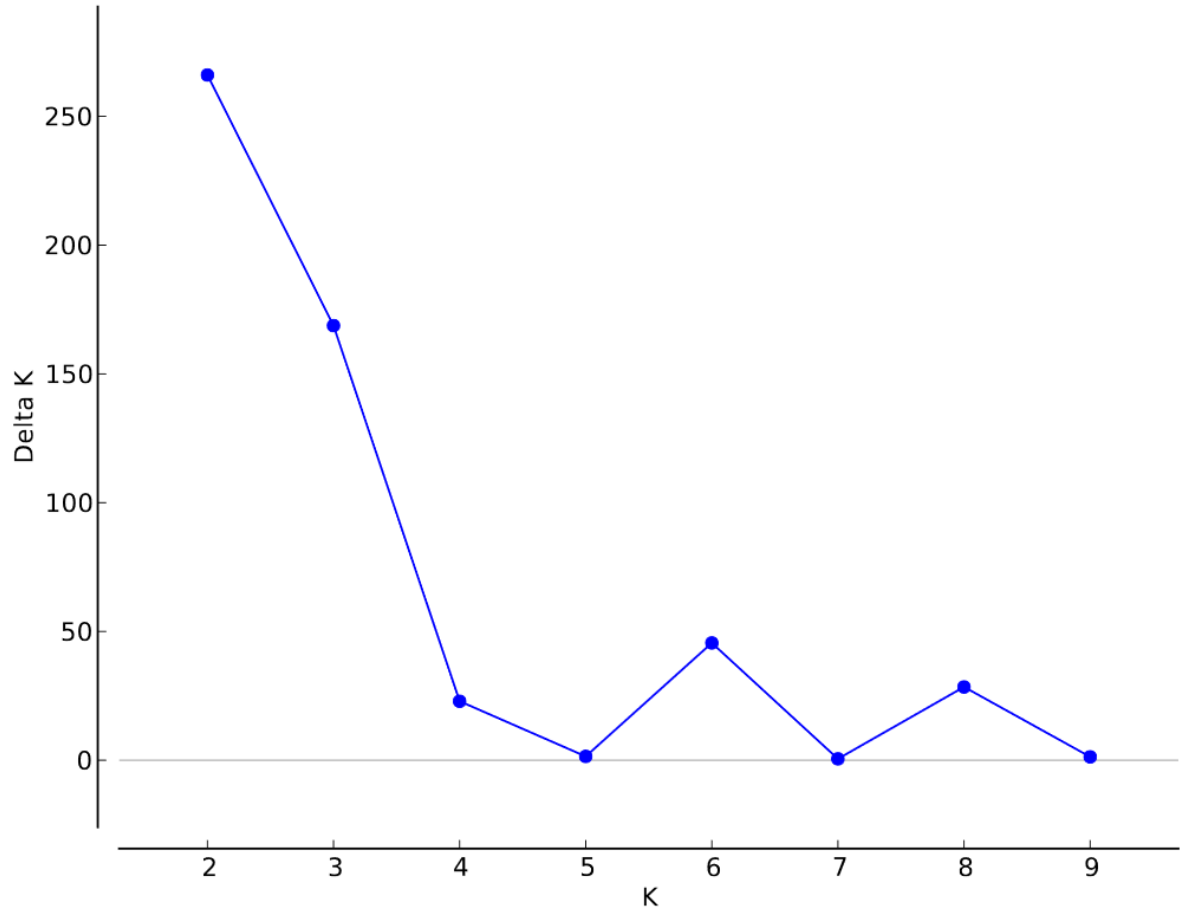

Figure SC4 Delta K values for *G. pulex* from Structure Harvester. The identification of the most likely number of genetic clusters K for K 1–9 was done using the Evanno method based on 16 microsatellite loci.

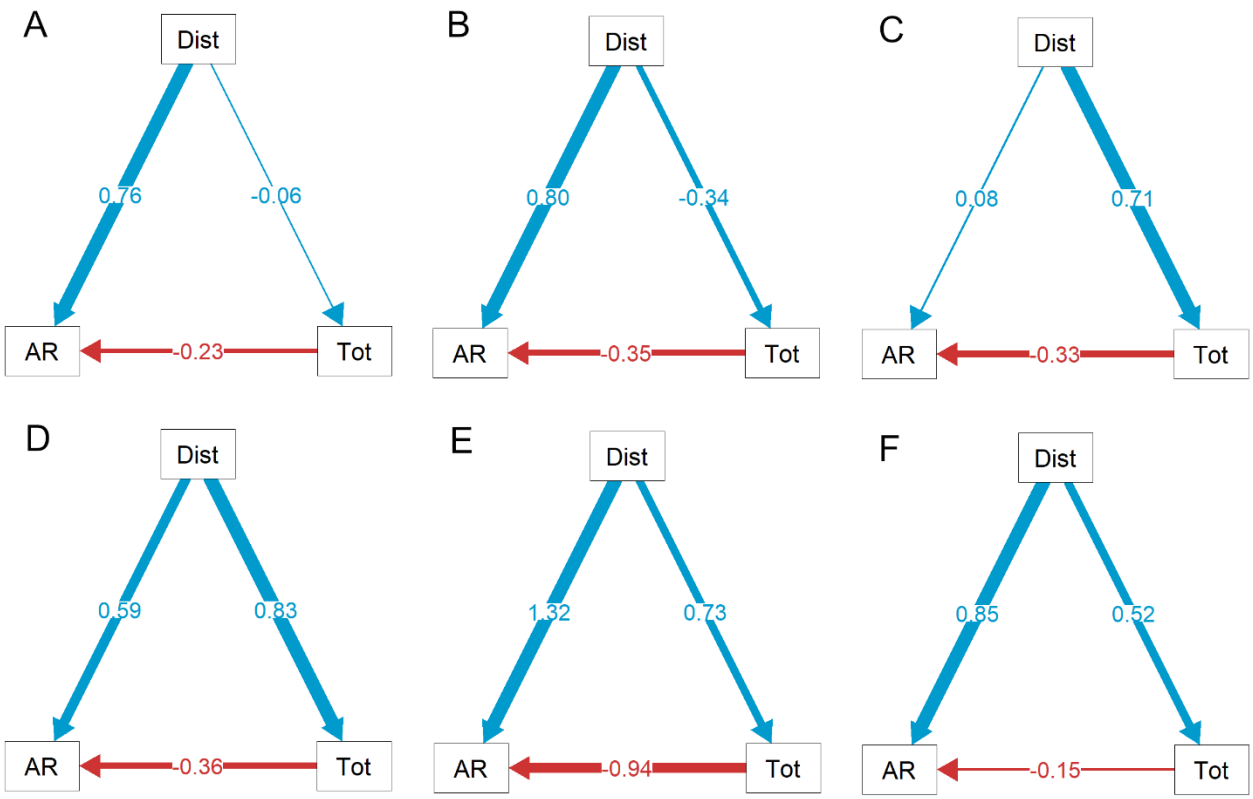

Figure SC5 Output of the standard equation models for each river (Altenau (A), Eine (B), Holtemme (C), Parthe (D), Saale (E), Wipper (F)) comparing the relationship between allelic richness (AR), distance of the sampled site from the source (Dist), and total concentration of AOM (Tot) detected at each sampling site. Blue color denotes positive relationship and the red color denotes negative relationship. The arrows width is scaled according to the value of each relation.

Supplementary Tables C

Table SC1 Studied sites at the rivers Altenau (A), Eine (E), Holtemme (H), Parthe (P), Saale (S), and Wipper (W). Sampling site codes, site names, sampling dates and times, sampling site coordinates, temperature, pH value, water oxygen concentration, conductivity, number of individuals per catch as a measure of abundance of *G. pulex* at sampling site, information of presence of WWTP effluents upstream of the sampling sites (WWTP upstream), and the number of *G. pulex* sampled for DNA analysis at each site are listed.

Site code	Site name	Sampling date and time	Coordinates (Lat, Long)	T (°C)	pH	O_2 (mg/L)	Conductivity (µS/cm)	Abun. (per catch)	WWTP upstream	Sampled *G. pulex* (DNA)
A1	Altenau. Upsteam of Schöppenstedt	11.11.2019 14:00	52.157923; 10.820882	6.9	7.31	10.9	953	100	No	30
A2	Altenau. Schöppenstedt. Upstream of WWTP	11.11.2019 13:00	52.141719; 10.762080	4.9	7.48	12.91	1059	60	No	30
A3	Altenau. Schöppenstedt. Downstream of WWTP	11.11.2019 12:00	52.143531; 10.757041	6.3	7.52	11.82	1049	120	Yes	30
A4	Altenau. Weferlingen	11.11.2019 11:00	52.151367; 10.688176	4.5	7.89	14.67	1197	150	Yes	30
A5	Altenau. Klein Denkte	11.11.2019 10:00	52.140427; 10.575466	3.7	7.84	12.44	1970	100	Yes	30
E1	Eine. Upstream of WWTP Schielo	9.1.19 8:00	51.616644; 11.211005	4.4	7.38	12.09	330	50	No	30
E1a*	Eine. Downstream of WWTP Schielo	9.1.19 10:00	51.612240. 11.219577	4.9	7.82	12.82	578	0	Yes	0
E2	Eine. Stangerode	18.12.18 14:00	51.650788; 11.348645	3.1	8.22	13	557	50	Yes	30
E3	Eine. Upstream of Aschersleben	18.12.18 13:00	51.737861; 11.439389	3.9	8.23	13.34	708	10	Yes	30
E4	Eine. Upstream of WWTP Aschersleben	18.12.18 12:00	51.743211; 11.481198	4	8.22	13.48	662	10	Yes	30
E5	Eine. Downstream of WWTP Aschersleben	18.12.18 11:00	51.748178; 11.493240	3.9	8.08	12.67	761	10	Yes	30
E6	Eine. Groß Schierstedt	18.12.18 10:00	51.748462; 11.510467	3.6	8.13	12.8	724	10	Yes	30
H1	Holtemme. Wernigerode	20.7.2017 9:00	51.847054; 10.791327	9.8	7.21	11.66	214	5	No	20
H2	Holtemme. Silstedt upstream of WWTP	15.3.2017 17:00	51.865251; 10.853865	7.5	7.62	11.91	201	50	No	20
H3	Holtemme Silstedt	15.3.2017 14:30	51.867732; 10.873714	8.3	7.55	12.52	278	200	Yes	20

	downstream of WWTP									
H4	Holtemme. Upstream of WWTP Halberstadt	16.3.2017 09:30	51.909947; 11.074887	5.2	8.53	12.16	316	500	Yes	20
H5	Holtemme. Groß Quenstedt.	16.3.2017 14:00	51.923896; 11.110006	9.1	8.26	12.92	426	200	Yes	20
H6	Holtemme confluence Bode	16.3.2017 16:30	51.963426; 11.182576	9.6	8.31	12.37	507	5	Yes	20
P1	Parthe. Glasten	10.10.2019 9:00	51.171061; 12.695489	10.5	7.06	7.8	701	100	No	30
P2	Parthe. Grossbardau	10.10.2019 10:00	51.211815; 12.703265	11.1	7.03	25.5	646	50	Yes	30
P3	Parthe. Borsdorf, Downstream of WWTP	10.10.2019 12:00	51.342064; 12.529571	11.3	7.55	4.87	555	10	Yes	30
P4	Parthe. Borsdorf, Downstream of WWTP	10.10.2019 14:00	51.355769. 12.532703	15.2	8.53	6.72	818	20	Yes	30
P5	Parthe. Dewitz	10.10.2019 15:00	51.381616. 12.526498	12.8	8.06	1.95	820	50	Yes	30
S1**	Saale. Kaulsdorf.	12.6.2018 17:30	50.613133; 11.393243	12	8.5	11.7	449	200	No	30
S2	Saale. Rudolstad, confluence with Schwarza	12.6.2018 16:00	50.684319; 11.323392	17.7	8.3	9.65	358	2	Yes	30
S3	Saale. Rudolstadt, upstream of WWTP	12.6.2018 13:30	50.719146; 11.352061	14.8	7.8	10.95	486	10	Yes	30
S4	Saale. Rudolstadt, at the WWTP	12.6.2018 11:30	50.720117; 11.377208	13.3	7.5	9.6	700	50	Yes	30
S5	Saale. Rudolstad, Downstream of WWTP	12.6.2018 10:00	50.718542; 11.398322	14.8	7.4	9.41	520	30	Yes	30
W1*	Wipper. Upstream of Wippra	14.10.2019 9:00	51.569797; 11.252111	12.9	7.59	8.5	423	50	No	30
W2	Wipper. Upstream of Friesdorf	14.10.2019 10:00	51.585998; 11.292676	13.5	7.85	8.09	469	20	Yes	30
W3	Wipper. Downstream of Bisenrode	14.10.2019 11:00	51.604347; 11.404161	14.1	7.82	8.11	492	20	Yes	30
W4	Wipper. Downstream of Vatterode	14.10.2019 13:00	51.606634; 11.462897	14.4	7.78	8.07	538	20	Yes	30
W5	Wipper. At the WWTP Grossörner	14.10.2019 14:00	51.627493; 11.507825	16.9	8.38	8.66	587	2	Yes	21
W6	Wipper. Before WWTP Hettestedt	14.10.2019 15:00	51.655010; 11.520962	15.8	8.29	8.98	577	1	Yes	10
W7	Wipper. After WWTP Hettestedt	14.10.2019 17:00	51.665774; 11.537412	16.2	7.63	7.47	678	50	Yes	30

* No *G. pulex* was found at the site E1a, therefore the genetic and AOM toxicity analyses could not be performed for this site.
** Site S1 is located downstream of the cascade of river dams, likely impassable for *G. pulex*. The distance from the source was therefore measured from the last dam before the first sampling site on the river.
*** Site W1 is located downstream of a large river dam, likely impassable for *G. pulex*. The distance from the source was therefore measured from this dam.

Table SC2 List of all detected compounds in *G. pulex* tissue and water samples with the application type of the compound, predicted logD values at pH 7.4, and, if available, EC_{50} value (EPA ecotoxicology database; https://www.epa.gov/chemical-research/ecotoxicology-database) used in the TU analysis.

See file eva13387-sup-0002-TableS2.csv available at https://doi.org/10.1111/eva.13387

Table SC3 List of concentrations of compounds detected in water samples from every site in ng/L, with information on types of application (Type), and minimal detection limits (MDL) of the detected compounds.

See file eva13387-sup-0003-TableS3.csv available at https://doi.org/10.1111/eva.13387

Table SC4 List of concentrations of compounds detected in amphipod tissue samples from every site in ng/g wet tissue, with information on types of applications (Type), and minimal detection limits (MDL) of the detected compounds.

See file eva13387-sup-0004-TableS4.csv available at https://doi.org/10.1111/eva.13387

Table SC5 List of toxic units for compounds found in *G. pulex* tissue samples from every site.

See file eva13387-sup-0005-TableS5.csv available at https://doi.org/10.1111/eva.13387

Table SC6 Primers used for the COI amplification, with information on primer name and reference, used concentration, and primer sequence.

Primer name and reference	Concentration	Sequence
LCO1490 (Folmer et al., 1994)	1.0 M	GGTCAACAAATCATAAAGATATTGG
HC02198 (Folmer et al., 1994)	1.0 M	TAAACTTCAGGGTGACCAAAAAATCA

Table SC7 Primers used for microsatellite loci amplification including the information of primer code, forward (F) and reverse primer sequence, repeated sequence, M13 or CAG primer multiplex tag, multiplex reaction in which primer was used, and a publication reference.

Code	Sequence	Repeat	Tag	Multi-plex	Ref.
gapu-8	F:GAGCGTCATCATTTCCATCC R:GCCAATCAGGGAACTGAGAA	(AT)^8	No	1	Gergs et al., 2010
Gapu-9	F:CTATGCCCAAGCTGGTTGTT R:TTCGCGTCATTCACTCGTAG	(ATT)^9	No	1	Gergs et al., 2010
Gapu-23	F:CAGCAAGTGGTGCAGCTAAA R:CAGCCACATCGAAGCTGTAA	(GCA)^11	No	1	Gergs *et al.*. 2010
Gapu-29	F:CCTGCTCAGTAACAGCCTCA R:TCAAATCGAGAAGGCTACAACA	(TTAA)^4/(AT)^4	No	2	Gergs et al., 2010
Gapu-30	F:AAGTCGTTGCCATTGCTCTC R:TCTTGGAGAGGGTGAGGTTG	(GT)^5/(ACA)5/(CAA)4+5	No	2	Gergs et al., 2010
Gammfos28	F:ACCTCTCCATCCCTGATGC R:CATCGACCCGTCAGTATGTG	(AC)13	No	2	Westram et al., 2010
Gp10	F:TGAAATCGCACCCACTTCG R:AGCTTCCAACAAGATTCCACC	(AC)^18	M13	1	Švara et al., 2019
Gp11	F:CATGCGCGACTAACCAGAC R:GGATGACTGCCATGTGTACC	(ACT)^14	M13	1	Švara et al., 2019
Gp13	F:GGGAATTTGGCCTAGCGTATG R:TGCAGTGGAGATGGTAGTCG	(TA)^22	M13	1	Švara et al., 2019

Gp18	F:GCACCATGGAGTCGATTTAGG R:AAGTCATTGCTTGACGACGG	(ATT)^9	M13	1	Švara et al., 2019
Gp28	F:TTGTAGACCCGGCACATCC R:TTCCCACGGATCTTGCACC	(AC)^12	M13	2	Švara et al., 2019
Gp30	F:AAACGACACAGTCTTGACTTC R:CCCTTCTTTATACCAAATAACATTGCG	(AT)^22	CAG	1	Švara et al., 2019
Gp31	F:CCTAACTAGGGGGAATCGGC R:TGTCACACGAGACCCTGATG	(ATAC)^7	M13	3	Švara et al., 2019
Gp37	F:TGGGTATGTTTCGAATGATGTCTAC R:TCCCTGCTCTAAGAAATTTGCG	(AT)^14	M13	3	Švara et al., 2019
Gp42	F:GTAAGCTCAACTCCACGGC R:TCATGGTTGTAATGTTTGGATCAG	(AAT)^8	CAG	3	Švara et al., 2019
Gp55	F:CCACATCTGGTCTACACTGGG R:TGCGGACGCAAAGATGAAC	(AAC)^11	M13	2	Švara et al., 2019
Gp68	F:TAACCTTGGGTGAGTGGCAG R:CCACCAGCGATTGTATGCAC	(ACGG)^8	CAG	2	Švara et al., 2019

Table SC8 List of parameters of genetic diversity analysis including sampling site name, number of genotyped amphipods (N), distance of sampling site from the river source (Distance (km)), rarefied allelic richness (AR), rarified private alleles (PA), average inbreeding coefficient of all individuals at each site (F_{is}), observed heterozygosity (H_o), expected heterozygosity (H_e), and effective population size (N_e).

Site	N	Distance (km)	AR	PA	F_{is}	H_o	H_e	N_e
A1	30	3.2	2.74	0.17	0.082	0.33	0.38	236.2
A2	30	8.4	2.85	0.18	0.117	0.37	0.43	27.3
A3	30	8.9	2.83	0.27	0.233	0.30	0.39	215.4
A4	30	13.8	2.8	0.2	0.131	0.33	0.40	287.2
A5	30	23.5	2.89	0.32	0.155	0.32	0.39	118
E1	29	3.6	2.34	0.11	0.247	0.31	0.43	26.7
E2	30	19	2.46	0.08	0.267	0.30	0.38	61.3
E3	30	34.3	2.7	0.17	0.243	0.33	0.40	70.9
E4	30	40.3	2.7	0.13	0.188	0.31	0.41	120.8
E5	30	41.4	2.65	0.25	0.181	0.29	0.38	503.3
E6	30	42.7	2.89	0.31	0.282	0.29	0.36	45
H1	19	8	2.77	0.22	-0.017	0.37	0.38	27.4
H2	19	15	2.58	0.15	0.026	0.36	0.36	55.3
H3	20	17	2.6	0.2	0.116	0.35	0.37	45.2
H4	20	31	2.66	0.28	0.148	0.33	0.36	186.5
H5	20	38	2.88	0.29	0.144	0.34	0.39	149.7
H6	20	42	2.43	0.16	0.243	0.26	0.36	30.2
P1	30	4.6	2.75	0.12	0.227	0.31	0.41	47.8
P2	30	10.4	2.88	0.26	0.19	0.31	0.40	76.4
P3	30	32.2	2.99	0.16	0.259	0.33	0.44	54.3
P4	30	34.2	2.87	0.07	0.22	0.33	0.42	51.2
P5	30	38.4	2.77	0.12	0.239	0.29	0.41	52.3
S1	30	7	2.76	0.21	0.073	0.40	0.43	40.2
S2	30	18.7	2.88	0.22	0.115	0.35	0.42	22.3
S3	30	25.3	2.81	0.2	0.182	0.36	0.43	39.4
S4	30	27.4	2.79	0.23	0.174	0.35	0.42	108.9
S5	30	29.1	2.94	0.26	0.17	0.36	0.44	371.2
W1	30	4.5	2.64	0.18	0.269	0.30	0.40	118.9
W2	30	8.8	2.77	0.14	0.121	0.31	0.40	113.8
W3	30	19.3	2.85	0.17	0.161	0.31	0.40	1371.3
W4	30	24.8	2.79	0.21	0.439	0.27	0.39	71
W5	21	29.3	2.78	0.23	0.245	0.29	0.38	20.8
W6	10	33.1	2.61	0.12	0.226	0.23	0.35	14.3
W7	30	34.9	2.84	0.19	0.2	0.31	0.39	34

Table SC9 Null allele rates (Null) and total detected alleles (Alleles) across sampled loci and rivers.

	Altenau		Eine		Holtemme		Parthe		Saale		Wipper	
Locus	Null	Alleles	Null	Alleles	Null	Alleles	Null	Alleles	Null	Alleles	Null	Alleles
g8	0.09	4	0.34	5	0.06	5	0.05	4	0.15	6	0.12	4
g9	0	4	0	1	0	2	0	1	0	2	0.05	6
g23	0	5	0	6	0	4	0.1	4	0	4	0	6
g29	0	2	0	4	0	3	0.06	2	0.19	3	0	2
g30	0	1	0	1	0	5	0	1	0	1	0	3
gf28	0	4	0.11	2	0	2	0	1	0	4	0	3
gp10	0	10	0	11	0	9	0.06	9	0	12	0.15	11
gp11	0	8	0.05	6	0.07	5	0.06	6	0	3	0.05	5
gp18	0.1	3	0	4	0	4	0	5	0	3	0.08	4
gp28	0	12	0	11	0	11	0.05	10	0	12	0	13
gp30	0.14	6	0.13	4	0	4	0.15	5	0.18	5	0.18	5
gp31	0	4	0	4	0	5	0	5	0	5	0	5
gp37	0.28	12	0.47	9	0.36	9	0.51	9	0.43	9	0.33	10
gp42	0.8	3	0	2	0	3	0.05	2	0.07	3	0	4
gp55	0	5	0.08	5	0	5	0.19	4	0	8	0.13	7
gp68	0	9	0	9	0	7	0	7	0	7	0	7

Table SC10 Tables of F_{st} values from pairwise comparison between sites within rivers. The upper F_{st} limit is listed in the upper right part of each table and the lower F_{st} limit is listed in the lower left part of each table.

	A1	A2	A3	A4	A5	E1	E2	E3	E4	E5	E6	H1	H2	H3	H4	H5	H6	P1	P2	P3	P4	P5	S1	S2	S3	S4	S5	W1	W2	W3	W4	W5	W6	W7
A1	0																																	
A2	0.01	0																																
A3	0.02	0.01	0																															
A4	0.02	0.01	0.01	0																														
A5	0.03	0.02	0.01	0.01	0																													
E1	0.21	0.2	0.2	0.18	0.19	0																												
E2	0.2	0.19	0.18	0.17	0.18	0.04	0																											
E3	0.18	0.17	0.17	0.15	0.16	0.05	0.01	0																										
E4	0.19	0.18	0.17	0.16	0.18	0.04	0	0.01	0																									
E5	0.2	0.19	0.18	0.17	0.17	0.05	0	0	0	0																								
E6	0.15	0.14	0.13	0.12	0.13	0.08	0.02	0.01	0	0.01	0																							
H1	0.16	0.16	0.17	0.14	0.14	0.09	0.1	0.09	0.09	0.08	0.1	0																						
H2	0.17	0.17	0.16	0.15	0.15	0.07	0.06	0.07	0.06	0.06	0.07	0.02	0																					
H3	0.15	0.15	0.14	0.13	0.13	0.05	0.06	0.06	0.06	0.05	0.06	0	0	0																				
H4	0.18	0.18	0.17	0.16	0.16	0.07	0.07	0.08	0.07	0.06	0.08	0.03	0	0	0																			
H5	0.14	0.14	0.13	0.11	0.11	0.08	0.08	0.08	0.07	0.07	0.07	0.01	0	0	0	0																		
H6	0.11	0.12	0.1	0.09	0.08	0.09	0.1	0.08	0.09	0.08	0.08	0.03	0.03	0.02	0.05	0.02	0																	
P1	0.12	0.1	0.09	0.11	0.11	0.17	0.18	0.15	0.16	0.15	0.12	0.15	0.14	0.12	0.14	0.12	0.13	0																
P2	0.14	0.12	0.11	0.13	0.12	0.16	0.17	0.14	0.14	0.14	0.11	0.12	0.11	0.09	0.11	0.1	0.1	0.03	0															
P3	0.12	0.11	0.1	0.12	0.1	0.13	0.14	0.1	0.12	0.11	0.08	0.1	0.1	0.07	0.09	0.08	0.08	0.03	0.02	0														
P4	0.1	0.09	0.07	0.1	0.08	0.14	0.15	0.11	0.14	0.13	0.1	0.11	0.11	0.08	0.11	0.09	0.07	0.04	0.02	0	0													
P5	0.12	0.1	0.09	0.11	0.09	0.16	0.17	0.13	0.15	0.14	0.12	0.12	0.13	0.1	0.13	0.1	0.08	0.05	0.03	0	0	0												
S1	0.22	0.18	0.18	0.17	0.16	0.21	0.2	0.17	0.18	0.2	0.14	0.2	0.21	0.18	0.21	0.18	0.19	0.17	0.16	0.14	0.14	0.16	0											
S2	0.16	0.13	0.12	0.12	0.1	0.23	0.22	0.17	0.2	0.2	0.14	0.19	0.2	0.17	0.21	0.17	0.16	0.15	0.13	0.11	0.1	0.12	0.04	0										
S3	0.18	0.15	0.14	0.14	0.13	0.24	0.22	0.18	0.2	0.21	0.15	0.21	0.22	0.19	0.23	0.19	0.18	0.16	0.15	0.13	0.12	0.15	0.02	0	0									
S4	0.16	0.15	0.14	0.12	0.12	0.22	0.21	0.16	0.19	0.19	0.13	0.19	0.21	0.18	0.21	0.17	0.16	0.17	0.16	0.14	0.14	0.16	0.08	0.02	0.03	0								
S5	0.15	0.11	0.12	0.1	0.1	0.21	0.2	0.16	0.18	0.18	0.13	0.17	0.2	0.17	0.21	0.16	0.15	0.15	0.13	0.12	0.11	0.13	0.06	0.01	0.02	0.01	0							
W1	0.11	0.08	0.05	0.09	0.09	0.2	0.15	0.14	0.13	0.16	0.09	0.19	0.15	0.15	0.17	0.14	0.14	0.11	0.1	0.11	0.07	0.1	0.15	0.11	0.11	0.13	0.1	0						
W2	0.09	0.08	0.05	0.07	0.08	0.15	0.13	0.1	0.11	0.12	0.07	0.14	0.12	0.1	0.13	0.11	0.09	0.08	0.06	0.07	0.04	0.07	0.15	0.1	0.11	0.11	0.09	0.02	0					
W3	0.13	0.11	0.08	0.1	0.09	0.19	0.12	0.11	0.1	0.11	0.06	0.17	0.14	0.13	0.15	0.12	0.12	0.12	0.1	0.11	0.08	0.1	0.15	0.1	0.11	0.11	0.09	0.02	0.03	0				
W4	0.14	0.11	0.09	0.11	0.09	0.24	0.18	0.15	0.16	0.16	0.11	0.21	0.19	0.17	0.2	0.16	0.15	0.14	0.13	0.13	0.11	0.13	0.16	0.09	0.11	0.11	0.09	0.04	0.05	0	0			
W5	0.13	0.1	0.08	0.12	0.1	0.25	0.17	0.15	0.16	0.16	0.1	0.22	0.19	0.18	0.2	0.18	0.16	0.14	0.11	0.12	0.1	0.11	0.19	0.12	0.14	0.15	0.12	0.03	0.05	0.01	0	0		
W6	0.13	0.11	0.09	0.12	0.12	0.27	0.2	0.17	0.19	0.19	0.12	0.25	0.22	0.2	0.22	0.19	0.18	0.15	0.14	0.14	0.12	0.14	0.21	0.13	0.16	0.16	0.14	0.06	0.05	0.04	0.03	0.01	0	
W7	0.12	0.1	0.08	0.09	0.1	0.24	0.18	0.16	0.16	0.17	0.11	0.2	0.17	0.16	0.18	0.15	0.14	0.13	0.12	0.14	0.11	0.14	0.19	0.12	0.14	0.13	0.12	0.04	0.04	0.02	0.02	0.02	0	0

Table SC11 Linear mixed-effect models for analyzed parameters with selected fixed effects (Distance from source (Dist), WWTP effluent upstream of the sampling site (WWTP), total concentration of AOM (Total), toxic unit values (TU), oxygen concentration (Oxy), pH value (pH), and conductivity (Cond)) and selection criteria (log Likelihood (logLik) and AIC criterion (AICc)).

Indicator	**Predictors (Fixed effects)**							**df**	**logLik**	**AICc**
	Dist	WWTP	Total	TU	Oxy	pH	Cond			
Allelic Richness								3	14.609	-22.3
		+	+					5	17.255	-22.1
		+						4	14.512	-19.5
			+					4	14.160	-18.8
				+				4	13.902	-18.3
Inbreeding (F_{is})								3	31.022	-55.2
				+				4	30.814	-52.1
		+						4	30.716	-51.9
Private alleles								3	37.929	-69.0
Abundance		+	+					5	-17.400	47.2
			+	+				5	-17.834	48.1
			+					4	-19.995	49.5
	+		+					5	-18.610	49.6
		+	+	+				6	-17.375	50.3
								3	-22.337	51.6
		+	+			+		6	-18.168	51.8
N_e								3	-17.708	42.3
		+						4	-17.031	43.6
		+		+				5	-16.909	46.2
			+					4	-18.437	46.4
						+		4	-18.456	46.5
				+				4	-18.735	47.0

Table SC12 Structural equation model output for each river and all rivers combined. A list of the analyzed parameters with a model estimate, standard errors (SE), z-values (z), and p-values (p) are included.

Wipper	**Estimate**	**SE**	**z**	**p**
Allel_Rich	~			
Distance_sourc	0.006	0.002	2.579	0.010
Total_log	-0.055	0.118	-0.470	0.639
Total_log	~			
Distance_sourc	0.010	0.007	1.366	0.172

Saale	**Estimate**	**SE**	**z**	**p**
Allel_Rich	~			
Distance_sourc	0.010	0.002	4.297	0.000
Total_log	-0.389	0.127	-3.062	0.002
Total_log	~			
Distance_sourc	0.014	0.006	2.114	0.034

Parthe	**Estimate**	**SE**	**z**	**p**
Allel_Rich	~			
Distance_sourc	0.004	0.005	0.791	0.429
Total_log	-0.164	0.341	-0.483	0.629
Total_log	~			
Distance_sourc	0.012	0.003	3.337	0.001

Holtemme	**Estimate**	**SE**	**z**	**p**
Allel_Rich	~			
Distance_sourc	0.001	0.006	0.144	0.886
Total_log	-0.068	0.116	-0.584	0.559
Total_log	~			
Distance_sourc	0.039	0.016	2.474	0.013

Eine	**Estimate**	**SE**	**z**	**p**
Allel_Rich	~			
Distance_sourc	0.010	0.001	8.191	0.000
Total_log	-0.428	0.121	-3.531	0.000
Total_log	~			
Distance_sourc	-0.003	0.004	-0.894	0.371

Altenau	Estimate	SE	z	p
Allel_Rich	~			
Distance_sourc	0.006	0.002	2.896	0.004
Total_log	-0.085	0.097	-0.879	0.380
Total_log	~			
Distance_sourc	-0.001	0.009	-0.140	0.889

All	Estimate	SE	z	p
Allel_Rich	~			
Distance_sourc	0.005	0.002	2.357	0.018
Abund	0.125	0.051	2.434	0.015
TUgam	0.033	0.015	2.222	0.026
Total_log	-0.257	0.056	-4.588	0.000
Abund	~			
Distance_sourc	-0.022	0.006	-3.498	0.000
TUgam	0.008	0.052	0.156	0.876
Total_log	0.630	0.161	3.924	0.000
TUgam	~			
Distance_sourc	0.057	0.019	2.981	0.003
Total_log	-0.182	0.556	-0.328	0.743
Total_log	~			
Distance_sourc	0.013	0.006	2.372	0.018

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