PROPAGATION OF CHEMICAL EFFECTS

ACROSS LEVELS OF BIOLOGICAL ORGANISATION AND HABITAT PATCHES OF SMALL STREAMS

by

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SUMMARY

The massive use of chemicals by humans is increasing pollution of the world's ecosystems. Yet, knowledge about exposure and effects of chemicals in real-world ecosystems remains limited. Prediction of chemical effects in the context of ecotoxicological research and chemical regulation continues to focus on organism- or population-level responses established under simplified conditions while aiming to protect the functioning of ecosystems. A unified, comprehensive framework for the prediction of chemical effects in real-world ecosystems is still lacking. A major limitation of ecotoxicological studies considered in predictive modelling is that they rarely consider spatial dynamics (e.g. gene flow or species dispersal) as relevant processes influencing the trajectory of populations or communities, respectively. For instance, the spatial propagation of pesticide effects from polluted to least impacted sites has been predicted in several modelling studies but has not yet been characterised in the field.

The thesis starts in Chapter 1 with a brief introduction to chemical pollution in ecosystems, chemical effect prediction in ecotoxicology, and pesticides in freshwater ecosystems, then outlines the main objectives of the thesis. Subsequently, Chapter 2 presents a conceptual study about the current prediction of chemical effects in ecotoxicology and potential future avenues to improve ecological relevance of effect predictions by addressing the integration of different levels of biological organisation (termed biological levels). The study shows that approaches and tools that currently contribute to the prediction of chemical effects can be attributed to three idealised perspectives: the suborganismal, organismal and ecological perspective. The perspectives focus on different biological levels and are associated with distinct scientific concepts and communities. They complement each other so theoretical and empirical links between them may enhance prediction by capturing the entire phenomenon of chemical effects, from chemical uptake to ecosystem effects. Complex experimental studies accounting for eco-evolutionary dynamics are needed to cross barriers between biological levels as well as spatiotemporal scales. Overall, the conclusions of Chapter 2 may help to develop overarching frameworks for predicting chemical effects in ecosystems, including for untested species. Chapters 3 and 4 present a field study combined with laboratory analyses on the potential propagation of pesticides and their effects from agricultural stream sections to the edge of least impacted upstream sections, that can serve as refuges for many species. The study examines exposure and effects for different biological levels at three site types, the pesticide-polluted agricultural sites (termed agriculture), least impacted upstream sites (termed refuge) and transitional sites (termed edge) in six small streams of south-west Germany. The results in Chapter 3 show that regional transport of pesticides can lead to ecologically relevant pesticide exposure in forested sections within a few kilometres upstream of agricultural areas (i.e. at both edge and refuge sites). As further demonstrated in Chapter 3, the tested indicators of community responses (Jaccard Index, taxonomic richness, total abundance, $\text{SPEAR}_{\text{pesticides}}$) together suggest a species turnover from upstream refuge to downstream agricultural sites and a potential influence of adjacent agriculture on the edge sites. In contrast, Chapter 4 does not identify any particular edge effect that distinguish edge organisms and populations in edge sites from those in more upstream refuge sites. *Gammarus fossarum* populations at edges show equal levels of imidacloprid tolerance, energy reserves (i.e. lipid content) and genetic diversity to populations further upstream. *Gammarus* spp. from agricultural sites exhibit a lower imidacloprid tolerance compared to edge and refuge, potentially due to energy trade-offs in a multiple stressor environment, but related effects do not propagate to the edges (Chapter 4). Notwithstanding, the results of Chapter 4 indicate bidirectional gene flow between site types, supporting the hypothesis that adapted genotypes – if present at locally polluted sites – could spread to populations at least impacted sites. Taken together, Chapters 3 and 4, illustrate that pesticides and their effects can potentially propagate to least impacted upstream sections, empirically novel findings to our knowledge. These results of this thesis can help in predicting or explaining population and community dynamics in least impacted habitats and can ultimately inform pesticide management as well as freshwater restoration and protection of biodiversity.

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1 INTRODUCTION AND OBJECTIVES

1.1 DECADES OF CHEMICAL POLLUTION IN ECOSYSTEMS

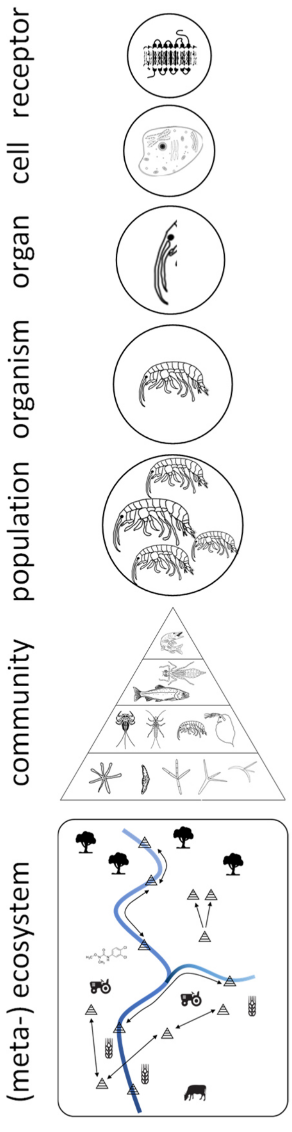
As industrialisation has progressed, ecosystems have experienced an increasingly strong and far-reaching human influence (Rockström et al., 2009; Steffen et al., 2007). Since 1970, the ecological footprint of humankind has exceeded Earth's rate of regeneration (WWF, 2020). Aquatic and terrestrial ecosystems around the globe have shown declines in biodiversity (Díaz et al., 2019; Reid et al., 2019; WWF, 2020) and degradation in ecosystem functioning, threatening the maintenance of ecosystem services, such as the provision of clean water (Díaz et al., 2019; Mace et al., 2012). Major direct drivers of declines in biodiversity and ecosystem functioning are overexploitation, change in land and sea use, climate change, invasion of alien species and chemical pollution (Díaz et al., 2019). Climate change and biodiversity loss are now recognised as significant global menace for the environment and humankind, while the scale and magnitude of the chemical crisis is still being assessed (Brack et al., 2022). Recent studies indicate that the chemical crisis has a global and irreversible dimension for numerous compounds, with implications for biodiversity and interactions with climate change as well as other stressors (Brack et al., 2022; Carney Almroth et al., 2022; Cousins et al., 2022; Diamond et al., 2015; Persson et al., 2022; Stehle & Schulz, 2015a).

Chemicals such as pesticides, pharmaceuticals or solvents have become a common mainstay of society, with diverse uses, for instance, in agriculture, medicine or industrial production. During their life cycle, they can enter the environment via a variety of entry paths, including direct discharges, surface runoff, aerial drift and atmospheric depositions from point or diffuse sources (Ahrens & Bundschuh, 2014; Carter, 2000; Hemond & Fechner, 2022; Stamm et al., 2008). Physico-chemical properties of a chemical interacting with geological, hydrological, and climatic processes govern chemical partitioning into various environmental compartments (air, water, sediment, organisms), which naturally also determines in which ecosystems (terrestrial or aquatic) and in what quantities chemicals occur (Hemond & Fechner, 2022; Nowell et al., 1999; Schäfer & Bundschuh, 2018). Once in an ecosystem, chemicals can affect different levels of biological organisation from suborganismal processes, over single organisms, populations, food webs and communities up to the functioning and services of the ecosystem itself (see Box 1).

Awareness of associations between chemical pollution and detrimental effects on the environment and humans has evolved first in the 20th century, ushering in a period of chemical policy, regulation, research, monitoring and management (Carson, 1962; Suter, 2008). This has led, for example, to legal frameworks for water policy, including binding rules for environmental quality standards (e.g., EC, 2013, 2000), and the construction of wastewater treatment plants (Lofrano & Brown, 2010) as well as efforts towards green and sustainable chemistry (Kümmerer & Clark, 2016). Despite these activities, the total quantity, diversity and geographic expansion of synthetic chemicals have continuously increased over the last five decades (Bernhardt et al., 2017), and more than 350,000 chemicals and mixtures of chemicals are registered for production and use to date (Wang et al., 2020). In addition, related industries, usages or risks are projected to grow in the upcoming decades (Civity Management Consultants, 2017; Delcour et al., 2015; Kattwinkel et al., 2011; Koleva & Schneider, 2010, 2009; U.N. Environment, 2019; Van Boeckel et al., 2015).

While the production and diversity of chemicals have increased rapidly, our knowledge about the complete chemical spectrum present in the environment is slowly becoming more comprehensive, largely due to technological progress, such as analytical multi-compound screening techniques (Brack et al., 2019; Escher et al., 2020; Moschet et al., 2014). For example, a wide range of pesticides, pharmaceuticals, consumer products and industrial chemicals are detected in different environmental compartments (e.g. aus der Beek et al., 2016; Halbach et al., 2021; Liess et al., 2021; Newell et al., 2020; Pelosi et al., 2021; Wilkinson et al., 2022). Knowledge about the temporal dimension of chemical pollution in terms of long-term averages and short-term peak exposures in ecosystems is also evolving, at least for data-rich regions such as Europe and North America (Brack et al., 2022; Vormeier et al., 2023). In contrast, our understanding of the effects of chemicals in complex real-world ecosystems is still severely tenuous and approaches to quantify or predict those are lacking (Box 1, and further discussed in Chapter 1.2).

Box 1: Chemical effects across levels of biological organisation



From the smallest to the largest scales, life is organised in defined structures that extend from molecules (e.g. receptors) over organelles to cells, tissues, organs, organ systems, organisms, populations, communities, ecosystems and finally to meta-ecosystems (or landscapes, Schäfer & Bundschuh, 2018). Chemical exposure can impact all of these levels of biological organisation (further referred to as biological levels; Clements, 2000; Schäfer & Bundschuh, 2018). Initially, however, chemicals always act at the molecular, biochemical or physiological levels – which are here collectively referred to as suborganismal levels (i.e. levels hierarchically below organism level). Suborganismal effects caused by chemicals include effects on the metabolism, cell signalling or hormone system (Schäfer & Bundschuh, 2018). For example, exposure to organochlorine chemicals has been associated with the induction of vitellogenin in male fish (Randak et al., 2009). Depending on the magnitude of these suborganismal changes, they can lead to effects on the entire organism, such as reduced fitness and growth, altered behaviour or increased mortality (Brodin et al., 2013; Schäfer & Bundschuh, 2018). Organism effects can then translate to the population level, measurable, for instance, as a reduction in population size and shifts in the age structure or the sex ratio of populations (Schäfer & Bundschuh, 2018). In the last decade, a link between chemical pollution and declines in populations has been suggested for aquatic and terrestrial ecosystems of different regions of the world (Beketov et al., 2013; Desforges et al., 2018; Kidd et al., 2007; Oaks et al., 2004; Rundlöf et al., 2015). Population level effects, in turn, can result in changes in the structure of communities or impact interspecific relationships (e.g. competition, Schäfer & Bundschuh, 2018). Such community effects may alter the functioning of ecosystems (e.g. nutrient cycling or organic matter processing) if a specific function is not compensated by an increase in tolerant organisms with the same function as the sensitive organisms (functional redundancy; Schäfer & Bundschuh, 2018). For instance, pesticide exposure has been repeatedly associated with effects on community structures and ecosystem functions in aquatic ecosystems (Rasmussen et al., 2008; Rumschlag et al., 2020; Schäfer et al., 2007, 2012). The impact on an ecosystem can eventually influence meta-ecosystem dynamics, such as flows of material, energy and organisms as well as temporal (evolutionary) dynamics (Schäfer & Bundschuh, 2018). The alteration of (meta-) ecosystem functions can endanger the services that ecosystems provide for humans (e.g. clean water provision, Díaz et al., 2019; Mace et al., 2012). With increasing hierarchical levels, the complexity of the system increases and stressor-response specificity (or causality) usually decreases as more and more processes are encompassed, which challenges the assessment of effects at these levels (Clements, 2000; Schäfer & Bundschuh, 2018).

1.2 CHEMICAL EFFECT PREDICTION IN ECOTOXICOLOGY

Understanding the effects of chemicals on suborganismal processes, organisms, populations, communities and ecosystems (Box 1) is a core topic of the scientific discipline of ecotoxicology and provides the foundation of risk assessment and management under chemical regulation (Calow & Forbes, 2003).

Developed as an equivalent of human toxicology, the general ecotoxicological approach to assess the effects of chemicals has been mainly based on summary statistics, so-called (no observed) effect concentrations ((NO)EC) estimated from quantitative dose-response or concentration-effect relationships (Calow & Forbes, 2003). Such relationships are typically established under controlled laboratory settings for single model species and chemicals (Forbes et al., 2017). The effect is usually measured as mortality, growth or reproduction and expressed as a fixed percentage (e.g. lethal or sublethal concentration affecting x % of tested organisms; LCx and ECx, respectively) (Calow & Forbes, 2003). Notwithstanding, such traditional ecotoxicological effect data are still insufficiently available for many chemicals and different taxa (Posthuma et al., 2019). For example, 88% of human pharmaceuticals are lacking comprehensive effect data (OECD, 2019) or 53% of studies focusing on pesticides and freshwater invertebrates are based on a single species, the standard test organisms *Daphnia magna* (Beketov & Liess, 2012). Vulnerable and ecologically relevant species, such as fungi with crucial roles in stream ecosystems, are not adequately represented in ecotoxicological studies (Zubrod et al., 2015). The main emphasis of traditional ecotoxicological effect assessments has been on freshwater systems and short-term (acute) exposures, whereas data for other ecosystems (e.g. terrestrial or marine) and long-term (chronic) exposures are still less common (Calow & Forbes, 2003). However, even if such traditional effect data were available for the full spectrum of chemicals and many species from different ecological systems, it is now widely recognized that the traditional ecotoxicological test approach is a considerable oversimplification of real-world ecosystem conditions and that derived data may not provide the desired protection from chemical effects (Calow & Forbes, 2003).

Protection targets are often vaguely defined in chemical or environmental legislation (e.g. it is not defined what “unacceptable effects” are, Forbes et al., 2017), but rather relate to populations, communities, food webs and ecosystems than to organisms of a single species (Clements, 2000; EFSA, 2016; Forbes & Galic, 2016; Raimondo et al., 2019). In real-world ecosystems, organisms and populations of a species are influenced by a variety of environmental factors (e.g. pH, temperature) that can differ considerably spatially and temporally but which are mostly disregarded in traditional ecotoxicity tests. In addition, repeated or parallel exposures to chemicals and multiple stressors (e.g. habitat destruction, climate change) can have an impact on the trajectory of populations in real-world ecosystems but are largely ignored in traditional ecotoxicity tests (Schäfer et al., 2023; Topping et al., 2020). Furthermore, focusing on single species in a test system neglects interactions between different species and trophic levels, whereby effects may cascade to higher biological levels such as communities or food-webs potentially resulting in effects on the entire ecosystems (Box 1, Brühl & Zaller, 2019; Kattwinkel & Liess, 2014; McMahon et al., 2012). Data from more complex experimental designs, such as micro/mesocosms or field studies, that attempt to account for ecological complexity are still largely underrepresented in ecotoxicological research and chemical regulation. A literature study found that less than 1% of all studies focussing on pesticides and freshwater invertebrates have been field studies in real ecosystems (Beketov & Liess, 2012). Finally, micro/mesocosm studies are themselves based on a small and biased set of species not representative of real-world communities (Forbes & Calow, 2002b) and due to their typically short duration and spatial restrictions largely ignore (similar to the laboratory tests) influence of spatial (e.g. species dispersal, gene flow) or temporal dynamics (e.g. adaptation, speciation) that can alleviate or magnify exposure and effects of chemicals (Govaert et al., 2021; Schäfer et al., 2023; Schiesari et al., 2018; Streib et al., 2022).

In regulatory chemical risk assessment, the various uncertainties in effect estimation (i.e. from laboratory – to field conditions, between different species, etc.) are assumed to be covered by a safety factor also called extrapolation, assessment or uncertainty factor, by which the estimated effect concentration is divided (Calow & Forbes, 2003). However, the use of a safety factor lacks a mechanistic foundation. Analyses have shown that the toxicity of a chemical may vary among species and between acute and chronic responses at a greater magnitude than covered by a fixed safety factor (Calow & Forbes, 2003; Forbes & Calow, 2002a).

To sum up, the understanding of chemical effects is still highest on the lower biological levels given that ecotoxicological studies historically focussed on these, but the related ecological relevance is tenuous (Beketov & Liess, 2012; Clements, 2000; Kramer et al., 2011; Schäfer & Bundschuh, 2018). The prevalent disregard of the multitude of processes and interactions relevant to ecosystems within the traditional ecotoxicological approach has likely contributed to unanticipated effects of chemicals in real-world ecosystems (e.g. Oaks et al., 2004; Rundlöf et al., 2015; Tian et al., 2021). To date, assessing and predicting the effects of chemicals on complex real-world ecosystems (Chapter 2), such as surface freshwaters (Chapters 3 and 4), remains one of the most important research challenges in ecotoxicology (Van den Brink et al., 2018). How this challenge is currently addressed in research and may be improved in the future is the topic of the conceptual study presented in Chapter 2 of this thesis.

1.3 PESTICIDES IN SURFACE FRESHWATERS

The previous Chapters (1.1 and 1.2) focused on chemical pollution in ecosystems in general. Among the different ecosystems, surface freshwaters (i.e. here lakes, ponds and streams) play a special role on our planet. They make up less than 0.01% of the world's water (Shiklomanov, 1993) and cover only around 3.5% of the earth's land surface (Downing et al., 2006, 2012), but contain almost 10% of global animal biodiversity (Balian et al., 2008) and approximately 40% of the global fish diversity (Lundberg et al., 2000). At the same time, more than 50% of humans on Earth live in proximity (< 3 km) to a surface freshwater body, whilst 90% live within 10 km (Kummu et al., 2011). This proximity arises from humans traditionally seeking easy access to freshwaters as these exhibit crucial roles for household, industrial, domestic and agricultural purposes, as well as aesthetics, culture and recreation (Jackson et al., 2001; McCool et al., 2008). Overall, freshwaters provide a high monetary value of ecosystem services to humans (de Groot et al., 2012). However, the proximity and intensive use of freshwaters come at a cost. Freshwater ecosystems around the globe are degraded or destroyed, and freshwater taxa are threatened globally (Collen et al., 2014; Dudgeon et al., 2006; Vörösmarty et al., 2010). In addition to excessive nutrient input or hydro-morphological changes, pesticides contribute considerably to the degradation of freshwater ecosystems (Liess et al., 2021; Schäfer et al., 2016; Stehle & Schulz, 2015a). For example, pesticide effects have been observed on different biological levels in freshwaters throughout the globe, including on populations of single species (Leonard et al., 2000; Liess & Schulz, 1999), entire communities (Beketov et al., 2013; Chiu et al., 2016; Hunt et al., 2017; Liess et al., 2021; Stehle & Schulz, 2015a) and ecosystem functions (Rasmussen et al., 2008; Rumschlag et al., 2020; Schäfer et al., 2007, 2012).

The particularity of pesticides is that these are chemicals designed to be hazardous to pests, pathogens or unwanted weeds and are intentionally applied on large areas in agricultural landscapes to maximize yields (Damalas & Eleftherohorinos, 2011; Kosnik et al., 2022). After the application of pesticides to agricultural land, only a small portion of the total amount applied may reach the intended target pests, while the majority can undergo various unintended environmental transport and fate processes (Pimentel, 1995). Through processes such as runoff or spray drift from agricultural areas and to a lesser extent, through discharges from wastewater treatment plants or urban runoff, pesticides enter surface freshwaters (Carter, 2000; Wittmer et al., 2010). In freshwater ecosystems, pesticides typically occur in complex mixtures. A recent field study detected up to 50 pesticides in a single water sample from a small stream (Schreiner et al., 2021).

Small streams, usually defined as first- and second-order streams (Strahler, 1952, 1957) show particularly high levels of pesticide contamination (Halbach et al., 2021; Knauer, 2016; Schulz, 2004; Stehle & Schulz, 2015b), given that (i) they typically drain landscapes with diverse land uses, (ii) they are often close to application areas and (iii) they exhibit a smaller ratio between water volume and riparian length resulting in a lower dilution potential relative to larger rivers (Lorenz et al., 2017; Szöcs et al., 2017; Wohl, 2017). At the same time, small streams are of high ecological relevance, as they (i) make up over 80% of the global as well as European stream length and, therefore riparian zones (Downing et al., 2012; Kristensen & Globevnik, 2014), (ii) provide unique habitats for diverse species, such as for recovery or reproduction purposes (e.g. Meyer et al., 2007), and (iii) govern connectivity at the watershed-scale as well as other ecosystem services (Biggs et al., 2017; Ferreira et al., 2022; Wohl, 2017; and references herein).

Despite their potentially high pesticide exposure and ecological relevance, small streams are less frequently sampled than larger ones and insufficiently protected by European law (Biggs et al., 2017; e.g. EC, 2000; Lorenz et al., 2017). Recent awareness of the importance of small streams (and other small water bodies) has spurred investigation into pesticide exposure and effects for agricultural patches of small streams. A recent comprehensive monitoring study for small agricultural streams in Germany identified pesticides as the main stressors for macroinvertebrates (Liess et al., 2021), a species group that plays an important role in the functioning of stream ecosystems (Wallace & Webster, 1996). However, agricultural stream sections are no isolated entities but they are in complex exchange with their biological, physical and chemical environment up- and downstream as well as with the surrounding terrestrial systems through flows of matter, energy or organisms (Loreau et al., 2003). Such exchanges may affect the chemical and ecological quality of adjacent habitats, even the least impacted ones, if pesticides or their effects propagate (as further discussed in Chapter 1.4). Least impacted habitats are defined as areas without significant human influence given an almost omnipresent anthropogenic influence on the planet (Chapters 3 and 4).

1.4 THE PROPAGATION OF PESTICIDES AND THEIR EFFECTS

Ecosystems in different states of pesticide pollution can influence each other via flows of air, water or organisms (Hageman et al., 2006; Harding et al., 2006; Richmond et al., 2018). For example, the rescue effect as part of the source-sink concept describes the phenomenon that organism movements (i.e. dispersal) from patches with positive per capita growth rate (called sources, in this case the least impacted patches) can enhance the persistence of populations in patches with negative per capita growth rate (called sinks, in this case the polluted patches) (Pulliam, 1988). Modelling, mesocosm and field studies suggest that organism dispersal between patches can enhance recovery or alleviate effects in a pesticide-polluted patch (Caquet et al., 2007; Hanson et al., 2007; Knillmann et al., 2018; Liess & Schulz, 1999; Orlinskiy et al., 2015; Schäfer et al., 2012, 2017; Trekels et al., 2011; Willson & Hopkins, 2013). Studies focusing on such benefits for polluted stream sections have received great attention. Conversely, pesticides or their effects may propagate from polluted to least impacted stream sections, processes that have been taken much less into focus. For example, pesticides can bioaccumulate at polluted sites and be released in the least impacted sites (e.g. biovector-transport; Richmond et al., 2018; Schiesari et al., 2018) or simply get transported in the air, water or soil (Chapter 3). Regarding pesticide effects, modelling studies suggest that the positive effect of recolonisation for pesticide-polluted patches can lead to a negative effect for adjacent source populations, such as their depletion through dispersal and dispersal mortality (Schäfer et al., 2017), and too high pesticide-driven population decline may threaten the persistence of meta-populations (Amarasekare, 2004; Willson & Hopkins, 2013). Such effects have been termed “carryover effects” or “action at a distance” (Schäfer et al., 2017; Spromberg & Scholz, 2011). In addition, organisms from polluted sites may propagate transgenerational (heritable) effects, such as increased pesticide tolerance, to least impacted populations through dispersal and successful genetic exchange (i.e. gene flow) with organisms from least impacted sites. Increased tolerance to pesticides has been repeatedly found in non-target freshwater populations in pesticide polluted stream sections compared to non-exposed populations (Becker & Liess, 2017; Bendis & Relyea, 2014; Shahid et al., 2018; Weston et al., 2013). Modelling studies suggest that gene flow can increase the frequency of tolerance-promoting alleles in nearby untreated fields (Caprio & Tabashnik, 1992; Comins, 1977), potentially contributing to pre-adaptations of untreated populations (Uyenoyama, 1986). For pest species, field studies suggest that pesticide resistance can spread through gene flow over long distances (Fraaije et al., 2005; Raymond et al., 1991; Torriani et al., 2009). To which extent this applies to non-target species in freshwater ecosystems remains unknown and to be characterised in the field (Chapter 4). Characterisation in the field is required at different biological levels, as the propagating effects may be present or detectable to varying degrees. Carryover effects, when present, are likely most pronounced at the transitions, here termed edges, between ecosystems, as these are often characterised by bi-directional expansions of the flows of organisms, matter and energy (Fischer & Lindenmayer, 2007). Edge effects are well known for terrestrial ecosystems (Magura et al., 2017; Ries et al., 2004; Wimp & Murphy, 2021), but largely lacking for freshwaters. Empirical evidence of the potential of pesticide effects to propagate from polluted stream sections to the edge of least impacted stream sections is important to advance our assessment of the costs of pollution for organisms, populations and communities in least impacted areas. A combined field and laboratory study is used in Chapters 3 and 4 of this thesis to address this research gap.

1.5 OBJECTIVES AND STRUCTURE OF THE THESIS

The overall research objectives of this thesis were (i) to identify future avenues to improve the prediction of chemical effects in ecosystems (Figure 1.1; Chapter 2) and (ii) to examine a potential edge effect of pesticides in stream ecosystems (Figure 1.1; Chapters 3 and 4). In doing so, the thesis focuses on different biological levels, as these can provide distinct valuable information and their simultaneous consideration may lead to deeper insights.

In detail, with Chapter 2 the thesis starts with a conceptual work about the prediction of chemical effects in ecosystems. Specific aims were:

- to provide an overview of current approaches and tools used for chemical effect prediction,
- to address the integration of biological levels and potential links between them in chemical effect prediction,

- and to identify related research gaps and provide guidance for future avenues in chemical effect prediction.

Chapter 3 examines the potential edge effect of pesticides, focusing on the exposure and the community level. Specific aims were:

- to scrutinise whether pesticides (i.e. concentration and toxicity) - and their effects at the community level (i.e. absolute abundance, structural and functional diversity of macroinvertebrate community) - propagate from agricultural stream sections to the edge of upstream least impacted stream sections.

Chapter 4 continues to examine the potential edge effect of pesticides, focusing on the level of organisms and populations. Specific aims were:

- to test whether pesticide effects on the population level (i.e. pesticide tolerance, genetic structure) and organism level (i.e. energy reserves) propagate from agricultural stream sections to the edge of upstream least impacted stream sections.

Finally, the general discussion in Chapter 5 elaborates on the individual findings of Chapters 2, 3 and 4 concerning overall relevance and novelty and suggests research needs for the future.

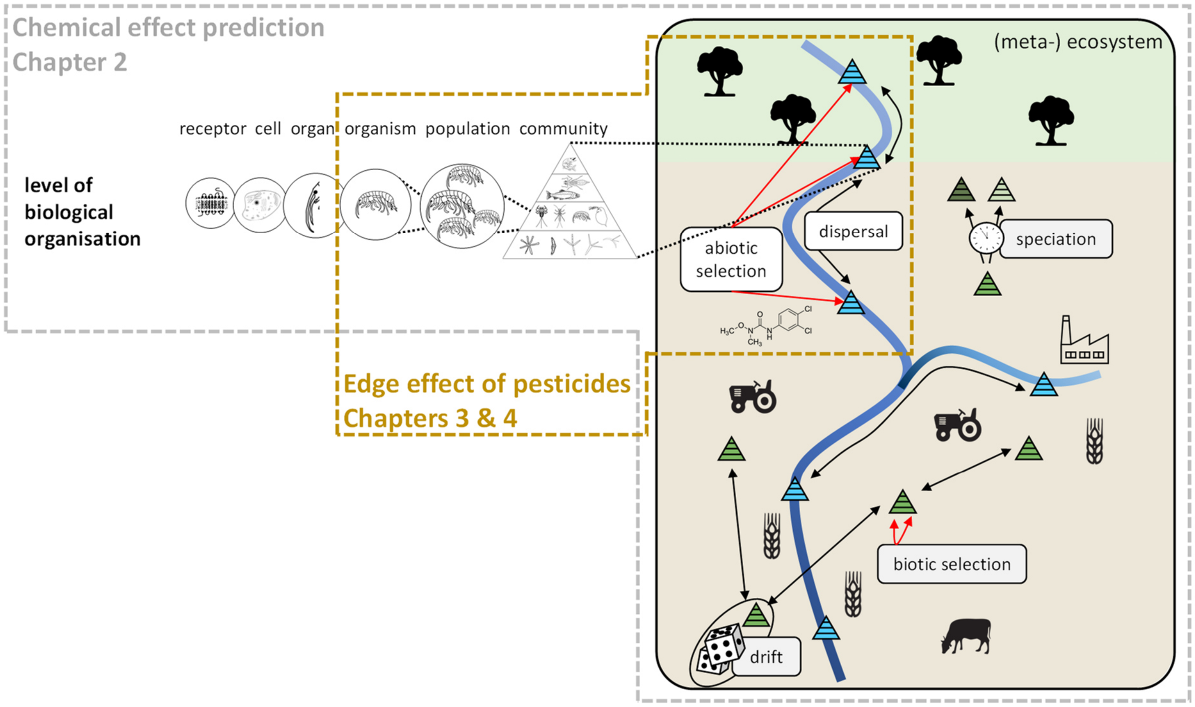


Figure 1.1 Simplified overview of the topics addressed by this thesis. The boxes delimited by the dashed grey and orange line represent the topics of Chapter 2 and of Chapters 3 and 4, respectively. The large box delimited by the solid black line represents an exemplary meta-ecosystem with green background colour referring to forest and brown background colour referring to agricultural areas. Triangles represent aquatic (blue) and terrestrial (green) communities. Arrows depict a bi- or unidirectional exchange of materials, chemicals and energy (black) or impact (red).

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2 THREE PERSPECTIVES ON THE PREDICTION OF CHEMICAL EFFECTS IN ECOSYSTEMS

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2.1 ABSTRACT

The increasing production, use and emission of synthetic chemicals into the environment represents a major driver of global change. The large number of synthetic chemicals, limited knowledge on exposure patterns and effects in organisms and their interaction with other global change drivers hamper the prediction of effects in ecosystems. However, recent advances in biomolecular and computational methods are promising to improve our capacity for prediction. We delineate three idealised perspectives for the prediction of chemical effects: the suborganismal, organismal and ecological perspective, which are currently largely separated. Each of the outlined perspectives includes essential and complementary theories and tools for prediction but captures only part of the phenomenon of chemical effects. Links between the perspectives may foster predictive modelling of chemical effects in ecosystems and extrapolation between species. A major challenge for the linkage is the lack of data sets simultaneously covering different levels of biological organisation (here referred to as biological levels) as well as varying temporal and spatial scales. Synthesising the three perspectives, some central aspects and associated types of data seem particularly necessary to improve prediction. First, suborganism- and organism-level responses to chemicals need to be recorded and tested for relationships with chemical groups and organism traits. Second, metrics that are measurable at many biological levels, such as energy, need to be scrutinised for their potential to integrate across levels. Third, experimental data on the simultaneous response over multiple biological levels and spatiotemporal scales are required. These could be collected in nested and interconnected micro- and mesocosm experiments. Lastly, prioritisation of processes involved in the prediction framework needs to find a balance between simplification and capturing the essential complexity of a system. For example, in some cases eco-evolutionary dynamics and interactions may need stronger consideration. Prediction needs to move from a static to a real-world eco-evolutionary view.

2.2 INTRODUCTION

Studies around the globe have established associations of chemical groups of concern, e.g. pesticides, pharmaceuticals and industrial chemicals, with population decline and biodiversity loss (Beketov et al., 2013; Brodin et al., 2013; Desforges et al., 2018; Kidd et al., 2007; Oaks et al., 2004; Rundlöf et al., 2015). Since 1970, synthetic chemical production has increased at a higher rate than other drivers of global change, such as CO_2 emissions or anthropogenic land-use change (Bernhardt et al., 2017). A recent study estimated more than 350,000 chemicals to be registered for production and use (Wang et al., 2020). In the environment, a wide range of chemicals of urban, agricultural and industrial origin can be detected, in particular in aquatic ecosystems that typically drain landscapes with diverse land use (Bradley et al., 2019; Moschet et al., 2014). For example, more than 400 chemicals with approximately 30 different modes of action (MoA) were found in three Central European rivers (Busch et al., 2016). A study on 19 agricultural streams in Eastern Europe found up to 50 pesticides in a single water sample (Schreiner et al., 2021). Simultaneous exposure to multiple chemicals can cause additive or synergistic effects compared to individual exposure (Cedergreen, 2014) depending on their MoA and interaction type. For example, mixtures including cholinesterase inhibitors or azole fungicides, both interfering with the metabolisation of other chemicals, tend to induce synergism (Cedergreen, 2014). Thus, predicting the effect of chemicals on ecosystems requires consideration of potential mixture effects and, consequently, knowledge on all chemicals occurring in an ecosystem. However, most studies focus on target chemicals, i.e. chemicals of concern. These often explained only a minor fraction of the total measured effect in *in vitro* bioassays, suggesting that relevant chemicals might be missed by the current targeted chemical analysis (Escher et al., 2020). Non-target chemical analysis can cover a wider range of chemicals (Brack et al., 2019). However, even if the full spectrum of chemicals was detected and quantified, predicting their ecological effects would be hampered by the absence of ecotoxicological data for many chemicals (Posthuma et al., 2019; Schäfer et al., 2013; Wang et al., 2020). Overall, the large number of synthetic chemicals in the environment combined with limited knowledge on their occurrence and effects in organisms compromise our ability to predict chemical effects in ecosystems. This in turn hampers ecosystem management and conservation and may contribute to underestimating chemicals as one of the drivers of biodiversity loss (Groh et al., 2022; Schäfer et al., 2019).

Predicting the impacts of chemicals in ecosystems is the key topic of the scientific discipline of ecotoxicology. Similarly, chemical regulation, operationalised as risk assessment and management, aims at protecting ecosystems from unacceptable impacts of chemicals. Protection goals typically target populations, communities, food webs and ecosystems (EFSA Scientific Committee, 2016; Forbes & Galic, 2016; Raimondo et al., 2019). However, ecotoxicology and chemical regulation have traditionally relied on summary statistics, so-called (no observed) effect concentrations ((NO)EC), derived from quantitative concentration-effect relationships established with single taxa and chemicals under controlled laboratory settings with optimal conditions for the test species (Forbes et al., 2017). The effect is typically measured as mortality compared to a control (also called lethal concentration killing x % of the test organisms, LC_x), but can also relate to sublethal measures such as growth or reproduction (called effect concentration, EC_x). For such data, simple mathematical models have demonstrated high predictability of toxic effects of mixtures (Kortenkamp & Faust, 2018). However, extrapolating such results to non-tested species and using them to predict impacts on spatiotemporally heterogeneous ecosystems remains a major challenge, which has recently been rated among the most important current research questions in ecotoxicology (Van den Brink et al., 2018). This challenge is due to organisms in nature being influenced by a range of environmental factors (e.g. pH, light), and multiple additional stressors (e.g. climate change, habitat degradation) (Birk et al., 2020; Côté et al., 2016) as well as complex eco-evolutionary dynamics in ecosystems driven by species dispersal, adaptation and species interactions such as predation or facilitation (Cadotte & Tucker, 2017; Govaert et al., 2021; Schäfer, 2019; Schiesari et al., 2018). Ecological studies have often dealt with such complexity by seeking to establish links between stressors and ecological responses based on field studies. Adopting this approach for chemicals is hampered by their sheer amount and potential interactions between chemicals in mixtures as well as with other environmental factors and stressors (Posthuma et al., 2020). Conversely, the reliance of chemical regulation on parameters such as experimentally derived (NO)ECs from few laboratory test species, coupled with a lack of mechanistic insight into the chemical MoA and ecosystem dynamics, has repeatedly led to ecological surprises in terms of unforeseen effects in ecosystems (Oaks et al., 2004; Rundlöf et al., 2015; Tian et al., 2021).

Here, we delineate three idealised perspectives that substantially contribute to date to prediction and cross-species extrapolation (Figure 2.1): 1) The suborganismal perspective, 2) the organismal perspective and 3) the ecological perspective. The suborganismal perspective uses molecular, cellular and biochemical information to predict chemical effects at the suborganism-level, rarely resulting in robust links to organism-level responses (Chapter 2.3, Kramer et al., 2011). The organismal perspective mainly focusses on effects at the organism and population level and also attempts to integrate chemical absorption (here uptake), distribution, metabolism and excretion (ADME) processes (Chapter 2.4). The ecological perspective provides concepts to predict the consequences of organism- or population-level effects in complex ecosystems, focussing on processes and underlying mechanisms in populations, communities, food webs and ecosystems (Chapter 2.5). We describe the perspectives, highlight challenges and identify links between the perspectives that may foster prediction and cross-species extrapolation in the following Chapters. We focus on process-based and mechanistic approaches and pay only limited attention to approaches based on data-driven (e.g. based on statistics and machine learning) relationships, e.g. interspecies correlation estimates (Dyer et al., 2006) or estimates based on species relatedness (van den Berg et al., 2021). In addition, our main focus is the prediction of ecological effects using biological approaches, whereas chemical-driven approaches, such as quantitative structure-activity relationships that rely on the chemical structure and physicochemical substance properties for prediction (Barron et al., 2015), are certainly also interesting and relevant but beyond our scope.

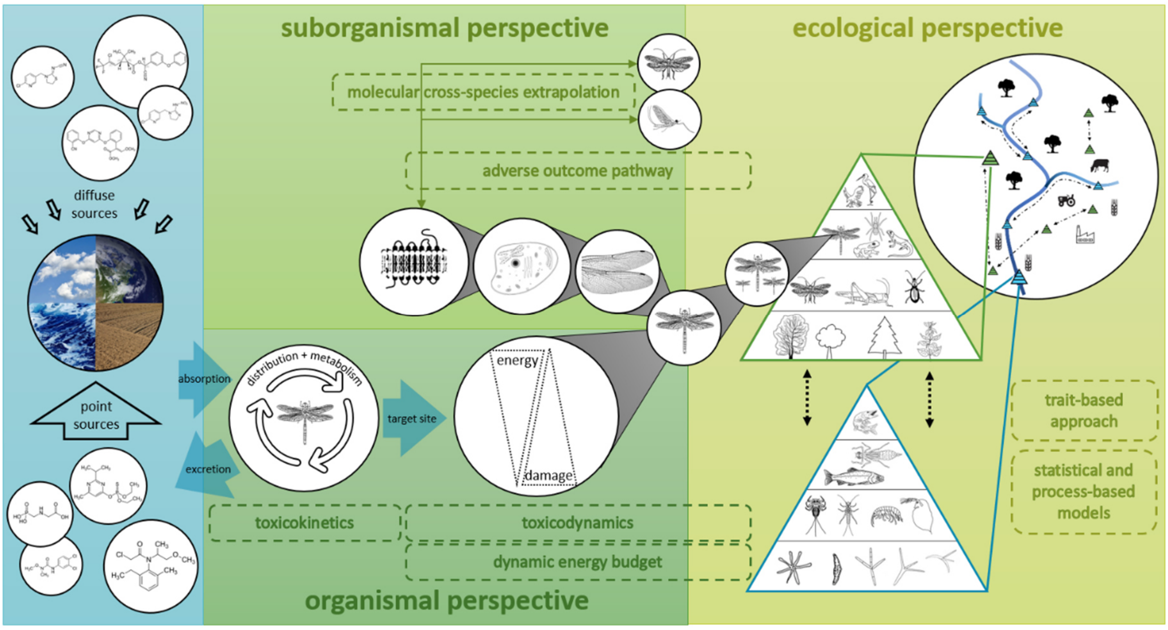

Figure 2.1 : Simplified representation of the interrelationship between the different perspectives to predict the effects of chemicals on ecosystems. The dashed boxes illustrate associated approaches and tools.

2.3 SUBORGANISMAL PERSPECTIVE

Although higher biological level effects are of ultimate interest in research and chemical regulation, they are always triggered by suborganismal processes such as a molecular initiating event (MIE, Figure 2.2). Responses to chemicals at the suborganismal level are largely considered as early warning signs of exposure or effects (Clements, 2000; Hagger et al., 2006). Although data from this level including single sensitive biomarkers are generally insufficient for robust effect prediction at the ecosystem level, they are pivotal for understanding mechanisms underlying chemical effects and represent a critical element of prediction and extrapolation. Since the early 2000s, a research area has emerged that relies on mechanistic understanding for the prediction of chemical effects through the use of emerging molecular, cellular and biochemical tools (de Nadal et al., 2011; Ouborg & Vriezen, 2007; Reusch & Wood, 2007).

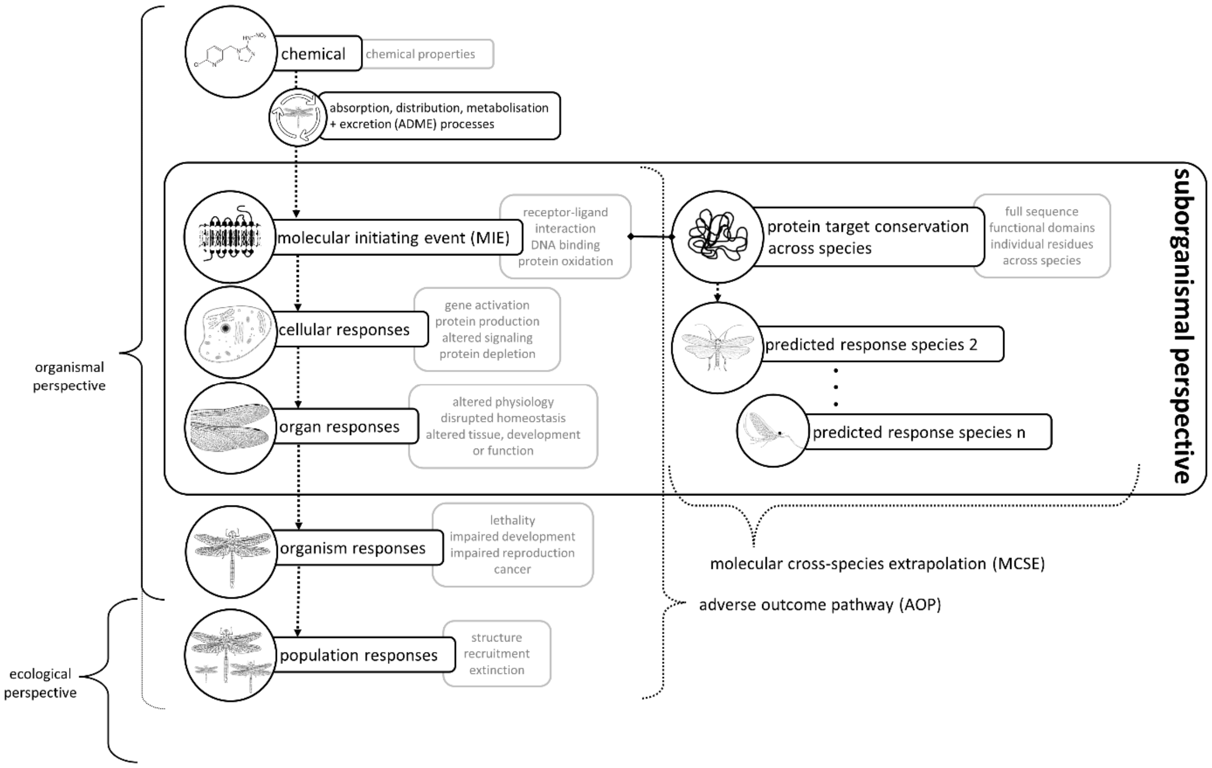

Figure 2.2: Main processes related to the suborganismal perspective. The box delineated by the solid black line depicts the main focus of the suborganismal perspective. Boxes in light grey provide more detailed information on terms. Arrows connect biological levels, whereas brackets indicate the processes and levels associated with the concepts of adverse outcome pathways (AOPs) illustrated based on Ankley et al. (2010) and molecular cross-species extrapolation (MCSE) and the other perspectives.

2.3.1 Adverse outcome pathways for prediction

In this context, adverse outcome pathways (AOPs, Figure 2.2) represent an important concept that structures toxicological knowledge originating from individual biomarkers as well as omics (e.g. genomics, transcriptomics, proteomics, lipidomics, metabolomics), thereby improving mechanistic understanding of all steps following chemical exposure from receptor to cell over organ to the whole organism (Ankley et al., 2010; OECD, 2013). AOPs describe the chain and network of events initiated by a chemical in an organism from triggering a MIE, cascading via key events to a physiological effect (Figure 2.2). Recent advances, such as *in vitro* testing, high-throughput screening, next-generation sequencing and omics-technologies, have simplified the identification of molecular target sites of chemicals, e.g. DNA, membrane or specific receptors and enable simultaneous testing of many effects at cellular and tissue level (LaLone et al., 2018; López-Osorio & Wurm, 2020; Villeneuve et al., 2019). Statistical tools can help increase the interpretability and applicability of omics-based data for prediction, whereas multi-level analysis approaches may enable the integration of such data from multiple biological levels (see Murphy et al., 2018b).

However, only if (causal) relationships of suborganismal responses (e.g. MIEs) with apical organism responses (e.g. survival or reproduction success) (Kramer et al., 2011) or with physiological MoA (pMoA) as defined in bioenergetic models (Chapter 2.4, Murphy et al., 2018a,b), can be established, AOPs can contribute to predicting effects at higher biological levels, e.g. population, community, food-web or ecosystem level. Given the considerable efforts required, only a few such relationships have been established to date. For example, the relationship between effects on the vitellogenesis and fecundity (eggs/female/day) in oviparous fish enabled population-level predictions for endocrine-disrupting chemicals (Miller et al., 2007; for further examples see Kramer et al., 2011). However, these relationships need to be quantitative to predict effects at higher biological levels. Dose and time data on initial and maximum induction, adaptation and recovery is required from low to high biological level responses to build quantitative links (Wu et al., 2005). For example, molecular and biochemical responses seem frequently similarly rapidly induced as physiological, cytological or behavioural responses (Wu et al., 2005). Longer recovery time has often been found for population and community responses (Wu et al., 2005). Regarding the dose, for baseline toxicants, for example, cellular - and organismal effects occurred at similar critical concentrations (Escher et al., 2019; Lee et al., 2021). Quantitative relationships can differ by several orders of magnitude between chemicals (Kimber et al., 2011; Sewell et al., 2018), species and environmental contexts. Moving from the current mainly qualitative scope of AOPs to quantitative AOPs (qAOPs) requires the incorporation of dose and time information into thresholds triggering MIEs and the sequence of key events (Sewell et al., 2018). To date, only a few probabilistic and mechanistic qAOP models were developed for effect prediction, with divergent characteristics and outcomes (reviewed in Spinu et al., 2020), and a need for guidance on the development and evaluation of qAOP models remains, particularly for application in a regulatory context (Spinu et al., 2020). Generally, improved databases, data-sharing initiatives, bioinformatics, data-mining tools as well as the use of standardised terminology will support the development of qAOPs based on existing data (Sewell et al., 2018) and the computational prediction of AOPs (Bell et al., 2016; Oki et al., 2016). qAOPs might allow the use of cellular-level data, which can be obtained efficiently from high-throughput assays, for the prediction of organism- and population-level effects (Ankley et al., 2010; Kramer et al., 2011; Villeneuve et al., 2019). In human toxicology, major developments are underway to use data from high-throughput assays as a robust anchor for higher-level (organ or organism) responses by establishing quantitative *in vitro* to *in vivo* relationships (Bell et al., 2020; Wetmore, 2015). Mammalian data can partly be extrapolated to other species with closely conserved pathways (see next Chapter 2.3.2), but generally the development of species-specific pathways in additional vertebrates, invertebrates and plants is needed (Villeneuve et al., 2019).

2.3.2 Molecular cross-species extrapolation

Molecular cross-species extrapolation (MCSE, Figure 2.2) can help extrapolate chemical sensitivity across species by comparing molecular target sites of chemicals, e.g. receptor protein sequence and conformation similarity among species. MCSE assumes that evolutionary conservation of molecular target sites implies chemical sensitivity (Gunnarsson et al., 2008). Where the structure and function of molecular target sites are similar across species, at least building-blocks of (q)AOPs can be reused, which enables more efficient AOP development for new species (Ashauer & Jager, 2018). Unfortunately, information on structure and function is rarely available (LaLone et al., 2016), but advances in sequencing technologies and molecular methods may rapidly expand the available data. Moreover, advances in web-based tools for MCSE such as the US EPA Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS) tool already enable the inclusion of some structural data of proteins (e.g. conserved functional domains and individual amino acid residue positions, Figure 2.2) (LaLone et al., 2013, 2016). Furthermore, a low degree of similarity in molecular target sites across species (e.g. using the tool SeqAPASS; LaLone et al., 2016) can already guide the selection of species for further laboratory tests (LaLone et al., 2013, 2018).

2.3.3 Major challenges and current developments

Despite major advances, the suborganismal perspective remains insufficient to predict the effects of chemicals in ecosystems. The steps from chemical uptake to reaching a molecular target site (termed toxicokinetic (TK) process, i.e. ADME) and the adaptive capacity of an organism before an MIE is initiated are not considered by AOPs or MCSE and remain a main focus of the organismal perspective. To close this gap, different approaches have been suggested, such as high-throughput physiologically based TK models in human toxicology (Breen et al., 2021), the aggregate exposure pathway framework (Teeguarden et al., 2016), the combined measurements of external and internal concentrations (i.e. circumvent TK processes; van den Berg et al., 2021) and linking AOPs with bioenergetic models (Chapter 2.4, Kramer et al., 2011; Murphy et al., 2018a,b).

Furthermore, suborganismal responses are usually recorded under standardised and simplified laboratory conditions, but the conditions in real systems can influence these responses. AOPs and MCSE consider usually a single chemical effect as independent of remaining physiological processes (Murphy et al., 2018a,b). However, depending on resource availability and other moderating factors, energy can be allocated differently, which influences the overall response. Energy trade-offs between physiological processes, such as maintenance (including metabolisation of chemicals), growth and reproduction, for instance under the impact of multiple stressors, are not considered. This limitation of AOPs could potentially be alleviated through integration with the organismal perspective (Chapter 2.4, Murphy et al., 2018a,b).

In addition, both chemical effect prediction and extrapolation across species should consider that chemicals can have multiple and non-specific target sites. Besides, a single MIE can result in multiple outcomes and common key events can be involved in multiple AOPs resulting in non-linear, branching AOPs (Sewell et al., 2018), with a high potential of reusing (q)AOP building-blocks. Finally, even if organism-level effects could be reliably predicted, prediction of higher-level effects up to the ecosystem level requires including ecological and evolutionary processes theoretically and empirically (Chapters 2.5,2.6&2.7).

2.4 ORGANISMAL PERSPECTIVE

Bioenergetic models, such as Dynamic Energy Budget (DEB) models can extend the prediction from the suborganism level to the organism and population level and at the same time provide the necessary background on ADME processes when integrated into toxicokinetic-toxicodynamic (TKTD) models (Figure 2.3). They allow one to predict the effects of realistic time-varying exposures in the field based on responses recorded under constant exposure conditions in the laboratory (Sherborne et al., 2020). At the same time, they can translate complex exposure patterns into effects at medium biological levels (e.g. organism, population), thereby providing an important element of effect prediction (Ashauer et al., 2011, 2016).

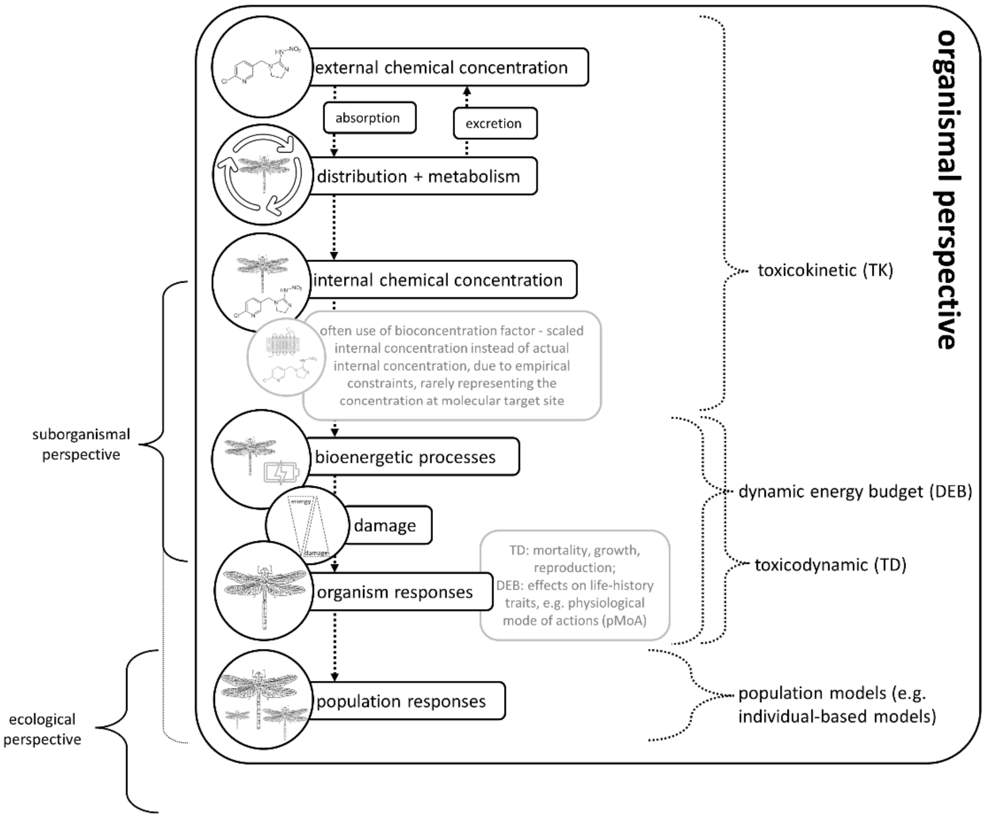

Figure 2.3: Main processes related to the organismal perspective. The large box delineated by the solid black line depicts the main focus of the organismal perspective. Boxes in light grey provide more detailed information on terms. Arrows connect processes or biological levels, whereas brackets indicate the processes and levels associated with each approach and the other perspectives.

2.4.1 TKTD models for prediction

TKTD models have a TK part that reflects the ADME processes of chemicals to predict a total internal concentration of a chemical in an organism (Figure 2.3). TK models can be modelled as one compartment (whole organism), multiple compartments (i.e. internal entities such as organs) or based on physiology (Grech et al., 2017) and are driven by chemical-specific (e.g. octanol-water partitioning coefficient) as well as organism-specific (e.g. lipid content) parameters. Toxicodynamic (TD) models focus on the effect of the concentration at a molecular target site on apical responses at the organism level, such as mortality, growth or reproduction (Figure 2.3). Here the derived TD model parameters can reflect biological processes and organism traits (Ashauer & Jager, 2018; Rubach et al., 2011). TKTD models allow for different complexities (e.g. include damage recovery) depending on the scope of prediction and data availability (Jager, 2020). This potential for different complexity has led to the emergence of a wide range of TKTD models. However, at least for the survival endpoint, they have been unified in the Generalized Unified Threshold model for Survival (GUTS), which models time-to-event or survival data (Jager et al., 2011). GUTS can be adapted to the available input data and to mechanistic assumptions when used to predict survival in a population. For example, the reduced GUTS (GUTS-RED), directly connects an external concentration to the scaled damage, without the need for internal concentrations (Jager, 2020; Jager et al., 2011).

2.4.2 DEB models for prediction

A commonly used bioenergetic model framework is based on DEB theory (Jager, 2020; Kooijman, 2009). The DEB theory consists of assumptions specifying how organisms obtain energy and matter from their environment to fuel their life cycle. The theory treats organisms as dynamic systems with explicit energy balances and can be applied to all species (Nisbet et al., 2000). Different variants of DEB models exist in ecotoxicology, which are coined under the term DEBtox or, according to a more recent publication, DEB-TKTD (Sherborne et al., 2020). Recent developments include a unified and simple DEB approach for practitioners (EFSA PPR et al., 2018; Jager, 2020; Sherborne et al., 2020). Generally, DEB-TKTD combines biological parameters of organisms (e.g. growth, feeding, and maintenance) and chemical exposure over time (Jager et al., 2006). The modelling of bioenergetic processes allows one to capture (sub)lethal effects of chemicals on life-history traits of individuals. Structurally, the DEB-TKTD model consists of TK and TD parts. The TK part is similar to other TKTD models discussed above (Figure 2.3). By contrast, the TD part differs from TKTD models. DEB-TKTD predicts the effects of a chemical inside an organism as stress costs of one of usually four pMoA (Figure 2.3), namely, assimilation, growth, maintenance, and reproduction (Ashauer & Jager, 2018), representing assimilation of energy from food, energetic costs for growing new body tissue, maintaining body functions including mitigating hazardous effects as well as producing offspring (Kooijman & Bedaux, 1996). Similar to GUTS, a reduced version (DEBkiss) of a full DEB-TKTD model has been developed, which simplifies bioenergetic processes and in turn requires fewer state variables and parameters (Jager et al., 2013). Another important simplification within the DEB-TKTD model framework is the introduction of “compound parameters” (comDEB(kiss)), such as maximum length, which replace hard-to-measure “primary parameters” (priDEB(kiss)) based on simplified model assumptions, e.g. a constant ratio between size and maturity (Sherborne et al., 2020). For the simplest model variant (comDEBkiss), each parameter relates to a measurable variable, and it can be fully parameterised based on standard laboratory tests, where growth and reproduction are measured over time (Jager, 2020; Sherborne et al., 2020). Generally, recent studies highlight the relevance of mechanistic understanding in DEB model parameters and processes for reliable prediction. If model parameters and processes have no phenomenological counterpart in the real world, they represent abstractions with unknown interpretability (Murphy et al., 2018a,b) and uncertainty. For instance, population-level responses can differ strongly depending on the suborganismal mechanisms involved (Martin et al., 2014). One way to alleviate this might be to incorporate AOPs in DEB models (Murphy et al., 2018a,b). For example, AOP key event responses could be integrated into a damage term that translates to DEB rates (Murphy et al., 2018a,b). As mentioned in Chapter 2.3, AOPs and gene expression data could thereby gain higher predictive applicability.

2.4.3 Major challenges and current developments

As outlined above, for both, TKTD and DEB models, simplified model variants have been introduced to achieve practical applicability for non-experts. However, it is challenging to find a compromise between depicting reality and keeping the model complexity (i.e. number of model parameters) and the data requirements computationally manageable. This compromise builds on assumptions, such as the constant ratio between size and maturity or the neglect of a reserve, ageing, body shape differences and starvation (Sherborne et al., 2020). For species or chemical MoAs where an assumption is not plausible, the model needs to be extended at the cost of simplicity (Sherborne et al., 2020).

Besides, another main challenge remains: For many taxa, key model parameter estimates required to build the model are lacking. Obtaining those by direct measurements in laboratory experiments after field sampling of the new species is resource-intensive and inappropriate for rare and endangered species (Petersen et al., 2008). Methods to reduce resource requirements in laboratory testing or to indirectly obtain key parameters for a model, such as parameter borrowing, pattern-oriented and artificial evolution approaches, are described elsewhere (Petersen et al., 2008). DEB model parameters have already been collected for more than 3200 species in the Add-my-Pet database (AmP, 2022), covering all major phyla (Lavaud et al., 2021). For species sharing the same receptor and pathway and compounds sharing the same MoA, model parameters should be the same and could be used for effect extrapolation to untested chemicals and species based on internal molar concentrations (Gergs et al., 2019; Jager & Kooijman, 2009). However, also the bioconcentration factor and the target site interaction efficiency of a chemical can make a difference in the outcome (Gergs et al., 2019; Jager & Kooijman, 2009), information that is rarely available. In addition, the actual internal concentration at the target site is difficult to measure for many species and the aggregation of ADME data for additional vertebrates, invertebrates and plants, as realised for mammals/humans, is still pending (Villeneuve et al., 2019). Finally, extrapolation of chemical effect concentrations to untested species has so far only been done for survival data based on GUTS (Gergs et al., 2019), but not for sublethal responses (Sherborne et al., 2020). The cross-species parameter correlation method used in this extrapolation enabled the prediction of chemical effects for new species, however, its predictive capability at the population level involving an individual-based model was still insufficient and underestimated the actual effect (Gergs et al., 2019).

Starting in 1994 many studies with TKTD and DEBtox modelling applied a rigorous approach to the estimation of model parameters and their uncertainty by likelihood profiling (Bedaux & Kooijman, 1994; Kooijman & Bedaux, 1996b). However, a more fundamental challenge was the propagation of uncertainty to the model outputs and subsequent predictions (Charles et al., 2022; Trijau et al., 2021). For the most common TKTD and DEB models, frequentist and Bayesian software packages have been developed in the last few years to solve this problem (Charles et al., 2022; Jager, 2021; Trijau et al., 2021). However, this remains a common challenge in community and ecosystem models (with some exceptions, e.g. Streambugs; Mondy & Schuwirth, 2017; Schuwirth et al., 2015), which are required to extend chemical effect prediction to the community or ecosystem level.

Overall, TKTD and DEB models can strongly improve our understanding and capacity for prediction, but they are largely limited to the organism or population level in laboratory settings. Even perfect knowledge at the associated level would be insufficient to reliably predict chemical effects on populations in real-world ecosystems and necessitates an ecological perspective, outlined below.

2.5 ECOLOGICAL PERSPECTIVE

A major aim of ecotoxicology and chemical risk assessment is to predict chemical effects at higher biological levels such as communities, food webs and ecosystems or biodiversity per se, which are generally within the domain of ecology. A rich but heterogeneous body of ecological theory has been developed to explain community, food web and biodiversity patterns and related processes at different spatial and temporal scales (Scheiner & Willig, 2011). In the last decade, generalising and unifying frameworks have emerged integrating previously separated lines of theory. Vellend's Theory of Ecological Communities identifies four processes that underly community dynamics (Vellend, 2010, 2016; Figure 2.4):

(1) Selection, which consists of a) environmental selection through environmental conditions including chemical pollution, and b) biotic selection through processes such as competition, predation or facilitation, that determine the occurrence and growth of a species in a habitat patch.

(2) Dispersal, which determines the recruitment to and from habitat patches.

(3) Drift, in terms of stochastic processes that influence the development of populations and communities.

(4) Speciation, i.e. formation of a new biological species, although mainly relevant on longer time scales.

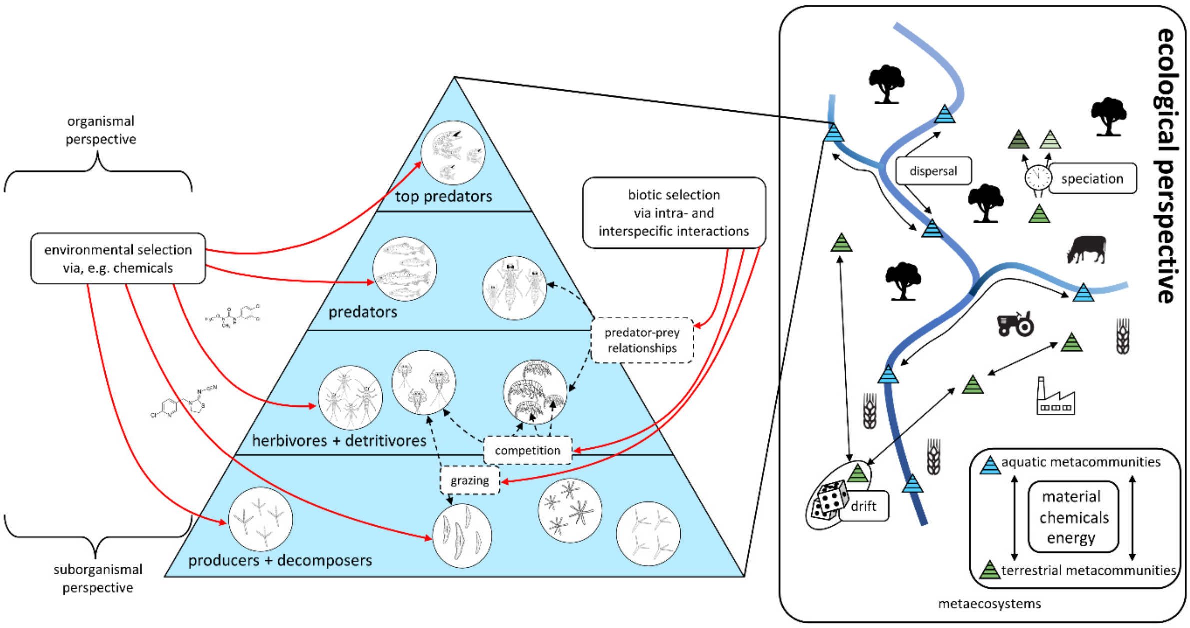

Figure 2.4: Main processes related to the ecological perspective. The large box delineated by the solid black line depicts the components of the ecological perspective in a metaecosystem. Triangles represent metacommunities, the left triangle zooms into an idealised aquatic metacommunity, represented as trophic pyramid. Arrows depict a bi- or unidirectional exchange in materials, chemicals and energy (black) or impact (red). Brackets indicate the processes associated with the other perspectives.

2.5.1 Chemicals and ecological processes

Many studies demonstrated that the first three processes are highly relevant for predicting chemical effects on ecological systems (Figure 2.4). Direct chemical effects constitute a form of (environmental) selection. In addition, environmental conditions such as temperature, moisture or pH, besides potentially modifying chemical concentrations and interactions, determine the occurrence of a biological species in a habitat patch exposed to a chemical and its fitness (Laskowski et al., 2010; Niinemets et al., 2017). Biotic processes such as competition or predation/cannibalism have also been shown to increase the effect of chemicals or to delay recovery (Kattwinkel & Liess, 2014; Liess & Foit, 2010; Viaene et al., 2015). Dispersal contributes to recovery from chemical effects via recolonisation (Trekels et al., 2011), but can also lead to a transfer of chemicals between food webs and propagate effects to non-polluted habitat patches (Richmond et al., 2018; Schäfer et al., 2017; Schneeweiss et al., 2022). Only few studies have specifically examined the relevance of stochastic ecological drift for chemical effects. However, a modelling study demonstrated that incorporating stochasticity improved the prediction of experimental data (Erickson et al., 2014). Moreover, stochasticity can profoundly modulate ecological dynamics

(Shoemaker et al., 2020). Although the fourth process, speciation, is mainly relevant on longer time scales, evolutionary processes at the population level (e.g. natural selection, gene flow, genetic drift and mutation) can already occur at similar temporal and spatial scales as ecological processes (Govaert et al., 2021). This phenomenon is often termed 'rapid evolution' and its ecological and functional significance has been shown already several decades ago (e.g. Gorokhova et al., 2002; Reznick et al., 1997). With respect to pesticide impacts, multiple studies found evidence for rapid evolutionary adaptation and lower sensitivity (Bass et al., 2015; Hawkins et al., 2019; Lucas et al., 2015; Palumbi, 2001; Powles & Yu, 2010). This adaptation can lead to co-tolerance against similar stressors – but it may also result in higher sensitivity of species to other stressors due to performance trade-offs (Luijckx et al., 2017; Orr et al., 2021). Theory, experiments and prediction should therefore consider eco-evolutionary dynamics (Govaert et al., 2021), which would allow to move from a static to a dynamic prediction framework. Further, genetic intraspecific variability, e.g. cryptic lineages, showed differential sensitivity (Becker & Liess, 2015; Feckler et al., 2014; Sturmbauer et al., 1999), which hampers the transferability of experimental results based on individual clones or low-diversity laboratory populations to natural populations. Overall, the Theory of Ecological Communities provides the key ecological processes that need to be considered when predicting the effects of chemicals in ecosystems, yet the approach is abstract and makes no prediction on the relevance of each of the processes for a specific ecosystem or species. A different amalgamating framework is provided by the Metacommunity Theory (Leibold et al., 2004). This explains and predicts patterns of community composition in connected habitat patches at local and regional spatial scales. Three of the processes (selection, drift, dispersal) outlined above are integrated with different weights into four non-exclusive paradigms of community organisation (for details see Leibold et al. 2004). Through the consideration of biogeochemical flows and complex food webs, this theory has been expanded to metaecosystems and meta food webs (Gounand et al., 2018; Loreau et al., 2003; Ryser et al., 2021; Figure 2.4). However, this theory also rather provides predictions of general patterns from assumptions of the relevance of the individual paradigms than predictions of the community composition in a specific habitat patch or region. With respect to chemicals, this theory has been applied to establish a conceptual framework for chemical effects across ecosystems and to explain patterns observed in experiments (e.g. Peng et al., 2018; Schiesari et al., 2018).

2.5.2 Use of traits in ecotoxicology

The trait-based approach, rooted in the so-called Assembly and Response Rule Framework (Keddy, 1992), has been most widely applied for explaining and predicting chemical effects in ecosystems. Loosely defined, traits are measurable characteristics of an individual (cf. Violle et al., 2007) and trait-based approaches aim at linking traits, instead of species, to environmental conditions at variable scales. Although it remains a challenge for ecologists to predict the specific set of species occurring in a habitat patch, trait-based approaches successfully predicted the trait composition of communities from environmental conditions (Shipley, 2010; Shipley et al., 2006). In the context of predicting the effects of chemicals, the sensitivity of a species to a chemical can be considered a trait. This trait forms the backbone of many applications in the context of ecotoxicology and risk assessment. For example, the sensitivity distribution across species, called SSD, is used to derive acceptable environmental concentrations of chemicals (Smetanová et al., 2014) and has been successfully linked to chemical effects in ecosystems (Posthuma & de Zwart, 2012). However, data on chemical sensitivity is lacking for most species. Hence, several approaches have aimed at predicting the chemical sensitivity trait, either by other traits (e.g. body size), phylogenetic relatedness or using tools related to the other two perspectives, i.e. suborganismal or organismal approaches (Malaj et al., 2016; Rubach et al., 2012; Van den Berg et al., 2019, 2021). Although useful for filling data gaps, these approaches often depend themselves on input data that is scarce or associated with high uncertainty. Though not related to physiological sensitivity, several other traits can be used to assess the vulnerability of taxa (Figure 2.4; De Lange et al., 2009). Examples of such traits are habitat preference (determining exposure), dispersal capacity (determining recolonisation and avoidance of chemical exposure) and growth rate (determining recovery). Several studies used a combination of traits to establish links between chemical pollution and species occurrence or community change (Badry et al., 2020; Delhaye et al., 2020; Kjær et al., 2021; Schäfer & Liess, 2013). Moreover, if links between traits that respond to environmental (including chemical) selection, so-called response traits, and traits that describe the effect of a species on ecosystem functioning, so-called effect traits, can be established, then these allow to predict changes in ecosystem functioning (e.g. Schäfer et al., 2012). Although trait-based approaches are valuable to identify vulnerable taxa and predict the trait composition of species in a habitat, their capacity to quantitatively predict the response to chemicals is hampered by data availability and insufficient consideration of eco-evolutionary dynamics.

Regarding data availability, traits with a strong mechanistic link to chemical effects, such as the capacity for production of heat shock proteins or the content of energy storage molecules (e.g. lipid, glycogen or proteins), average biomass or body size have rarely been measured for many organism groups that are particularly at risk from chemical effects such as invertebrates or fungi (Rubach et al., 2011). Measuring such traits would require major coordinated efforts, though the other perspectives might aid in predicting traits (Gergs et al., 2019; Pecquerie et al., 2011). Finally, intraspecific trait variation can be important (see for example De Laender et al., 2014) but, depending on the organism group, is often ignored due to a lack of data. Regarding eco-evolutionary dynamics, the trait-based approach is currently static in many respects. First, traits may vary over the lifetime of a species (Lancaster & Downes, 2010). Second, the average trait of a population may change with environmental conditions, which includes adaptation processes to chemicals (Dinh et al., 2016; Shahid et al., 2018), whereby the future response to the same or other stressors is moderated (Orr et al., 2021; Vinebrooke et al., 2004). Third, biotic selection and dispersal are often insufficiently accounted for in trait-based approaches (Cadotte & Tucker, 2017).

2.5.3 Statistical and process-based models

A wide range of statistical and process-based models have been developed, rooted in a variety of ecological concepts and theories, that can predict the effects of chemicals. Joint species distribution models (JSDMs) are a group of statistical models that have attracted wide attention in ecology (Ovaskainen & Abrego, 2020). If larger monitoring data sets with measurements of species and chemicals as well as all relevant environmental factors are available, JSDMs can quantify the relevance of chemicals for the species distribution and predict the occurrence of species in non-measured patches in the landscape (Brown et al., 2018; Ovaskainen et al., 2017). The modelling requires knowledge on ecological processes such as biological interactions, which can be estimated from the data, though such estimates may not be reliable (Dormann et al., 2017, 2018). As larger field data sets are required, JSDMs are unsuitable for novel chemicals that have not been authorised for use. Many process-based models have been developed and used to predict the response of populations, communities and ecosystems to chemicals (see reviews by: Galic et al., 2010; Rohr et al., 2016; Schmolke et al., 2010). Most of these models derive from classical ecological models and consider individuals as the smallest unit, thereby ignoring suborganismal processes related to the MoA (Accolla et al., 2020; Rohr et al., 2016). Notwithstanding, process-based models have successfully been used to quantitatively predict the responses of populations, communities and food webs to chemicals (Kattwinkel et al., 2016; Lei et al., 2008; Mondy & Schuwirth, 2017; Topping et al., 2003). The main challenge for process-based modelling remains model validation and the spatially explicit modelling of communities and food webs, which still faces conceptual, practical and partly computational constraints.

2.5.4 The challenge of multiple stressors

Chemical stressors often co-occur with other stressors (Holmstrup et al., 2010; Schäfer et al., 2016) and stressors can interact resulting in additive, antagonistic or synergistic effects (Piggott, Townsend, et al., 2015b). This represents a challenge to most ecological approaches. A link to the other perspectives may aid in predicting multiple stressor effects. For example, combining analytical and bioanalytical tools (e.g. *in vitro* bioassays) provides the opportunity to analyse mixtures of chemicals found in the environment for their combined risk and to identify risk drivers retrospectively (Escher et al., 2020). Further, knowledge about a chemical's MoA and species sensitivity traits can guide model selection when trying to unravel mixture effects (Spurgeon et al., 2020). A few qAOPs assessed mixture effects and considered additional stressors (Chu, 2018; Spinu et al., 2020). Also bioenergetic models can incorporate mixture toxicity (Ashauer et al., 2007, 2015; Bart et al., 2021) including chemical interactions (e.g. synergism, antagonism) (Cedergreen et al., 2017) and provide a framework to deal with multiple stressors through the inclusion of other physical (e.g. temperature) or biological stressors (Galic et al., 2018; Goussen et al., 2020). However, data for relevant stressors, such as pH, nutrients or habitat degradation are largely lacking (Goussen et al., 2020). Finally, to reliably predict multiple stressor effects our understanding of the relative importance of stressors must improve (Goussen et al., 2020) including the development of theoretical concepts (De Laender, 2018; Schäfer & Piggott, 2018).

2.6 THE SCOPE OF PREDICTION – A QUESTION OF SCALE

Prediction can have different scopes including biological levels, environmental contexts, as well as spatial and temporal scales (see Scenario-based guide to prediction in the Text A.1 and Figure A.1). The three perspectives focus on different biological levels, which typically imply a certain spatial and temporal scale (Figure 2.5).

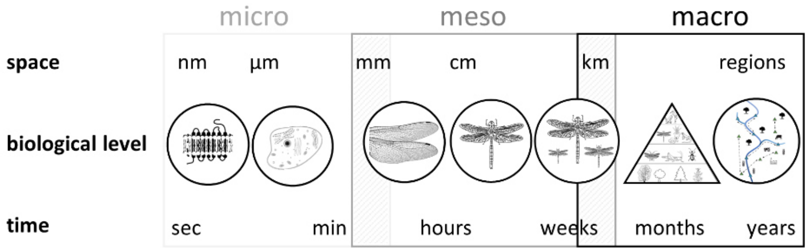

Figure 2.5: The expression of spatial and temporal scales varies between the biological levels and is also influenced by the type of organism and chemical. This illustration is simplified.

The suborganism level is rather associated with small spatial and short temporal scales. Changes in populations, organisms or in parts of organisms, which is the focus of the organismal perspective, typically cover larger (mm to m) and longer (hours to weeks) scales. Community, food-web and ecosystem levels usually cover large spatial and long temporal scales (Figure 2.5). However, the scale is also influenced by the type of organism and chemical. For example, microorganisms have much shorter generation times than macroorganisms. Thus, chemical-driven changes in an individual mammal can persist longer than changes in (meta-)communities of microorganisms. Accordingly, temporal and spatial scales of (eco)toxicological test methods, of species traits and of stressors are interrelated, resulting in several species groups being underrepresented in certain test methods (Schuijt et al., 2021). For example, *in vitro* bioassays have been realised mainly for mammalian cell lines, whereas whole organism tests as well as biomarkers mainly for selected fish and invertebrate species (Schuijt et al., 2021). To assess the effect of chemicals on underrepresented species groups (e.g. fungi), extrapolation approaches (e.g. MCSE, trait-based) are certainly fruitful, but further test systems still need to be developed. Similarly for chemicals, test systems (e.g. high-throughput *in vitro* tests) for several major MoA still need to be developed (Schuijt et al., 2021; Villeneuve et al., 2019). Overall, prediction for larger spatial and temporal scales (e.g. regional level over months to decades), for example with larger scale process-based models, is particularly challenging because empirical data on chemical effects often originates from experiments on much smaller spatial and temporal scales (Schneider, 2001). This means that calibration and parameterisation may be biased. Simultaneous recording data at multiple biological levels and over large and long scales may provide empirical relationships on which models can be built (see Chapter 2.7).

2.7 DATA AND EXPERIMENTAL ADVANCES TO IMPROVE PREDICTION

Each of the three perspectives provides essential elements for the prediction of chemical effects in ecosystems. Strengthening the links between these perspectives would considerably foster predictive modelling of chemical effects in ecosystems and effect extrapolation between species. A major prerequisite is data that simultaneously cover different biological levels as well as spatial and temporal scales. Below, we outline promising approaches and associated types of data that would strengthen the links between the perspectives as well as focus and coordinate the effort in data collection:

First, record suborganism- and organism-level responses to chemicals and test for relationships with chemical groups and traits of organisms (Figure 2.6 - 1). Traits have been relevant predictors of variability in chemical sensitivity at the level of organisms or populations (van den Berg et al., 2021; Wiberg-Larsen et al., 2016). Yet, suborganismal processes involved in chemical uptake, transformation and effects may also relate to organism traits (Gergs et al., 2015, 2019). Moreover, expression profile data (omics), which are important for constructing the cellular portion of AOPs, might vary in tissues as a function of chemical MoA and selected organism traits (e.g. metabolic rate) and grasping such relationships would contribute to a fundamental understanding of toxic effects (López-Osorio & Wurm, 2020). Although trait data has increasingly become available for various taxa, substantial work remains to standardise trait data (Gallagher et al., 2020) and data on traits that are likely mechanistically involved in chemical effects are still scarce. Prioritising traits and potential relationships with (sub-)organismal responses based on expert knowledge for a specific ecosystem type (e.g. marine, terrestrial), chemical MoA and organism group, would limit the required measuring effort (Kearney et al., 2021; McGill et al., 2006). Pioneering studies have focussed on easily measurable traits such as body mass that is strongly related to the metabolic rate (Baas & Kooijman, 2015; Gergs et al., 2015; Ryser et al., 2021). Even purely correlative relationships between (sub-)organism responses with chemical MoA and traits would foster our predictive capacity (Kramer et al., 2011; Murphy et al., 2018a,b) and extrapolations across species. Studies recording responses to chemicals at different (sub-)organism levels would ideally be done under variable spatiotemporal and environmental conditions to evaluate the generality of potential relationships, including with traits (McGill et al., 2006).

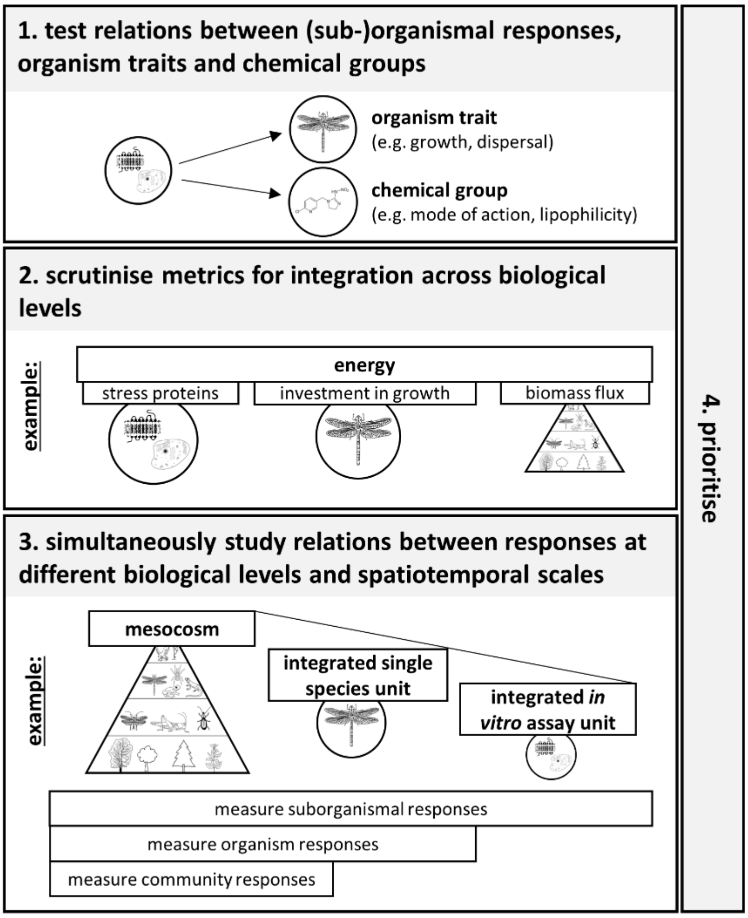

Figure 2.6: Overview of the four main aspects to improve prediction of chemical effects in ecosystems.

Second, scrutinise metrics that are measurable at many biological levels such as energy for their potential to integrate across levels (Figure 2.6 - 2). Analysis of bioenergetics has been suggested as a common currency to integrate across biological levels given that generic energy flow rules apply universally and that bioenergetics is involved in the regulation of structural and functional responses at all levels (Fischer et al., 2013; Forbes et al., 2017; Forbes & Galic, 2016; Segner et al., 2014; Sokolova, 2021). At the suborganism level, proteins associated with the protection from stress (e.g. heat shock proteins), antioxidant, pro- and anti-apoptotic proteins may indicate stress-induced bioenergetic state transitions (Reusch & Wood, 2007; Sokolova, 2013). Similarly, biomarkers such as the aerobic scope (difference between maximum and basal metabolic rate), energy reserves, energy uptake, mitochondrial capacity or cellular energy allocation can be measured (Goodchild et al., 2018; Schuijt et al., 2021; Sokolova, 2021). At the organism level, the investment in growth or reproduction may indicate bioenergetic stress responses (Schuijt et al., 2021; Segner et al., 2014), whereas on the population level population growth rate, density, age structure and biomass are potential candidate metrics (Forbes et al., 2017; Schuijt et al., 2021). At the community level, the measurement of energy budgets of a representative sample of individuals requires a high replication in experiments due to the invasiveness of the method. However, this could allow the use of energy budgets in process-based community models, thereby establishing links between the organismal and the ecological perspective. At this level, energy fluxes could be measured as biomass flux, directly (e.g. trapping organisms and organic matter) or indirectly using trophic relations established with stoichiometry and stable isotope measurements (Graf et al., 2020; Kato et al., 2004; Paetzold et al., 2005). The latter would prohibit downscaling to the energy budget of individuals. Structural metrics such as species abundance, diversity and biomass or the trophic organisation are usually assigned to the community level, while functional metrics such as primary production and element cycling rates are assigned to the ecosystem level (Schuijt et al., 2021). The bioenergetic-AOP framework quantitatively links energetic responses across biological levels (Goodchild et al., 2018). Thereby, correlations have been found between cellular energy allocation and whole-organism growth, metabolic rate and the scope for growth as well as with the non-traditional response of locomotion, enabling the incorporation of suborganismal insights into bioenergetic models. Further relationships (preferably causal, quantitative) between energetic responses from different biological levels could strengthen predictive ecotoxicology.

Third, conduct complex experiments to simultaneously study responses to chemicals at different biological levels and spatiotemporal scales. The ultimate challenge is to evaluate if and how low-level responses manifest themselves in higher-level responses. A combination of micro- and mesocosm experiments, covering a range of environmental and biotic selection factors would help to link different biological levels. For instance, different biological levels over time and space could be manipulated. Such experiments include nested designs that contain experimental subunits from different biological levels, by e.g. including single species subunits in multi-species mesocosm experiments to quantify the effects of a chemical in the absence of interspecific interactions (Figure 2.6 - 3). Species for such subunits could be selected to cover a wide range of traits. The results could also foster cross-species extrapolation based on traits (Van den Berg et al., 2021). Single and multispecies micro- and mesocosm experiments have been proven to be valuable tools to understand the response to a perturbation within a local spatial scale (Beermann, Elbrecht, et al., 2018; Beermann, Zizka, et al., 2018; Piggott, Salis, et al., 2015; Piggott, Townsend, et al., 2015a). To link local processes to a landscape context, another experimental design represented by linked experimental units has been successfully implemented in the laboratory using vial microcosms for aquatic (Altermatt et al., 2015) and terrestrial (Gilarranz et al., 2017) units (Figure 2.6 - 3). These designs can be further extended, theoretically and empirically, to include cross-habitat exchanges that enable to monitor flows of energy, material and organisms (dispersal) as well as changes in the genetic and trait composition of patches, simulating real-world meta-community or -ecosystem dynamics (Ryser et al., 2021; Harvey et al., 2020). Furthermore, repeated experiments could highlight consistency or changes in chemical effects over time and space (Belanger et al., 1994; Schreiner et al., 2018). Finally, disentangling eco-evolutionary dynamics and interactions, as mentioned in the following aspect of this Chapter, will require large, collaborative experiments such as described in Govaert et al. (2021).

Within these experimental designs, a wide range of data from different biological levels can be collected allowing to screen for links between the perspectives. For example, omics data can be collected in experiments of most levels of complexity (i.e. ranging from single-species laboratory to multi-species mesocosm experiments), but also in field surveys (Van Aggelen et al., 2010; Williams et al., 2011). Data from complex experiments with multiple factors of environmental and biotic selection or from field surveys covering a wide range of conditions allow to evaluate, how these higher levels influence the suborganismal response (Figure 2.6 - 3). The sampling plan should be informed by the scale of the response and may require high temporal resolution sampling of suborganismal responses (Figure 2.5). Recorded response data should be supplemented by detailed information on the test organisms (e.g. age, size, sex, origin) as well as on the experimental conditions (e.g. pH, temperature) to enable a wide range of predictions and modelling. Finally, experiments would be most informative if following a gradient design with a high number of chemical concentrations to establish robust concentration-response curves (Kramer et al., 2011; Kreyling et al., 2018).

Fourth, identify those processes most critical in light of the biological level and spatiotemporal scale of prediction, as well as in light of the taxa, chemicals and potentially characteristics of the (eco)system under scrutiny (Figure 2.6 - 4). Important processes and missing data might be identified and prioritised, respectively, in a collaborative effort, for example in workshops with participants from multidisciplinary backgrounds (Forbes et al., 2020). Also novel data science tools including artificial intelligence might strongly expand our prioritisation capacity in future (Pichler et al., 2020; Scowen et al., 2021). For example, the AOP framework should aim at "informed simplicity", i.e. capture the essential, measurable events that lead to a relevant organism-level toxicity endpoint rather than a comprehensive description of all biological aspects involved (Knapen, 2021). Similarly, depending on the application, reduced DEB and TKTD variants, that can easily be applied, may be sufficient. For the ecological perspective, several approaches allow to prioritise critical processes and, if necessary, to simplify (e.g. aggregation; for details see Text A.1). An example of a process that has rarely been considered within any of the perspectives, but can be relevant at all biological levels and may provide links between them, is evolutionary adaptation. An example of such a link are potential relationships between genes responsible for adaptation and fitness-related phenotype metrics such as shifted survival time, time to first brood or total number of offspring (Kramer et al., 2011). Novel evolutionary genomic techniques, such as restriction site-associated DNA sequencing (RADseq), shotgun population variation profiling (PoolSeq), transcriptome sequencing (RNAseq) or whole-genome resequencing, can identify genetic locations of adaptation even for non-model species, which lack prior genomic information (Weigand & Leese, 2018). Specific traits may help identify species with high adaptation potential, such as high de novo mutation rates, as mutations may emerge faster in species with large populations, short generation times and regular exposure (Doria et al., 2022; Hawkins et al., 2019). In addition, CRISPR-Cas9-based reverse genetic approaches in conjunction with in vitro metabolism and genome scans enable to test the relevance of certain genes for resistance (Denecke et al., 2017; Douris et al., 2020; Wang et al., 2018). Adaptation data from the (sub-)organism level may be further linked to changes in the diversity and frequency of species on the community and ecosystem level. DEB models have the potential to analyse the evolution of organismic traits and to identify adaptations including underlying mechanisms (Beaudouin et al., 2012; Goussen et al., 2015). If there is strong indication of rapid adaptation of specific taxa to selected chemicals in the environment under focus, eco-evolutionary dynamics and interactions should be quantified and integrated into the prediction framework, otherwise they may be neglected. Ultimately, the challenge is to reduce the uncertainty in predicting chemical effects in ecosystems while maintaining or making feasible the data collection effort and model complexity.

2.8 CONCLUSION

Ideally, a prediction framework would trace the chemical from its uptake into an organism and its transformation, over the triggering of biochemical reactions inside the organism to physiological effects and the propagation to the population, community, food web and ecosystem level (Figure 2.1). Yet, each idealised perspective captures only a part of this framework (Figure 2.1). The perspectives are complementary and rather reflect different phenomenological approaches than scientific disciplines, but still have own scientific concepts and communities. Each of the perspectives has its own scale, which also delineates its limitations for comprehensive prediction (Figure 2.5). Our predictive capacity for effects of chemicals generally decreases with increasing biological levels, given the emergent dynamics inherent to ecological systems at the level of communities, food webs and ecosystems. Predictions seem feasible on a rather coarse level when focussing on organism groups, traits, compound groups and qualitative responses (e.g. sensitivity categories) (Bracewell et al., 2019; Halstead et al., 2014; Rumschlag et al., 2020). However, quantitative predictions (e.g. of densities) become almost impossible when dealing with specific organisms (intraspecific trait variation) and chemicals in a complex system, a circumstance that is shared with ecology (Ovaskainen & Abrego, 2020). Overall, a balance has to be found between simplification and capturing the essential complexity of a system, aiming at "informed simplicity" for modelling complex systems (Knapen, 2021). The effort in data collection must be focused and coordinated. In this context, novel data science tools including artificial intelligence might strongly expand our capacity in future (Pichler et al., 2020; Scowen et al., 2021). We emphasise that the three idealised perspectives that substantially contribute to date to the prediction of chemical effects and effect extrapolation across species are still largely separated. We see the potential that theoretical and empirical links between the suborganismal, organismal and ecological perspective could enhance predictive modelling of chemical effects in ecosystems and extrapolation between species.

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3 POTENTIAL PROPAGATION OF AGRICULTURAL PESTICIDE EXPOSURE AND EFFECTS TO UPSTREAM SECTIONS IN A BIOSPHERE RESERVE

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3.1 ABSTRACT

In the last decades, several studies have shown that pesticides frequently occur above water quality thresholds in small streams draining arable land and are associated with changes in invertebrate communities. However, we know little about the potential propagation of pesticide effects from agricultural stream sections to least impacted stream sections that can serve as refuge areas. We sampled invertebrates and pesticides along six small streams in south-west Germany. In each stream, the sampling was conducted at an agricultural site, at an upstream forest site (later considered as "refuge"), and at a transition zone between forest and agriculture (later considered as "edge"). Pesticide exposure was higher and the proportion of pesticide-sensitive species ($\text{SPEAR}_{\text{pesticides}}$) was lower in agricultural sites compared to edge and refuge sites. Notwithstanding, at some edge and refuge sites, which were considered as being least impacted, we estimated unexpected pesticide toxicity (sum toxic units) exceeding thresholds where field studies suggested adverse effects on freshwater invertebrates. We conclude that organisms in forest sections within a few kilometres upstream of agricultural areas can be exposed to ecologically relevant pesticide levels. In addition, although not statistically significant, the abundance of pesticide-sensitive taxa was slightly lower in edge compared to refuge sites, indicating a potential influence of adjacent agriculture. Future studies should further investigate the influence of spatial relationships, such as the distance between refuge and agriculture, for the propagation of pesticide effects and focus on the underlying mechanisms.

3.2 INTRODUCTION

Several studies over the past two decades have assessed pesticide pollution of freshwater habitats (Liess et al., 2021; Schäfer, 2019; Schulz, 2004). They found that pesticides regularly exceed ecological quality thresholds (Szöcs et al., 2017) and are a major factor shaping macroinvertebrate communities in stream sections draining arable land (Beketov et al., 2013; Chiu et al., 2016; Hunt et al., 2017; Liess et al., 2021; Miller et al., 2020; Schäfer et al., 2012). Through flows of matter, energy and organisms, agricultural stream sections can be connected to least impacted stream sections (Loreau et al., 2003), which, in the face of almost ubiquitous human influence, are defined as sites that are relatively free from human influence, for example without agricultural land use. Via these flows, either pesticides or their effects might propagate to the adjacent ecosystems (Schiesari et al., 2018). For example, pesticides can be transported to adjacent ecosystems, via flows of air, water or organisms (Hageman et al., 2006; Harding et al., 2006; Richmond et al., 2018). Among these flows, organisms occupy a special position, as they can move against the flow direction of water and air. The flow of organisms between patches of different states of pollution can moderate pesticide concentrations of the patches (e.g. biovector-transport; Richmond et al., 2018; Schiesari et al., 2018). In addition, dispersing organisms can alleviate or exacerbate the effects of pesticides. For instance, organisms from less or non-polluted patches can recolonise polluted patches, thereby fostering recovery of vulnerable species and alleviating the pollutants effects on communities (Orlinskiy et al., 2015). A field study attributed the recovery of eight out of eleven invertebrate populations from an insecticide pulse to immigration from less or non-affected connected patches (Liess and Schulz, 1999). We refer to these patches as "refuges". An analysis of multiple field studies suggested that certain presumed pesticide-vulnerable taxa can occur even in highly polluted stream sections if upstream refuges are present (Knillmann et al., 2018). The authors attributed their occurrence to dispersal and stress-resistant traits, such as asynchronous life cycles and resistant aquatic or terrestrial life stages. Similarly, a meta-analysis of field studies found that the presence of non-polluted upstream refuges supports the persistence of pesticide-sensitive species in polluted downstream sections (Schäfer et al., 2012). Together, these studies suggest that through dispersal from refuge stream sections the effects in polluted stream sections can be alleviated.

However, the dispersal processes that alleviate pesticide effects in polluted downstream sections may incur costs for the refuge populations. For example, the propagation of effects from polluted to non-polluted systems was predicted in several studies with metapopulation models (Chaumot et al., 2003; Spromberg et al., 1998; Willson and Hopkins, 2013). A metapopulation model focusing specifically on pesticide effects in streams estimated a reduction of up to 25 % population size of a freshwater insect in the non-polluted patch (Schäfer et al., 2017). This reduction occurred as a result of density-dependent depletion of source organisms via dispersal and associated mortality. Besides, pesticide effects may propagate to least impacted habitats, when organisms disperse from polluted patches to non-polluted stream sections and genetically exchange with refuge organisms, but related studies are scarce. Empirical studies quantifying to which extent the effects of pollutants such as pesticides propagate to least impacted upstream sections are lacking. Therefore, our understanding of the spatial dynamics of pesticide effects is low, compromising our ability to predict or explain community dynamics in least impacted habitat patches. Assuming that effect propagation is relevant, such knowledge would inform pesticide management and might contribute to the conservation and protection of biodiversity.

Following the concept of edge effects in landscape ecology (Fischer and Lindenmayer, 2007), the transition zone, termed edge, between ecosystems is characterised by the bi-directional extension of flows of organisms, matter and energy into the adjacent system. Thereby, the edge of one ecosystem is most influenced by the adjacent ecosystem. Hence, edge habitats are frequently characterised by the biotic and abiotic conditions, including pesticide pollution, of the adjacent ecosystems. For example, changes in resource availability, quality and/or structure at edges can drive community responses at edges of terrestrial habitats (Ries et al., 2004; Wimp and Murphy, 2021). But also the repeated disturbance by anthropogenic activities, such as pesticide drift from agriculture, has been found to lower the diversity-enhancing properties of edges in comparison with edges maintained by natural processes in terrestrial landscapes (meta-analysis, ground beetles in forested edges; Magura et al., 2017). To our knowledge, studies on potential edge effects in freshwaters are lacking.

We examined six small streams for pesticide exposure and pesticide effects in agricultural sites and related upstream edges and refuges. To detect potential exposure and effects, we compared edges of refuges (hereafter “edge”) to agricultural downstream areas (hereafter “agriculture”) and the core zones of refuges (hereafter “refuge”). The study was conducted in south-west Germany in a region where streams originate in forested areas of a biosphere reserve and subsequently run through an agricultural landscape with presumed high pesticide inputs and effects on invertebrates. Given that refuges mainly drain forested areas without pesticide use, we hypothesised the absence of pesticide exposure at edges and refuges, whereas, we hypothesised high pesticide exposure in the downstream agricultural sites (hypothesis 1). In addition, we hypothesised that dispersal processes result in the propagation of pesticide effects to the edge sites, measurable as a reduction in pesticide-sensitive invertebrate species at edges compared to refuges further upstream (hypothesis 2).

3.3 MATERIAL AND METHODS

3.3.1 Study area

We conducted the study in the summer of 2019 in Rhineland-Palatinate, south-west Germany. The study region is located in the transition area between low mountain ranges and lowlands. We selected six streams (Figure 3.1; from south to north: Russbach, Otterbach, Hainbach, Modenbach, Triefenbach, Kropsbach). Within each stream, we took samples at three different sampling sites, i.e. “refuge”, “edge” and “agricultural” sites. The agricultural sites were predominantly characterised by vineyards. Refuge sites were defined as sites without known pesticide use in the upstream catchment and were located in the upstream forest section of each stream. The edge sites were located at the edge of the forest in the transition zone to agriculture (Figure 3.1). The site types were characterised by different elevations, with the forested refuge sites being about 100 – 200 m higher than the agricultural sites (Table B.1). The terrain around the agricultural sites is slightly flatter than the terrain of the forested refuge and edge sites but still characterised by low hills. The distances between the agricultural sites and the edge sites ranged from 3.2 to 4.8 km, except for two streams with 1.4 and 1.6 km (Table B.1). The distances between the edge and the refuge sites were 1.1 to 1.5 km, except for one stream (0.6 km; Table B.1). The variability in distances owes to differences in stream courses, lengths and accessibilities that influenced site selection. The distance between refuge and edge was selected to exceed the maximum gammarid dispersal, a dominant organism group in this region and assuming that pesticide effects propagate via dispersal. *Gammarus* spp. can reportedly disperse up to 6 m per day and have an average life expectancy of six months (Elliott, 2003), resulting in an upper dispersal limit of approximately 1 km under normal conditions (e.g. no catastrophic drift). However, depending on the network structure, genetic exchange in the stream catchment can also take place over greater distances (Alp et al., 2012).

The present study supplemented the national pesticide monitoring of Germany (“Kleingewässer-Monitoring” or “KgM”) in 2019 (Liess et al., 2021). A subset of our sites (six agricultural sites and the Modenbach refuge site) was part of the KgM monitoring and hereafter referred to as “subset of KgM sites”.

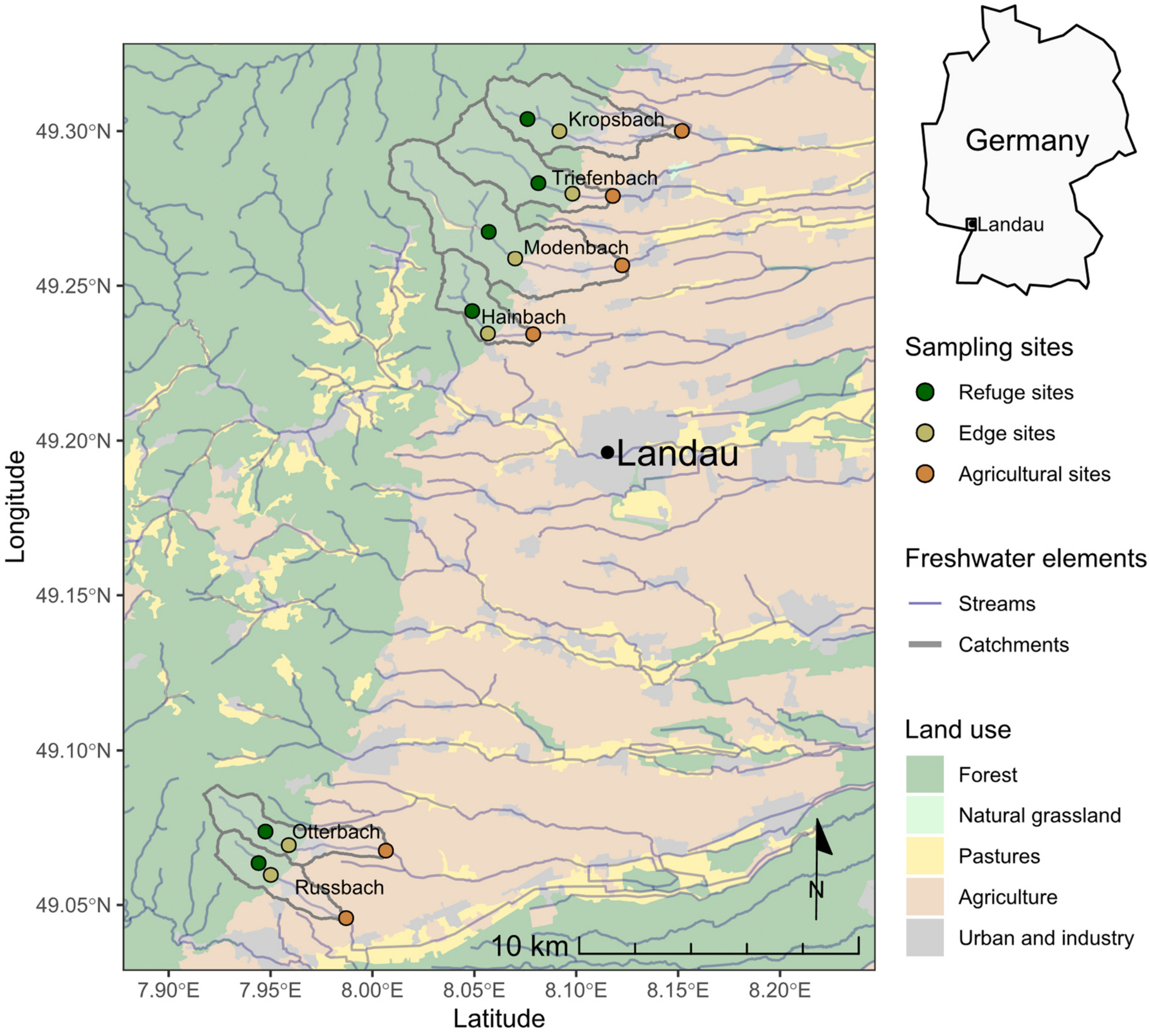

Figure 3.1: Overview of the sampling sites, i.e. refuge, edge and agricultural sites at the six streams and their catchments, in Rhineland-Palatinate, Germany, with different land use categories based on the CORINE land cover 2018 (Copernicus Land Monitoring Service, 2019).

3.3.2 Habitat characterisation

We recorded physicochemical habitat properties to control for factors other than pesticide exposure that may shape invertebrate assemblages. We measured water temperature, electrical conductivity, dissolved oxygen and pH using a multi-parameter portable meter (WTW® Multi 3630 IDS Set G; Xylem Analytics, Rye Brook, US) and flow using a flow meter (Höntzsch, Waiblingen, Germany) at all sampling sites directly after pesticide field-sampling in June 2019 (11./12.). In addition, we recorded stream depth and width and measured concentrations of ammonium-nitrogen (NH4-N), nitrate-nitrogen (NO3-N), nitrite-nitrogen (NO2-N) and phosphate-phosphor (PO4-P) using a portable spectrophotometer (DR1900, Hach Lange, Düsseldorf, Germany) and the corresponding cuvette tests (Hach Lange, Düsseldorf, Germany). If nutrient measurements were below the limit of quantification (LOQ), we set values to 0.5 times the LOQ.

3.3.3 Sampling and chemical analysis of pesticides

To characterise pesticide exposure and test hypothesis 1, we took grab samples of surface water at all sampling sites (n = 18) in early (11./12.) June 2019 and analysed their pesticide concentrations. In addition, grab samples were taken every three weeks from the beginning of April to the end of July (n = 28) at the subset of KgM sites (Halbach et al., 2021; Liess et al., 2021). Using these KgM data allowed us to assess the representativeness of the June sample for the baseline toxicity during the main pesticide application season (Szöcs et al., 2017). Details of chemical analyses are described in Halbach et al. (2021). Briefly, after filtering with a syringe filter (glass fibre filter with 0.45 µm regenerated cellulose acetate; Altmann Analytik, Munich, Germany), the samples were analysed for 74 pesticides (in Rhineland-Palatinate, 2019) via direct injection of the aqueous samples into LC-MS/MS (Agilent 1290 infinity liquid chromatography system; Agilent Technologies, Santa Clara, USA; coupled to a QTrap6500+tandem mass spectrometer equipped with an electrospray ionization interface; Sciex, Framingham, USA) and multiple-reaction-monitoring (Halbach et al., 2021). To control instrumental performance, we spiked 1 mL of the filtered samples with internal standards. For further details such as on quantification and qualification procedures see Halbach et al. (2021).

3.3.4 Determining macroinvertebrate community composition

To characterise community composition and test hypothesis 2, macroinvertebrate communities were sampled quantitively in early (03.-06.) June 2019, the period when pesticide effects on the macroinvertebrate community are most likely (Liess et al., 2021; Liess and Ohe, 2005; Szöcs et al., 2017). Sampling was done following the standardised multi-habitat sampling method (Meier et al., 2006) with minor modifications. Briefly, we collected five Surber kick-samples (0.124 m² area, 0.5 mm mesh; HYDRO-BIOS Apparatebau, Altenholz, Germany) within a stream section of approximately 25 m, sampling each substrate relative to its abundance. The sampled organisms were preserved in ethanol until identification in the laboratory. We identified all macroinvertebrates with a stereomicroscope (4.7x-42.8x SZX9; Olympus, Tokyo, Japan) to the lowest taxonomic level attainable (see Table B.2 for identification literature). Species-level was achieved for most taxa of the orders Ephemeroptera, Trichoptera, Amphipoda, for some Diptera and for some taxa of the phylum Mollusca.

For the subset of KgM sites, community data originated from the KgM where the same multi-habitat method was employed (Liess et al., 2021). We accounted for differences in the number of habitat samples per site (KgM; 10 samples, non-KgM: 5 samples) by standardising the abundance to the same area sampled (i.e. 1 m²). Note that the current study was conducted in sandstone streams characterised by relatively homogeneous stream beds with fine substrate (Table B.3) and consequently a low habitat diversity (Fernández et al., 2015; Voß and Schäfer, 2017). Hence, we suggest that both samplings captured the main habitats and communities. However, to exclude potential bias, we restrict comparisons of the taxonomic richness to refuge and edge sites (same type of habitat sampling) and focus on a metric ($\text{SPEAR}_{\text{pesticides}}$) that is based on relative community composition, which should be relatively unbiased to sampling intensity.

3.3.5 Data processing and analysis

3.3.5.1 Estimating pesticide toxicity

To estimate the toxicity of pesticide exposure for invertebrates, we calculated the logarithmic sum of toxic units (sumTU), which corresponds to the potential mixture toxicity of all detected pesticides within one sample (Sprague, 1969).

They were calculated as:

$$\text{sumTU} = log10\left(\sum_{i=1}^{n} \frac{C_i}{EC_{50_i}}\right)$$

where n is the total number of pesticides targeted by analytical measurements, C_i is the measured environmental concentration of pesticide i and EC_{50_i} is the effect concentration of pesticide i that affected 50% of test organisms in acute standard laboratory tests for the most sensitive freshwater invertebrate. We obtained effect concentrations (EC_{50i}) from Standardtox (Version 0.0.1; Scharmüller, 2021; Scharmüller et al., 2020), which constitutes a collection of quality-checked and aggregated ecotoxicological test results from the ECOTOX Knowledgebase (US EPA, 2021). We selected effect concentrations corresponding to active ingredients and 24 to 96 h test duration and used the geometric mean if multiple toxicity data were available for the most sensitive species. Data gaps were filled from the Pesticide Property DataBase (PPDB) (Lewis et al., 2016) (see Table B.4 for EC_{50i} data and corresponding references). Concentrations of spinosad a and d were summed up and considered as spinosad during TU calculation because of an absence of individual EC_{50} data. We consider sumTU's above -3 to be associated with ecological effects on freshwater invertebrates as suggested by Schäfer et al. (2012), whereas we consider sumTU's below -4 as indicative for reference sites free from pesticide effects as suggested by Becker et al. (2020).

3.3.5.2 *Comparing invertebrate communities between site types*

To compare the invertebrate communities between refuge, edge and agricultural sites, we calculated the taxonomic richness (i.e. number of different taxa per site) and total abundance (i.e. number of macroinvertebrate individuals per m^2). Moreover, we assessed the compositional similarity of the macroinvertebrate communities using the Jaccard index, a commonly used similarity index (Chao et al., 2004; Le et al., 2021). The Jaccard index was calculated as:

$$\text{Jaccard index} = \frac{n_{ab}}{(n_a + n_b - n_{ab})}$$

where n_{ab} is the number of taxa that both sites a and b have in common, whereas n_a and n_b are the number of taxa present in sites a and b, respectively. The Jaccard index ranges from zero (no shared taxon) to one (identical composition).

Moreover, we particularly focused our analysis on a relative measure of community composition, the SPEAR$_{\text{pesticides}}$, which has been developed to identify pesticide effects (Knillmann et al., 2018; Liess et al., 2021; Liess and Ohe, 2005). This metric has been successfully applied in multiple studies to link estimated pesticide toxicity to the loss of pesticide-sensitive species in communities (Chiu et al., 2016; Hunt et al., 2017; Liess et al., 2008; Schäfer et al., 2007). We calculated the SPEAR$_{\text{pesticides}}$ according to Liess and Ohe (2005) and with the updates of Knillmann et al. (2018) and Liess et al. (2021) as:

$$\text{SPEAR}_{\text{pesticides}} = \frac{\frac{\sum_{i=1}^{n} log\,(4x_i + 1) * y_i}{\sum_{i=1}^{n} log\,(4x_i + 1)} * 100}{SPEAR_{reference}}$$

where n is the total number of taxa in a sample, x_i is the abundance of taxon i given as individuals per m^2 and y_i is the risk classification parameter for taxon i (1 – at risk, 0 – not at risk). The risk classification was retrieved from the online SPEAR$_{\text{pesticides}}$ calculation tool "indicate" (Version 2.2.1, Indicate, 2021) and is based on species traits. We excluded the trait "dependence on the presence of refuges" from the risk classification given that this trait has been implemented to remove the effects of refuges (Knillmann et al., 2018). As suggested by Liess et al. (2021), we standardised $\mathrm{SPEAR}_{\mathrm{pesticides}}$ values to a $SPEAR_{reference}$ value (44) determined for reference sites in the framework of the KgM (for details see Liess et al., 2021). The standardised $\mathrm{SPEAR}_{\mathrm{pesticides}}$ ranges from zero to one and represents the proportion of pesticide-sensitive species present in a site relative to reference sites.

3.3.6 Statistical analysis

To compare estimated pesticide toxicity and community composition across the three site types (refuge, edge, agriculture), we modelled sumTU, taxonomic richness, total abundance, Jaccard index and $\mathrm{SPEAR}_{\mathrm{pesticides}}$ separately as response variable explained by site type. Given that sites within a stream are likely more similar than between streams, we accounted for the nested structure of the data using linear mixed models (LMM) with stream as random factor (Zuur et al., 2009). Similarly, we modelled $\mathrm{SPEAR}_{\mathrm{pesticides}}$ as response variable explained by sumTU and site type using a LMM with stream as random factor to evaluate the relationship between the proportion of pesticide-sensitive species and estimated pesticide toxicity. All statistical analyses and figures were produced in R version 4.1.2. (R Core Team, 2021). For LMM, we used the lme4 package 1.1-27.1 (Bates et al., 2015). LMM were fitted using restricted maximum likelihood (REML). To test for significance of single effects in LMM, we applied a type III analysis of variance with Kenward-Roger's method available in the lmerTest package 3.1-3 (Kuznetsova et al., 2017). This method has been shown to perform well for small sample sizes (Luke, 2017). We tested for pairwise differences between sites using the Kenward-Roger estimation of degrees of freedom and adjustment by the Tukey method available in the emmeans package 1.7.2 (Lenth, 2022). For visualisation, we used the ggplot2 package 3.3.5 (Wickham, 2016) and the effects package 4.2-1 (Fox and Weisberg, 2019, 2018). We provide all raw data and the R script on GitHub at https://github.com/rbslandau/schneeweiss_refuge_1. Pesticide- and site-specific results are provided in tables B.4 to 7.

3.4 RESULTS

3.4.1 Habitat characteristics of refuge, edge and agricultural sites

The environmental conditions were similar at edge and refuge sites. The agricultural sites were on average slightly deeper and approximately 2 °C warmer. Similarly, the nutrient concentrations and conductivity (1.6-1.8 fold) were higher, whereas the dissolved oxygen was lower (4-5 %) in agricultural compared to edge and refuge sites (Table 3.1).

Table 3.1: Environmental variables characterising refuge, edge and agricultural sites (measured for six streams). Nutrient concentrations indicate the amount of nitrogen or phosphor in the respective compound (i.e. NH4-N, NO3-N, NO2-N, PO4-P). If nutrient measurements were below the limit of quantification (LOQ), we set values to 0.5 times the LOQ (LOQ of NH4-N: 0.015-2 mg/L; NO3-N: 0.23-13.5 mg/L; NO2-N: 0.015-0.6 mg/L; PO4-P: 0.05-1.5 mg/L).

Variable [unit]	Site type	Minimum	Maximum	Median	Mean	SD
Stream width [m]	Refuge	0.80	2.70	1.17	1.48	0.76
	Edge	1.18	2.90	1.83	1.92	0.62
	Agriculture	0.85	2.40	1.66	1.69	0.54
Stream depth [cm]	Refuge	4.50	25.00	13.50	13.25	7.87
	Edge	6.00	17.00	10.00	10.67	3.88
	Agriculture	11.00	28.00	12.50	15.33	6.44
Flow velocity [m/s]	Refuge	0.07	0.40	0.15	0.19	0.13
	Edge	0.09	0.39	0.21	0.22	0.12
	Agriculture	0.10	0.26	0.19	0.19	0.06
Water temperature [°C]	Refuge	11.10	15.60	13.65	13.32	1.53
	Edge	12.90	14.50	13.70	13.75	0.60
	Agriculture	14.50	16.30	15.70	15.60	0.64
Dissolved oxygen [%]	Refuge	89.10	95.00	91.75	92.02	2.59
	Edge	91.40	95.00	93.45	93.43	1.35
	Agriculture	81.10	91.90	89.10	87.92	4.22
Conductivity [µS/cm]	Refuge	124.00	207.00	145.00	157.67	36.41
	Edge	117.00	218.00	186.00	172.00	38.66
	Agriculture	188.00	393.00	243.00	274.17	93.48
pH	Refuge	6.49	7.84	7.38	7.26	0.51
	Edge	5.75	7.93	7.51	7.20	0.81
	Agriculture	6.73	8.22	7.66	7.61	0.54
Ammonium [mg/L]	Refuge	0.01	0.05	0.03	0.03	0.02
	Edge	0.01	0.30	0.04	0.08	0.11
	Agriculture	0.05	0.97	0.07	0.22	0.37
Nitrate [mg/L]	Refuge	0.38	2.53	0.93	1.11	0.73
	Edge	0.50	1.57	0.95	0.98	0.34
	Agriculture	0.34	2.98	1.49	1.50	0.88
Nitrite [mg/L]	Refuge	0.01	0.04	0.03	0.02	0.01
	Edge	0.01	0.05	0.02	0.02	0.02
	Agriculture	0.02	0.08	0.04	0.04	0.02
Phosphate [mg/L]	Refuge	0.03	0.07	0.03	0.03	0.02
	Edge	0.03	0.30	0.04	0.09	0.11
	Agriculture	0.03	0.19	0.06	0.07	0.06

3.4.2 Estimated pesticide toxicity in refuge, edge and agricultural sites

Agricultural sites were characterised by a higher level of pesticide exposure compared to edge and refuge sites both in terms of number of detected pesticides and total concentrations (Figure 3.2 A,B). The average number of detected pesticides was approximately 3.5-fold higher in agricultural sites than in edge and refuge sites (6, 6 and 22 pesticides in refuge, edge and agricultural sites; Table B.8). Similarly, the total pesticide concentration was approximately 100-fold higher (0.002, 0.003 and 0.391 µg/L in refuge, edge and agricultural sites; Table B.8). The estimated toxicity in terms of sumTU ranged from -3.7 to -0.9 at agricultural sites (Figure 3.2 C; Table B.8) with five sites exceeding sumTU of -3, suggesting ecological effects on invertebrates (threshold definition in Chapter 3.3.5.1). This level of toxicity was significantly higher compared to edge and refuge sites (LMM, p=0.015, Table B.9,10; pairwise differences of site type: agriculture – refuge: p = 0.03, agriculture – edge: p = 0.02, edge –

refuge: p = 0.95). The estimated pesticide toxicity at edge and refuge sites only occasionally exceeded the sumTU of -3 (Figure 3.2 C), but, when considering additional samples covering April to July (at KgM sites), the quality criterion for reference sites (sumTU below -4; Chapter 3.3.5.1) was violated in 7 of the 16 grab samples (Figure B.11). The estimated pesticide toxicity for June (sampling temporally closest to invertebrate sampling) was similar to other samplings throughout the season of pesticide application from April to July (One-way ANOVA with sumTU as response and sampling date as predictor, p=0.97; Table B.10, Figure B.11). Although the number of detected compounds and the total concentrations of herbicides and fungicides were generally higher than those of insecticides, the estimated toxicity to invertebrates was driven by insecticides in all site types (Figure B.12; Table B.13). Except for two herbicides (chloridazon, prosulfuron), all pesticides showed common occurrence at the three site types or showed a gradient in occurrence from agricultural to edge to refuge sites (Table B.14). The estimated toxicity at agricultural sites of this study (median sumTU = -1.93 of 2019 June samples) was slightly higher than in other regions of Germany monitored in the KgM, comprising catchments with a wide variety of crop types (median sumTU = -2.65 of 2019 June samples, calculated from the KgM raw data following the toxicity estimation detailed in the methods).

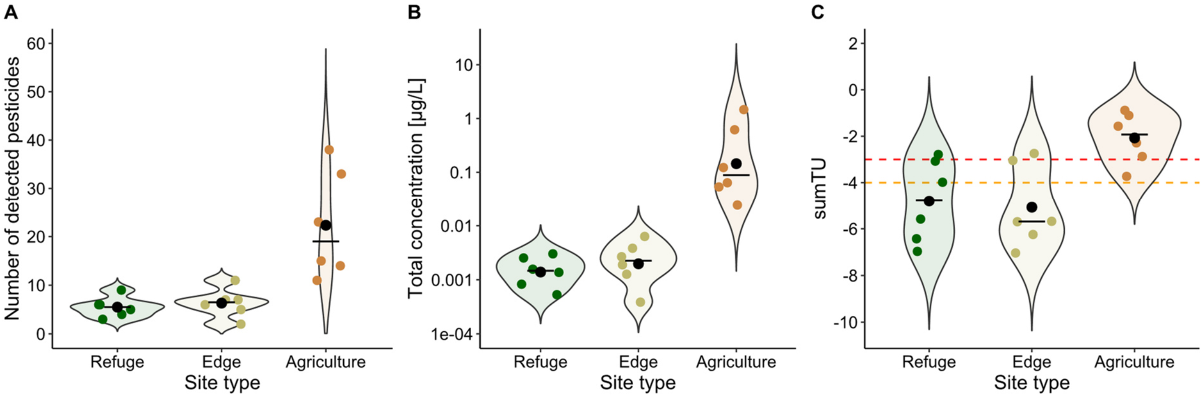

Figure 3.2: Violin plots (Wickham, 2016) visualising the number of detected pesticides (A), the total pesticide concentration in µg/L (B, on a logarithmic scale) and the estimated invertebrate toxicity (logarithmic sum toxic unit, sumTU) (C) analysed in surface water grab samples from refuge, edge and agricultural sites. Each coloured dot represents a single sample taken in each of the six streams, with the colours representing the site types. The black dot and line represent the mean and the median, respectively, for the six streams per site type. The red and orange lines represent the thresholds for potential effects on invertebrates (-3) and reference sites (-4), respectively (for details see Chapter 3.3.5.1).

3.4.3 Community composition in refuge, edge and agricultural sites

Taxonomic richness and total abundance were similar across site types (LMM, factor site type not significant at p = 0.35 and p = 0.66 for richness and abundance, respectively; Table B.9,10). Notwithstanding, a slight trend towards an increase in average taxonomic richness from refuge over edge to agricultural sites was observed (35, 39 and 41; Figure 3.3 A; Table B.8). Similarly, average total abundance increased from refuge over edge to agricultural sites (approximately 5800, 7600 and 12300 individuals/m²; Figure 3.3 B; Table B.8). The higher values in agricultural and edge sites were driven by few individual dipteran species (e.g. from *Tanytarsini Gen.* spp.) and gammarids (Figure B.15). Communities of refuge and edge sites were more similar, in terms of the Jaccard index, to each other than to agricultural sites (Figure 3.3 C; Table B.8; LMM, factor pairwise site type comparisons significant at p = 0.03), but edge and agricultural communities were on average slightly more similar than agricultural and refuge communities (0.4 vs 0.3; Table B.8), though not significant (pairwise differences of pairwise site type comparisons: agriculture – refuge vs edge – refuge: p = 0.02, agriculture – edge vs edge – refuge: p = 0.20, agriculture – edge vs agriculture – refuge: p = 0.36). The abundance of pesticide-sensitive taxa in terms of $\text{SPEAR}_{\text{pesticides}}$ values differed across site types (Figure 3.3 D, LMM, factor site type significant at p = 0.005; Table B.9,10) with significantly lower values in agricultural sites compared to edge and refuge sites (approximately 50 % lower, pairwise differences of site type: agriculture – refuge: p = 0.006, agriculture – edge: p = 0.016, edge – refuge: p = 0.838).

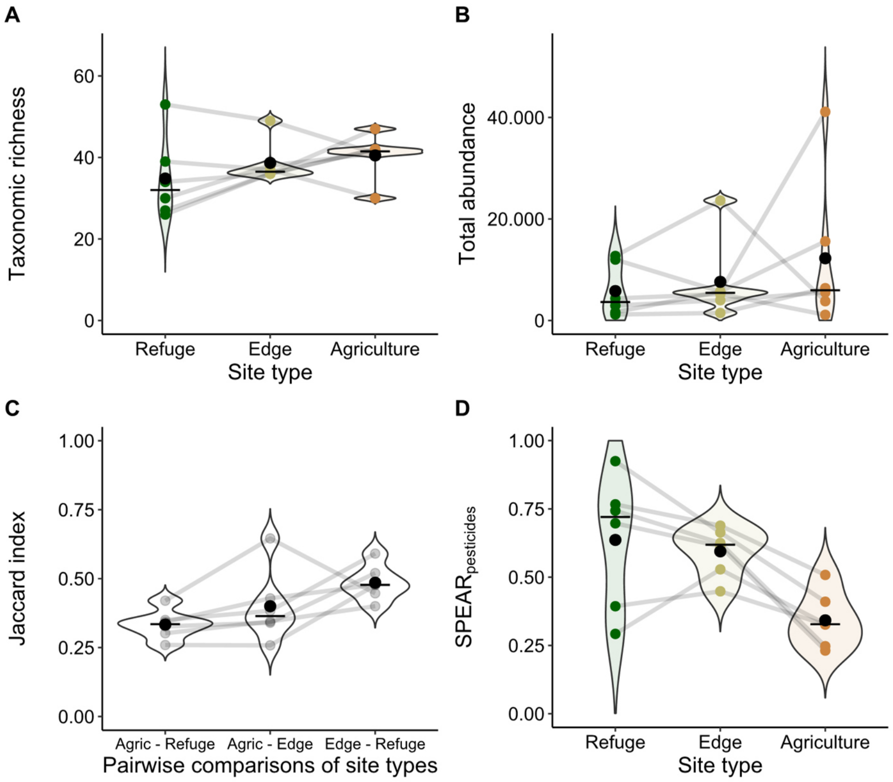

Figure 3.3: Violin plots (Wickham, 2016) visualising taxonomic richness (A), total abundance of individuals per m^2 (B), Jaccard index (C), and $\text{SPEAR}_{\text{pesticides}}$ (D) calculated for quantitative macroinvertebrate community samples at refuge, edge and agricultural sites. Each coloured dot represents a single sample taken in each of the six streams, with the colours representing the site types and grey lines connecting site types in the same stream. The grey dots represent pairwise comparisons of site types. The black dot and line represent the mean and the median, respectively, for the six streams per site type.

3.4.4 $\text{SPEAR}_{\text{pesticides}}$ - sumTU relationship

Site type described a significant amount of variation in the $\text{SPEAR}_{\text{pesticides}}$ values (LMM, factor site type significant at p = 0.04; Table B.9,10), whereas sumTU did not (LMM, factor sumTU not significant at p = 0.86). Note that the factor site type captured different segments of the toxicity gradient (see Chapter 3.4.2.). When including sumTU and the interaction term (both not significant) in the model, agricultural sites showed a decrease of $\text{SPEAR}_{\text{pesticides}}$ with increasing sumTU, whereas edge and refuge sites showed a mild increase, with edge sites exhibiting slightly lower values of sensitive species (Figure 3.4).

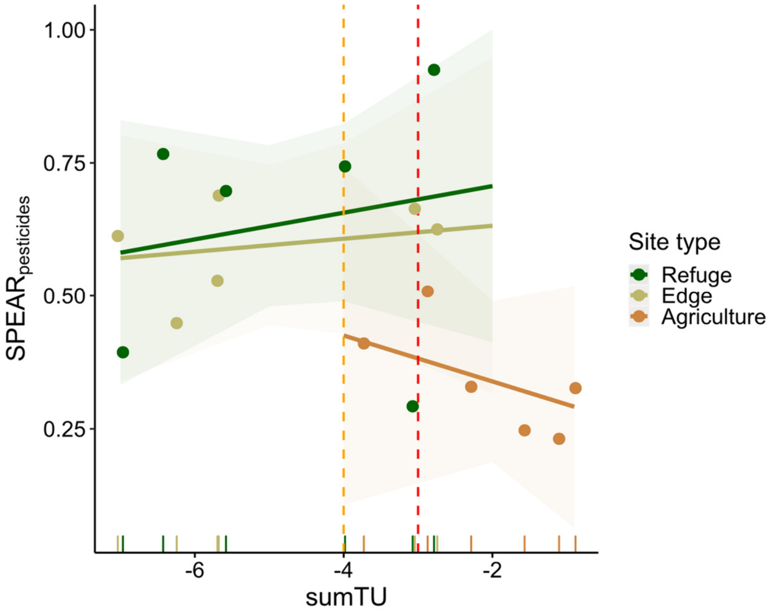

Figure 3.4: Linear mixed model regression lines (Fox and Weisberg, 2018; Wickham, 2016) visualising SPEAR$_{pesticides}$ in response to the estimated invertebrate toxicity (logarithmic sum toxic units, sumTU), site type and the interaction between them. The model includes stream as random factor. Each coloured dot represents a single sample taken in each of the six streams, with the colours representing the site types. The red and orange lines represent the thresholds for potential effects on invertebrates (-3) and reference sites (-4), respectively (for details see Chapter 3.3.5.1).

3.5 DISCUSSION

3.5.1 Pesticides occur in forested sections

We hypothesised the absence of pesticide exposure at edge and refuge sites and a high pesticide exposure at downstream agricultural sites (hypothesis 1). We found, in line with this hypothesis, differences in estimated pesticide toxicity between site types, with agricultural sites displaying significantly higher values than edge and refuge sites. This can be explained by the proportion of agricultural land use in the catchment being a dominant driver of pesticide exposure in small streams (Rasmussen et al., 2011; Schreiner et al., 2021; Szöcs et al., 2017). Nevertheless, contrary to our hypothesis, at refuge and edge sites we estimated occasionally relevant pesticide toxicity, defined as a sumTU above -3 (definition Chapter 3.3.5.1). Throughout the sampling period from April to July, only 56% of samples taken at refuge and edge sites met the quality criterion for reference sites (sumTU below -4; Figure B.11). In general, the estimated pesticide toxicity levels were similar across the whole sampling period (based on KgM sites), though they varied strongly between single samplings in individual sites which is presumably driven by single substances (Figure B.11). Hence, we consider the June sampling as largely representative of the general baseline exposure. The baseline exposure was determined independently of weather conditions using regular three-weekly grab sampling. This sampling method is likely to miss rain-driven pesticide pulses, thereby underestimating pesticide toxicity (Rabiet et al., 2010; Spycher et al., 2018; Xing et al., 2013). However, given that runoff from surfaces treated with pesticides is a major route of precipitation-driven pesticide input (Leu et al., 2004; Liess et al., 1999; Szöcs et al., 2017), in particular in our region characterised by terrain with steep slopes, we suggest that concentrations and toxicity were in particular underestimated in agricultural sites. Overall, we conclude that agricultural sites exhibited significantly higher pesticide toxicity than edge and refuge sites.

Interestingly, most pesticides were detected more frequently in the agricultural sites than in refuge and edge sites in June (Table B.14). Therefore, we suggest that agricultural land use was also the main source of contamination for refuge and edge sites. We consider it unlikely that the pesticide concentrations are a legacy of past use because exposure should then be more stable and independent from current use patterns. However, companion studies displayed clear temporal trends linked to current use (Halbach et al., 2021; Weisner et al., 2022) and a recent large-scale study for Germany also demonstrated clear seasonal trends, very likely linked to pesticide use (Szöcs et al., 2017). We exclude forest-related pesticide use as a main source of contamination for refuge and edge sites, given that, to our knowledge, the use of pesticides in forests in this region has been limited to rare cases of local application and, in the case of insecticides, to other (i.e. pyrethroids) than the detected compounds over the last decade (Landtag RLP, 2019). We expect that non-agricultural pesticide use (e.g. in urban areas such as roads or residential areas) contributes negligibly to the pesticide residues in the refuges because urban areas were absent or covered only a minor proportion of area in the upstream catchments of edge and refuge sites. Notwithstanding, a recent study suggests that already very low proportions of urban land (< 5 %) in the catchment can result in pesticide residues in streams of undeveloped areas (Sandstrom et al., 2022). Hence, urban use may have contributed to the exposure. Due to the topography of the study region (forested refuge sites are located at approximately 100 – 200 m higher elevation than agricultural sites, Table B.1), surface water runoff, erosion, drain flow, or leaching can be excluded as transport pathways for pesticide residues originating from downstream agricultural pesticide use. We suggest that compounds were most likely transported in the air to the forested sites, which were located in the proximity of only 0.6 to 1.5 km to the forest-agricultural edges. In the air, pesticides can generally be transported as vapor, droplets or associated with particles or dust and field measurements can provide information on the relative importance of these processes (Plimmer, 1990). Transport as vapor was generally most relevant for persistent and relatively volatile compounds (Daly and Wania, 2005; Hageman et al., 2006; Plimmer, 1990). Most of the compounds driving estimated toxicity in refuge and edge sites (Table B.13) were persistent in soils but non-volatile (dimension of henrys law constants: 10^{-4} to 10^{-11} Pa m³/mol, Lewis et al., 2016). They may have been transported as droplets or associated with particles or dust. Several previous studies point to the importance of the transport of pesticides via air and rain into untreated areas (Décuq et al., 2022; Kreuger et al., 2006) and also into protected (forested) areas in Germany (Kruse-Plaß et al., 2020). However, within our study the transport paths remain subject to speculation and a study measuring different transport paths would be required for clarification.

Overall, we suggest that regional transport of pesticides can lead to pesticide exposure in forest sections within a few kilometres upstream of agricultural areas. Several earlier studies also reported pesticide exposure in sites with low or negligible agriculture in upstream catchments. For instance, a large-scale study in Germany reported water quality threshold exceedances, i.e. considerable pesticide exposure, even for sites without agriculture in the catchment (Szöcs et al., 2017). Moreover, several studies reported pesticide exposure in seemingly pristine regions throughout the world (Daly and Wania, 2005; Guida et al., 2018; Hageman et al., 2006; Le Noir et al., 1999; Usenko et al., 2005; Wang et al., 2019; Zhan et al., 2021). Future studies, ideally in catchments with known agricultural and non-agricultural pesticide use, should further scrutinise the influence of pesticides transported to least impacted ecosystems.

3.5.2 Adjacent agricultural habitats influence edge communities

We hypothesised that dispersal processes result in the propagation of pesticide effects to the edge area (hypothesis 2). We found a significantly lower proportion of pesticide-sensitive species at agricultural sites compared to edge and refuge sites. However, the proportion of pesticide-sensitive species in edges was only slightly, and statistically non-significantly, lower compared to refuges further upstream. An analysis of over 100 sites of which our sites represent a subset (Liess et al., 2021) found that pesticides are the main driver of the community composition depicted by $\text{SPEAR}_{\text{pesticides}}$. Hence, we suggest that pesticides are also an important driver in the agricultural sites in our study, given that they displayed high estimated pesticide toxicity. Nevertheless, in contrast to previous studies (Schäfer et al., 2012), $\text{SPEAR}_{\text{pesticides}}$ was not associated with sumTU in our study. The sample size was relatively small (18 sites, but from only 6 streams) and may have prohibited to detect an association between $\text{SPEAR}_{\text{pesticides}}$ and estimated pesticide toxicity. More importantly, our study was primarily designed to capture differences between site types rather than a pesticide toxicity gradient across streams. Indeed, the factor site type explained a considerable amount of variation in $\text{SPEAR}_{\text{pesticides}}$ and was closely associated with the pesticide gradient. In refuge and edge sites, the estimated toxicity barely crossed the threshold where effects are likely (-3), whereas in agricultural sites most values were above this threshold. Indeed, in the agricultural sites, the $\text{SPEAR}_{\text{pesticides}}$ and sumTU were clearly negatively related (Figure 3.4). In edge and refuge sites, where the sumTU gradient ranged from approximately -7 to -3, the association between $\text{SPEAR}_{\text{pesticides}}$ and sumTU was very weak and rather positive as identified previously (Liess et al., 2021). This suggests that the effect threshold applied, i.e. sumTU of -3, is largely protective, which matches the findings of previous studies (Orlinskiy et al., 2015; Schäfer et al., 2012). The $\text{SPEAR}_{\text{pesticides}}$ values varied to a similar extent for edge and refuge sites and pesticide levels were comparable.

Despite the difference of pesticide-sensitive species in edges compared to refuge was only minor and non-significant, the other metrics (Jaccard Index, taxonomic richness, total abundance) also indicated an influence of the agricultural sites on the edges. For instance, the results for the Jaccard index show that edge communities share on average slightly more taxa with agricultural communities than refuge communities with agricultural communities, though not significant. Similarly, there was no statistical evidence that taxonomic richness and total abundance differed across sites, but they showed a rather increasing trend from refuge over edge to agriculture. This contrasts with the decreasing trend of pesticide-sensitive species in terms of $\text{SPEAR}_{\text{pesticides}}$. The loss of species with pesticide-vulnerability traits (“losers”), such as long generation time and high physiological sensitivity (Liess and Beketov, 2011) seems to be balanced by an increase in species with tolerance traits (“winners”), resulting in a species turnover rather than a loss (Dornelas et al., 2019). We conclude that the loss of pesticide-sensitive species may not always translate to a loss in species diversity. However, the loss of pesticide-sensitive species can be associated with the loss of functional and genetic diversity, and may affect ecosystem functioning and stability (Cadotte et al., 2011). In line with our findings, a loss of functional diversity with a simultaneous increase in taxonomic diversity was found for fish communities in anthropogenically disturbed habitats (Villéger et al., 2010). In contrast, another field study in our region found a decline in the taxonomic diversity of macroinvertebrate communities with environmental stress, whereas functional diversity remained stable (Voß and Schäfer, 2017). However, this study was conducted in autumn and hence associated with a relatively small taxon pool (Voß and Schäfer, 2017). Generally, the relationship between functional diversity and taxonomic richness is complex and context-dependent (Cadotte et al., 2011). If colonisation matches species loss, functional diversity can change whereas species diversity remains stable (Cadotte et al., 2011). In our study, agricultural sites are bidirectionally connected (with stream sections up- and downstream), while upstream refuges near the stream source are only unidirectionally connected with downstream sections. This particular spatial context may allow for higher net immigration based on mass effects in terms of density-dependent organism dispersal from adjacent habitats with high reproductive success (Shmida and Wilson, 1985), as well as higher gains from drift into agricultural sites compared to refuge sites. In addition, specific habitat characteristics at the agricultural sites such as higher nutrient input as well as higher water temperature (Table 3.1) may support a higher diversity and abundance (van Klink et al., 2020). Furthermore, the dense shading of the upstream forested sites could hinder higher diversity and abundance while favouring the presence of specialists. However, the agricultural sites also exhibited diverse riparian vegetation, including trees, and we did not monitor shading of sites.

To sum up, although indicators for taxonomic and functional diversity established different relationships with site type, they both suggested an influence of agricultural stream sections on the edges. Given a relatively low sample size and related uncertainty, further research is needed to quantify the extent and unravel the mechanisms of how pesticide effects propagate to refuges. For example, it remains open, whether pesticide effects in edge and refuge sites may be detected at lower levels of biological organisation, such as the organism and sub-organism level. Furthermore, effect propagation is likely to depend on spatial characteristics, such as the distance between agriculture and refuge patches as well as the size of the refuge, as found in previous studies (Orlinskiy et al., 2015; Trekels et al., 2011; Willson and Hopkins, 2013). In our field survey, the distances between refuge, edge and agricultural sites varied slightly for the six streams and refuges were large in proportion to the agricultural land involved within the catchments (Figure 3.1; Table B.1). Future studies are required, to understand the influence of spatial patterns and relationships for the propagation of pesticide effects. Finally, given that the information provided by taxonomic richness and the functional metric ($\text{SPEAR}_{\text{pesticides}}$) differed, we suggest that both, taxonomic and functional metrics, should be considered when studying pesticide effects in the context of biodiversity change (Cadotte et al., 2011; Dornelas et al., 2019).

3.6 CONCLUSION

We found significantly higher potential pesticide toxicity (sumTU) and altered functional community composition ($\text{SPEAR}_{\text{pesticides}}$) associated with pesticide exposure at agricultural compared to edge and refuge sites. Notwithstanding, at some edge and refuge sites, which were considered as being least impacted, we estimated unexpected pesticide toxicity exceeding thresholds where field studies on the association of estimated pesticide toxicity and invertebrate community composition suggested adverse effects on freshwater invertebrates. We conclude that the regional transport of pesticide residues can result in ecologically relevant pesticide exposure in forest sections within a few kilometres upstream of agricultural areas. In addition, we found that the majority of edge sites were characterised by a slightly lower abundance of pesticide-sensitive species (lower $\text{SPEAR}_{\text{pesticides}}$) compared to refuge sites, indicating a potential influence of adjacent agriculture. However, future studies are required to unravel the extent to which pesticide effects propagate to refuges and to scrutinise underlying mechanisms, and particularly to understand the spatial patterns and relationships for the propagation of pesticide effects. Furthermore, pesticide effects in edge and refuge communities need to be studied in the future at lower levels of biological organisation, such as the organism and sub-organism level.

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4 POPULATION STRUCTURE AND INSECTICIDE RESPONSE OF *GAMMARUS* SPP. IN AGRICULTURAL AND UPSTREAM FORESTED SITES OF SMALL STREAMS

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4.1 ABSTRACT

Exposure to pesticides may cause adaptation not only in agricultural pests and pathogens, but also in non-target organisms. Previous studies mainly searched for adaptations in non-target organisms in pesticide-polluted sites. However, organisms may propagate heritable pesticide effects, such as increased tolerance, to non-exposed populations through gene flow. We examined the pesticide tolerance - as one of the pre-assumptions of local adaptation - of the freshwater crustacean *Gammarus* spp. (at genus level reflecting the gammarid community). The pesticide tolerance was quantified in acute toxicity tests using the insecticide imidacloprid. Gammarids were sampled at pesticide-polluted agricultural sites (termed agriculture), least impacted upstream sites (termed refuge) and transitional sites (termed edge) in six small streams of south-west Germany. Furthermore, we examined the population genetic structure of *Gammarus fossarum* and the energy reserves (here lipid content) of *G. fossarum* as well as of *Gammarus* spp. at the three site types (i.e. agriculture, edge and refuge).

We found significantly lower imidacloprid tolerance of *Gammarus* spp. from agricultural sites compared to edge and refuge sites, potentially due to higher environmental stress at agricultural sites, as indicated by a slightly lower lipid content per mg gammarid tissue. We found no differences in pesticide tolerance between edge and refuge populations, indicating no propagation of pesticide effects to edges. The genetic structure among *G. fossarum* populations showed significant differentiation between streams, but not within a stream across the site types. We suggest that high gene flow within each stream hindered population differentiation and resulted in similar (pre)adaptations to local stress levels between site types, although they exhibited different pesticide pollution. Further studies on target genes (e.g. conferring pesticide tolerance), population structure and fitness of different phenotypes in particular among non-target organisms are required for adjacent pristine ecosystems to detect potential propagations of pesticide effects.

4.2 INTRODUCTION

Pesticides are applied over large areas in agricultural landscapes, where they can affect populations, communities and food webs in non-target ecosystems such as streams (Beketov et al., 2013; Hunt et al., 2017; Liess & Von Der Ohe, 2005; Rundlöf et al., 2015; Schäfer, 2019). Exposure to pesticides can set off adaptation processes in agricultural pests and pathogens (Hawkins et al., 2019; Melander, 1914; REX Consortium, 2013; Tabashnik et al., 2014) and also in non-target organisms such as freshwater taxa (Becker & Liess, 2017; Bendis & Relyea, 2014; Shahid et al., 2018; Weston et al., 2013). A study reported an up to 3.6-fold average increase in insecticide tolerance across 17 freshwater invertebrate taxa (Becker & Liess, 2017). Similarly, amphipods from pesticide-exposed streams exhibited a 3-fold higher insecticide tolerance compared to non-exposed populations (Shahid et al., 2018). In both studies, the authors suspected a predominant genetic basis underlying the tolerance development. First, the taxa were likely exposed regularly over decades (Shahid et al., 2018), which is usually associated with genetic adaptation (Hua et al., 2013). Second, the tolerance was similar during and after the pesticide spraying season, suggesting transgenerational effects that may be explained by genetic adaptation (Becker & Liess, 2017; Shahid et al., 2018). Finally, genetic adaptation to pesticides has been detected or suspected frequently in pest species (Bass et al., 2015; Karatolos et al., 2010; Rinkevich et al., 2012; Weill et al., 2003), and for non-target species (Boyd & Ferguson, 1964) including freshwater invertebrates (Bendis & Relyea, 2014; Major et al., 2018; Weston et al., 2013).

Overall, the development of increased tolerance in freshwater invertebrates may at least partly be based on genetic adaptations that occur through selection at gene loci affecting survival (e.g. target site encoding or metabolic resistance genes) (Bickham, 2011; Hawkins et al., 2019). Directional selection can leave a long-term fingerprint on the gene pool (i.e. selective signatures in the genomes) (Hawkins et al., 2019), such as allele or genotype frequency changes in selected (e.g. pesticide-tolerant) populations (Bickham, 2011; Chung et al., 2012; Hoffmann & Daborn, 2007; Rusconi et al., 2018), potentially translating into strong population differentiation (Laporte et al., 2016; Pavey et al., 2015). Strong small-scale population differentiation within the same catchment has been demonstrated for *Gammarus fossarum* (Weigand et al., 2020), also in association with differences in pesticide susceptibility (Grethlein et al., 2022). In addition, reduced genetic diversity has been found for strains carrying resistant alleles (Estep et al., 2015; Lynd et al., 2010; Weetman et al., 2015) and in taxa inhabiting polluted sites (Bach & Dahllöf, 2012; Coors et al., 2009; Švara et al., 2022; Weston et al., 2013). A loss of genetic variation can affect numerous ecological processes at the population, community and ecosystem levels (Hughes et al., 2008; Medina et al., 2007). Furthermore, increased tolerance may be associated with fitness costs, if the tolerance traits are genetically correlated with fitness traits (negative pleiotropy) or if the tolerance-related energy allocation to defence mechanisms leads to trade-offs in energy allocation (Kliot & Ghanim, 2012; Posthuma et al., 1993; Shirley & Sibly, 1999; Vigneron et al., 2015; Xie & Klerks, 2004).

To date, adaptations in non-target species have been mainly investigated in pesticide-polluted sites. However, the exchange of organisms may propagate heritable pesticide effects, such as increased tolerance, to non-exposed populations, for example, if related genes are transferred (i.e. gene flow) and if fitness costs are low. Historically theoretical and empirical research mainly focused on constraints or disruptive effects of gene flow on adaptation whereas to date an increasing number of studies suggests that local adaptation may develop or be maintained despite gene flow and that local adaptation can even be promoted by gene flow (Crispo, 2008; Fitzpatrick et al., 2015; Garant et al., 2007; Lenormand, 2002; Moody et al., 2015; Muir et al., 2014; Tigano & Friesen, 2016; Zhang et al., 2021). For example, already early (finite) population models suggested that gene flow can retard adaptation in treated fields, but can also increase the frequency of tolerance-promoting alleles in nearby untreated fields (Caprio & Tabashnik, 1992; Comins, 1977) and may contribute to pre-adaptation of untreated populations (Uyenoyama, 1986). For pest species, field studies suggest that insecticide resistance can spread through insect migration and related gene flow even between continents (Raymond et al., 1991). Similarly, fungicide resistance can spread over long distances (Fraaije et al., 2005; Torriani et al., 2009). To which extent this applies to non-target species in freshwater ecosystems remains unknown. Such knowledge is required to reliably predict or explain non-target population and community dynamics in non-exposed habitats, which may inform pesticide management and contribute to biodiversity protection.

We examined pesticide tolerance as one of the pre-assumptions of local adaptation of the widely occurring freshwater crustacean *Gammarus* spp.. We collected organisms from downstream pesticide-polluted agricultural sites (here termed agriculture), non-polluted upstream sections that are directly connected (here termed edges) and sites in forested sections further upstream (here termed refuges) in six small streams in south-west Germany. Based on between-sites distances and the maximal upstream dispersal distance of *Gammarus* spp. (for details see Chapter 4.3.1), we expected gene flow between site types. The highest levels of exchange (including genetic exchange) were expected for the edge areas following the edge concept (Fischer & Lindenmayer, 2007). Given the regular pesticide exposure at agricultural sections in our study region, we hypothesised local adaptation processes in terms of higher pesticide tolerance of *Gammarus* spp. and that these propagate via gene flow to the edge habitats (hypothesis 1). Pesticide tolerance was quantified in acute toxicity tests (similar to the studies of Becker & Liess, 2017; Grethlein et al., 2022; Shahid et al., 2018; Švara et al., 2021) using the insecticide imidacloprid. Further, the regular pesticide exposure in agricultural sites may leave a long-term fingerprint in the gene pool of populations despite considerable gene flow. This is hypothesised to result in a higher population differentiation between agricultural and refuge populations than between agricultural and edge populations (hypothesis 2a) and a decrease in genetic diversity from refuge over edge to agricultural sites (hypothesis 2b). Following the concept of resource allocation (Sibly & Calow, 1986, p. 7), we hypothesised that a higher tolerance is associated with a higher allocation of energy to defence mechanisms, resulting in lower energy reserves in tolerant organisms compared to less tolerant organisms (hypothesis 3).

4.3 MATERIALS AND METHODS

4.3.1 Study area

We sampled at the three different site types "refuge", "edge" and "agricultural" of six streams (i.e. a total of 18 sampling sites) in summer 2019 in Rhineland-Palatinate, south-west Germany. Vineyards dominate land use near agricultural sites and forests characterise the catchment areas of upstream refuge and edge sites. The edge sites were located in the transition zone between agriculture and forest. The forested refuge sites were about 100 to 200 m higher than the agricultural sites (Table C.1). Refuge and edge sites were about 0.6 to 1.5 km apart, whereas edge and agricultural sites were about 1.4 to 4.8 km apart (Schneeweiss et al., 2022). The distance between refuge and edge sites was selected based on estimates of the maximum gammarid upstream dispersal (i.e. approximately 1 km/lifespan see Schneeweiss et al., 2022; based on Elliott, 2003), as we focus on gammarids in this study and assume that potentially acquired pesticide tolerance propagates via upstream dispersal. Analytical testing of stream water confirmed significant and repeated pesticide contamination that is likely to result in adverse effects on invertebrates at the agricultural sites (for details see: Liess et al., 2021; Schneeweiss et al., 2022). Edge and refuge sites occasionally also had relevant pesticide toxicity, defined as log sum toxic units above -3 (Schäfer et al., 2012; Schneeweiss et al., 2022). Accordingly, forested upstream sites correspond to "least impacted" sites relatively free of human influences, rather than completely "pristine" sites. However, the focus of this study was to examine site types with strong differences in pesticide stress. This was met by the site conditions because edge and refuge sites exhibited overall significantly lower pesticide exposure and toxicity than agricultural sites (average log sum toxic unit -4.8 , -5.1 and -2.1 in refuge, edge and agricultural sites, respectively; Schneeweiss et al., 2022). The estimated toxicity to invertebrates was driven by insecticides in all site types (Schneeweiss et al., 2022). Further details on the study area, site selection and environmental conditions at the site types can be found in (Schneeweiss et al., 2022).

4.3.2 Gammarid sampling

Sampling of gammarids for measurement of pesticide tolerance, population genetics and energy reserves was conducted from mid-June to mid-July 2019. This is a period when field populations likely have been exposed to high pesticide concentrations (Halbach et al., 2021; Szöcs et al., 2017; Vormeier et al., 2023), which may lead to pesticide tolerance. For determining pesticide tolerance, we collected *Gammarus* spp. (Crustacea, Amphipoda) alive and transported them under cool and aerated conditions to the laboratory. All living organisms were cautiously handled and used in tests on the same day of sampling without acclimation, because this reduced time in captivity and did not lead to relevant mortality (Kefford et al., 2005). We opted for random sampling to obtain a representative sample of the field communities within a genus (species level not identifiable by eye without harm) and therefore omitted prior identification and sorting (e.g. for size and sex; Kefford et al., 2005). Most *Gammarus* spp. were *Gammarus fossarum,* but *Gammarus pulex* and *Gammarus roeselii* were also common, particularly at the downstream agricultural sites (Table C.2; Figure 4.1 C). We estimated the relative abundance of the tested *Gammarus* species based on the representative subsample taken for population genetics and considered the potential for interspecific differences by including the relative abundance of species as a random factor in the statistical modelling (see Chapter 4.3.6.1). For population genetic analyses, we randomly sampled approximately 40 *Gammarus* spp. per sampling site and stored them in ≥ 96 % ethanol (Roth, Karlsruhe, Germany) at -20°C. Within each sample, we identified and counted *G. roeselii* individuals microscopically. *G. pulex* and *G. fossarum* were distinguished via genetic analysis following the procedure described in Chapter 4.3.4, given ambiguities in visual identification. The *G. fossarum* individuals (n = 20, where abundances permitted; Table C.2) were further processed for population genetic analysis. For the analysis of energy reserves, we randomly collected approximately 20 *Gammarus* spp. per sampling site, which were stored in liquid nitrogen in the field and then at -80 °C in the laboratory until lipid analysis (Chapter 4.3.5).

4.3.3 Experiments to determine pesticide tolerance

We conducted acute toxicity tests with *Gammarus* spp. principally based on the procedure described in (Kefford et al., 2005). Briefly, we exposed organisms for 96 h to the neonicotinoid insecticide imidacloprid (formulation Confidor® WG70 – LOT: DE79858094D, Bayer CropScience AG, 40789 Monheim, Germany). The formulation consisted of 700 g/kg active ingredient (AI) imidacloprid. In the following, concentrations refer to the AI concentrations. We selected this insecticide since it was of major relevance in terms of toxicity to the most sensitive freshwater invertebrate (highest toxic unit), high detection frequency, and widely occurring in the region under investigation in 2018 (Liess et al., 2021). The analyses of 2019 stream water samples confirmed the major relevance of imidacloprid for the region and period under investigation (Liess et al., 2021; Schneeweiss et al., 2022). Specifically, imidacloprid was detected in 2019 in refuge, edge and agricultural sites at average concentrations of 1, 0 and 102 ng/L and in 20%, 0% and 73 % of samples, respectively (edges only sampled in June, pesticide raw data from (Schneeweiss et al., 2022) at https://github.com/rbslandau/schneeweiss_refuge). For logistical reasons, we implemented test setups for each stream consecutively (for test start per stream see Table C.3). In each assay, we applied six increasing imidacloprid concentrations (7, 21, 63, 189, 567 and 1701 µg/L) together with pure medium controls (0 µg/L). A seventh concentration (1134 µg/L) was applied when testing the last three streams to increase the reliability of the concentration-response relationship (Table C.5). The concentration range was selected to capture the complete concentration-response curve based on toxicity values from the literature for *Gammarus* spp.. The tests were run in medium Elendt M7 (Annex 2 of OECD, 2012), which we prepared on the day before the test setup in ultrapure water. Test vessels (volume of 150 mL) were aerated and kept at 16 °C, mimicking stream temperature (on average 13.3 °C, 13.7 °C and 15.7 °C at refuge, edge and agriculture, respectively; Schneeweiss et al., 2022). We tested four vessel replicates per concentration each with five test organisms per vessel. We added 4*4 cm of stainless-steel mesh to each test vessel to provide a substrate for hiding and clinging to. Given that these taxa are photosensitive, the tests were run in complete darkness. We recorded mortality every 24 h until 96 h after test start. After 96 h exposure, we additionally recorded immobility (no swimming after disturbance with forceps).

At both the beginning and the end of the test, we measured water temperature, dissolved oxygen, electrical conductivity and pH in at least one replicate per concentration using a multi-parameter portable meter (WTW® Multi 3630 IDS Set G; Xylem Analytics, Rye Brook, USA; Table C.3,4). We took one 10 mL water sample per test concentration at the beginning of each test setup for the chemical analysis of imidacloprid. In addition, we took 10 mL triplicate water samples every 24 h during the test of the highest test concentrations (i.e. 1700 µg/L) to detect potential degradation of imidacloprid. For details on chemical analysis see Text C.1. Nominal and measured imidacloprid concentrations showed good agreement (Table C.5), we report nominal concentrations hereafter. Imidacloprid concentrations remained relatively stable throughout the test (Figure C.1).

4.3.4 Population genetic analysis

We analysed a fragment of the mitochondrial cytochrome c oxidase subunit one (CO1) gene in 20 *G. fossarum* individuals (where abundances permitted; Table C.2). Briefly, we cut off animals` heads with a sterile scalpel and extracted the total DNA from them using a slightly modified salt-extraction protocol after Aljanabi (1997; for modifications see Text C.2). We stored DNA extracts at -20 °C until further examination. We used a nanodrop spectrophotometer (NanoDrop 1000, NanoDrop products, Wilmington, Delaware, USA) to measure the amount and quality of extracted DNA and, if necessary, diluted to final DNA extraction stocks of 10-20 ngDNA/µL. We amplified an approximately 650 base-pair (bp) long fragment of CO1 with the primers LCO1490-JJ and HCO2198-JJ (Astrin & Stüben, 2008). These primers are based on the standard primers LCO1490 and HCO2198 (Folmer et al., 1994) and have been successfully used for *Gammarus* spp. (Katouzian et al., 2016). We used the following polymerase chain reaction (PCR) protocol per reaction: 1.5 µL PCR Buffer (1x), 1.2 µL dNTPs (0.2 mM), 0.75 µL each primer (0.5 µM), 0.125 µL Takara Ex Taq® (0.625 U/µL; TAKARA BIO INC., Shiga, Japan), 0.75 µL DNA template, filled to 15 µL with sterile water (PCR grade, autoclaved). We set the PCR setting for amplification as follows: initial denaturation at 94 °C for 60 s; 35 cycles of denaturation at 94 °C for 30 s; annealing at 51 °C for 45 s, and extension at 65 °C for 60 s; final extension at 65 °C for 5 min (after Katouzian et al., 2016 with minor modifications). After a PCR product quality check using gel electrophoresis, PCR products were purified and sequenced by SEQ-IT GmbH & Co. KG (Kaiserslautern, Germany).

4.3.5 Measuring energy reserves

The energy reserve analysis was performed in two separate runs, one with a random sample of the field *Gammarus* spp. community and one with *G. fossarum* only (identified via microscopy and genetic analysis of two legs per individual following the procedure described in Chapter 4.3.4). For each run and sampling site, five shock-frozen gammarids (each of the five individuals is treated as a replicate on the measurement subunit) were freeze-dried for 24 h and subsequently weighed to the nearest 0.001 mg. We then quantified the total lipid content of each gammarid after Van Handel (1985) with minor modifications as described in Text C.3 and Zubrod et al. (2011).

4.3.6 Data processing and statistical analysis

All statistical analyses and figures were produced in R (version 4.2.0.; R Core Team, 2022). For visualisation, we used the ggplot2 package (version 3.3.6; Wickham, 2016). We provide all raw data and the R script on GitHub at https://github.com/rbslandau/schneeweiss_tolerance.

4.3.6.1 Factors explaining pesticide tolerance

To compare the pesticide tolerance between site types (i.e. agricultural, edge, refuge), we modelled the mortality and immobility (which includes dead organisms) of *Gammarus* spp. after 96 h imidacloprid exposure as response variables explained by the imidacloprid concentration (nominal, log-transformed with half of the lowest non-zero test concentration added to avoid infinite numbers, see Becker et al., 2020), site type and an interaction term. We chose a binomial distribution with a logit link function, given that the response was binary (dead/alive). We accounted for the nesting of site types within a stream by using stream as random factor (categorical, six levels) in a generalised linear mixed model (GLMM) (Zuur et al., 2009). Furthermore, vessel replicates related to each sampling site and concentration, representing a measurement subunit in our study design, were added as random factor (categorical, 120-135 levels). To examine potential interspecific differences in tolerance, we also added the relative abundance of *G. fossarum* (categorical, two levels: ≥80% = high, ≤50% = low; Table C.2) as a random factor to the models, but singular fits required changing to fixed factor (only two levels; e.g. Oberpriller et al., 2022). Moderate collinearity between the covariates site type and the relative abundance of *G. fossarum* required removing the latter covariate from the full models (Pearson correlation coefficient = -0.7; VIF = 2.5; Zuur et al., 2013). We constructed a second, similar model, keeping the *G. fossarum* abundance instead of the site type as fixed predictor to illustrate the explanations given in the discussion chapter. For GLMM, we used the lme4 package (version 1.1-29; Bates et al., 2015). To test for the significance of single effects in GLMM, we applied a type II Wald Chi-squared (X^2) test available in the car package (version 3.1-0; Fox & Weisberg, 2019). We calculated lethal concentrations that killed 50% of the test organisms (LC_{50}) as well as concentration-response relationships per site type by applying the estimated parameters of each model to the logistic link function or its inverse, respectively.

4.3.6.2 Estimation of genetic differentiation

We edited and aligned sequence chromatograms in the software Geneious Prime (version 2022.0.1; http://www.geneious.com; Kearse et al., 2012). Details on the sequence data preparation, quality control and taxonomy assignment are described in Text C.4. We computed and visualised population genetic metrics using the software R (version 4.2.0.; R Core Team, 2022) and following mainly a workflow provided by Toparslan et al. (2020). Briefly, we extracted haplotypes and plotted a minimum spanning (haplotype) network using the pegas package (version 1.1-2; Paradis, 2010). A hamming distance matrix (“N”) was used for the haplotype network. We calculated the genetic diversity as haplotype- and nucleotide diversity by applying the methods of Nei & Tajima (1981) and Nei (1987), respectively, both of which are implemented in the pegas package (version 1.1-2; Paradis, 2010). We modelled the haplotype diversity as a response explained by site type using a linear mixed model (LMM) with stream as random factor (Zuur et al., 2009). For LMM, we used the lme4 package (version 1.1-29; Bates et al., 2015) and fitted the models using restricted maximum likelihood (REML). We applied a type III analysis of variance with Kenward-Roger’s method available in the lmerTest package (version 3.1-3; Kuznetsova et al., 2017) to test for the significance of single effects in the LMM. This method has been shown to perform well for small sample sizes (Luke, 2017). Additionally, we calculated the fixation index (F_{ST}) after Nei (1987) between all populations as a measure of genetic population differentiation using the hierfstat package (version 0.5-11; Goudet & Jombart, 2022) for haploid genetic data. To determine potential drivers of genetic distance (i.e. site types, streams or sampling sites), we performed an analysis of molecular variance (AMOVA) as described in Excoffier et al. (1992) using the pegas package (version 1.1-2; Paradis, 2010). For the AMOVA, we used a hamming distance matrix as a measure of genetic distance.

4.3.6.3 Factors explaining energy reserves

For comparing the energy reserves between site types, we modelled the lipid content and dry weight per gammarid separately as response explained by site type using a LMM with the stream and replicate (related to each sampling site) as random factor (Zuur et al., 2009). To avoid growth effects, we previously normalised the lipid content to the gammarids' dry weight (µg/mg gammarid) (Zubrod et al., 2011). For the *Gammarus* spp. experimental run, we constructed similar models using the *G. fossarum* abundance instead of the site type as fixed predictor (categorical, two levels: ≥80% = high, ≤50% = low; Table C.2).

4.4 RESULTS

4.4.1 The pesticide tolerance of gammarids in refuge, edge and agricultural sites

The mortality of *Gammarus* spp. was explained by "Concentration" (GLMM, $p < 0.001$; Table C.6,7) and by the factor "Site type" (GLMM, $p = 0.02$; Table C.6,7), where agricultural sites exhibited higher mortality than edge and refuge sites (Figure 4.1 A; pairwise differences: agriculture – refuge: $p = 0.03$, agriculture – edge: $p = 0.07$, edge – refuge: $p = 0.93$). The estimated LC_{50} was 492, 439 and 224 µg/L for refuge, edge and agricultural sites, respectively (Table C.8). The relative *G. fossarum* abundance was high (≥ 80%) at all refuge and edge sites, however, it was low (≤ 50%) at four of the six agricultural sites (Figure 4.1 C). The model including the relative abundance of *G. fossarum* as a covariate (Figure 4.1 B) indicated interspecific differences in tolerance (GLMM, $p = 0.005$; Table C.6,7). The immobilisation of *Gammarus* spp. was similar across all site types (GLMM, $p = 0.78$) and can be seen in the Appendix (Figure C.2; Table C.6,7).

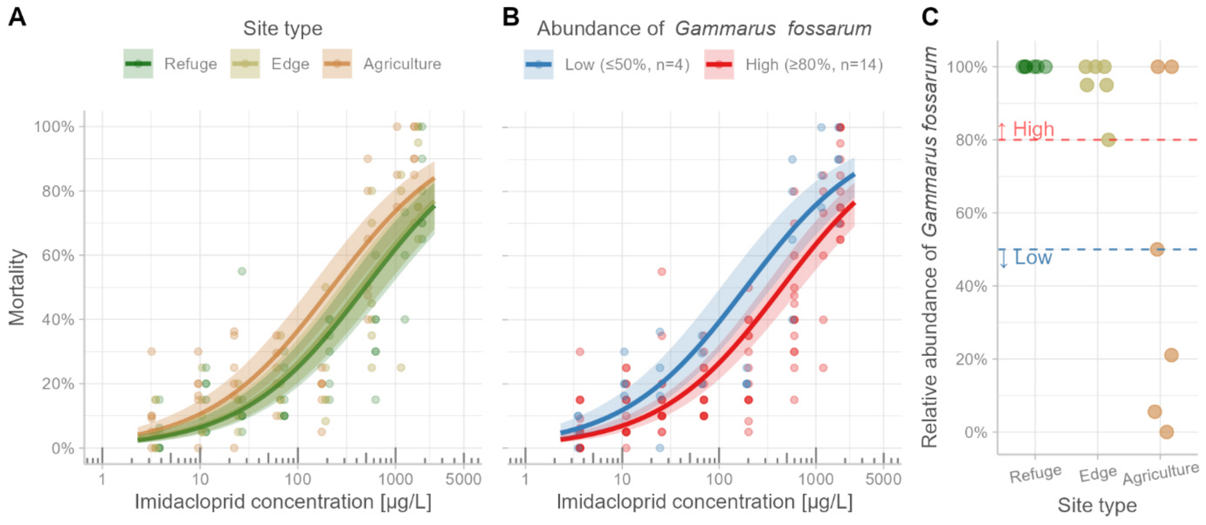

Figure 4.1: Concentration-response relationship visualised for the mortality of *Gammarus* spp. individuals after 96 h of exposure to imidacloprid, visualised for the tested concentration range (A, B; test concentrations were log-transformed with half of the lowest non-zero test concentration added to avoid infinite numbers) and the relative abundance of *Gammarus fossarum* per site type (C). Each coloured dot represents the mean of replicates per concentration and sampling site (with different degrees of transparency for overlapping dots). The lines represent the average estimate of mortality per exposure concentration for a typical stream with the shadowed areas indicating the 95 % confidence intervals (uncertainty of random effects not taken into account). Colours represent the site types (A, C) or the relative abundance of *G. fossarum* (B). Note that in (A) the line of the edge is slightly covered by the line of the refuge.

4.4.2 Population genetics of *Gammarus fossarum* populations from refuge, edge and agricultural sites

We generated 397 CO1 sequences for *Gammarus* spp. from 18 sampling sites, 69 for *G. pulex* (seven sampling sites) and 328 for *G. fossarum* (17 sampling sites). Quality was sufficient for 309 sequences of *G. fossarum* (for threshold definition see Text C.4) for further population structural analysis. In the 511 bp alignment (293 sequences, 16 removed due to insufficient length) for *G. fossarum*, we detected 18 variable sites, all of which were synonymous substitutions (Table C.9). The individuals were clustered into 15 haplotypes, five of which had a frequency below 1% (Table C.9). The most common haplotype was found among 24.6% of individuals belonging to 6 sampling sites (Table C.9,10). At 12 of the 17 sampling sites, populations exhibited only one or two haplotypes (Table C.10, Figure 4.2), whereas the refuge and agricultural site of one stream (Triefenbach) exhibited seven haplotypes (Table C.10, Figure 4.2). The distribution of haplotypes was comparable across refuge, edge and agricultural sites, as indicated by the haplotype network (Figure 4.3). Note that H5 and H4 have been found at edge and refuge of Kropsbach where no samples could be taken at agricultural sites due to the absence of *G. fossarum*. The genetic diversity of *G. fossarum* populations measured as haplotype- and nucleotide diversity was similar across site types (Figure 4.4; LMM, factor site type not significant at $p = 0.4$; Table C.11,12). F_{ST} values ranged from 0 - 0.2 among site types within the same stream and from 0.43 - 1 among sites of different streams (Figure C.3; Table C.13). The AMOVA revealed that most of the total variance in the CO1 gene was found among streams and no variance among site types (Table C.14). For *G. pulex*, the 67 sequences belonged to five streams and two site types (agriculture and edge). For a 352 bp alignment (61 sequences, six removed due to insufficient length), we detected no variable sites and all individuals clustered into one haplotype (Table C.15). Accordingly, there was no genetic diversity or distance among populations, site types and streams for the *G. pulex* individuals. A brief discussion of the preliminary data for *G. pulex* can be found in Appendix Text C.5.

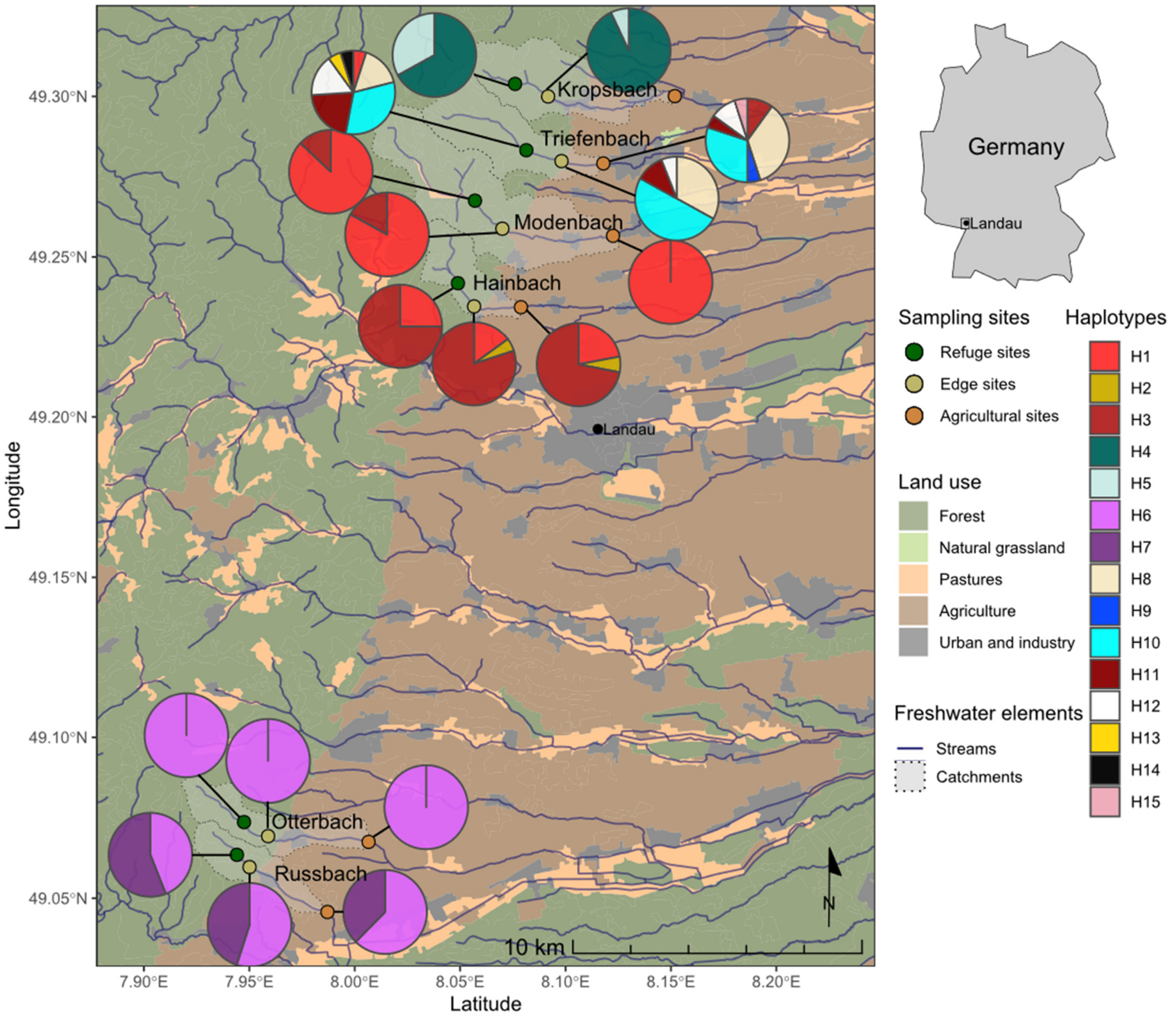

Figure 4.2: Overview of the sampling sites, i.e. refuge, edge and agricultural sites at the six streams and their catchments, in Rhineland-Palatinate, Germany, with different land use categories based on the CORINE land cover 2018 (Copernicus Land Monitoring Service, 2019). H1 to H15 refer to the haplotypes presented in Table C.10. The map was used from Schneeweiss et al. (2022) and slightly formatted.

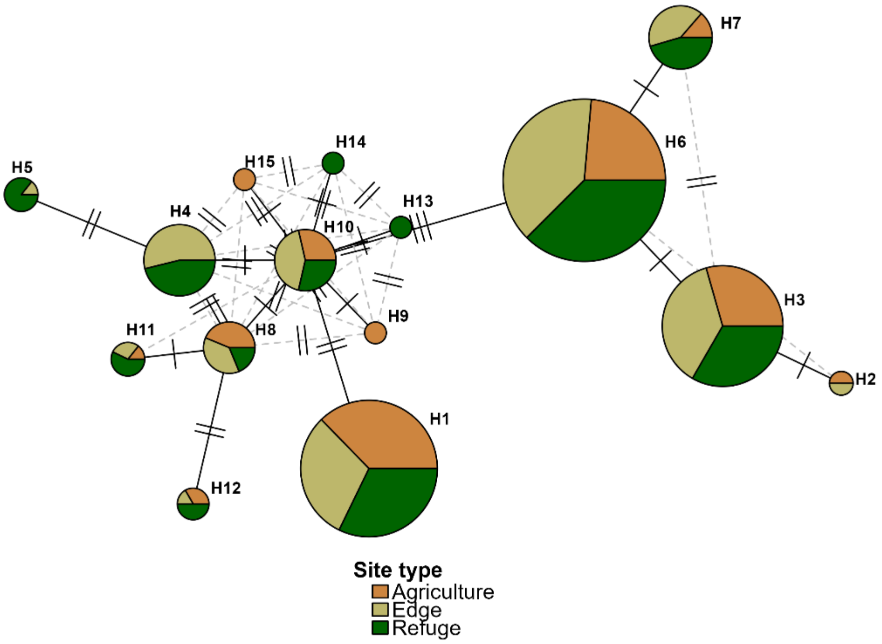

Figure 4.3: Minimum spanning network created from *Gammarus fossarum* CO1 sequences using the pegas package 1.1-2 (Paradis, 2010). The circles represent sampled haplotypes (H1-H15) and their dimensions are scaled based on the number of sequences given in Table C.9 and 10. Short vertical lines on the connecting lines between haplotypes represent mutations. Colours represent site types.

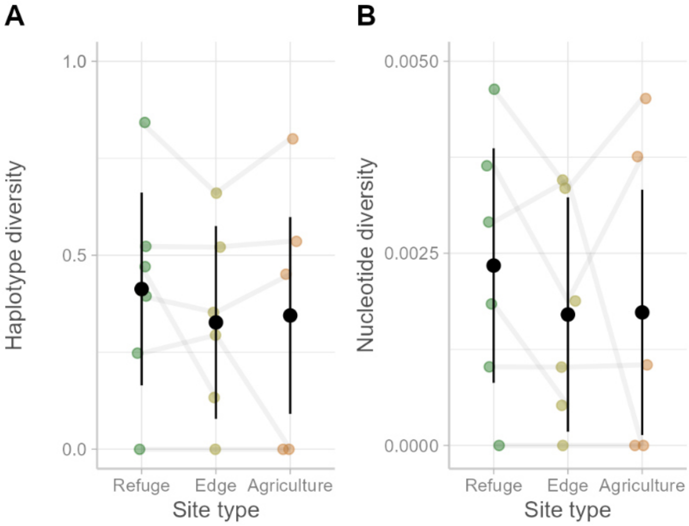

Figure 4.4: Dot plots (Wickham, 2016) visualising the haplotype- (A) and nucleotide diversity (B) of *Gammarus fossarum* populations. Each coloured dot represents a population sampled in each of the six streams, with the colours representing the site types. The black dots and ranges represent the predicted mean and 95 % confidence intervals, respectively. The factor site type was not significant at p= 0.4 (Table C.11, 12).

4.4.3 The energy reserves of gammarids at refuge, edge and agricultural sites

The energy reserves, in terms of average lipid content per mg gammarid tissue [µg/mg gammarid], decreased from refuge over edge to agriculture for *Gammarus* spp. (113, 101 and 88 µg/mg gammarid; Figure 4.5 A), although not significantly (LMM, p = 0.296; Table C.16,17). Similarly, for the model considering the relative abundance of *G. fossarum*, we observed a decrease in energy reserves from sites with high to sites with low *G. fossarum* abundance (106 and 82 µg/mg gammarid; Figure 4.5 B), albeit also statistically non-significant (LMM, p = 0.143; Table C.16,17). It should be noted that we have previously normalised the lipid content to the gammarids' dry weight (µg/mg gammarid) and that the dry weight itself was significantly related to the site type (LMM, p = 0.029; Table C.16,17) and even stronger to the relative abundance of *G. fossarum* (LMM, p = 0.003; Table C.16,17; separate models; Figure 4.5 C, D). Both the lipid content and dry weight were similar across site types for the second experimental run involving *G. fossarum* individuals only (LMM, factor site type statistically not significant at p = 0.302 and 0.712, respectively; Table C.16,17; Figure C.4).

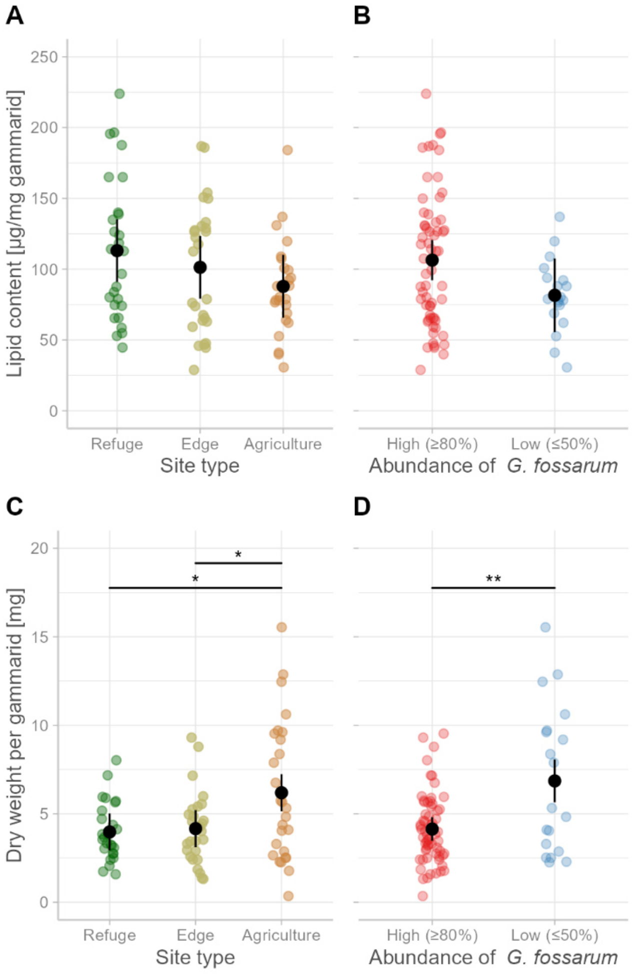

Figure 4.5: Dot plots (Wickham, 2016) visualising the total lipid content [µg] normalised by the dry weight per gammarid [mg] (A, B) and the dry weight per gammarid [mg] (C, D). Each coloured dot represents a replicate on the measurement subunit (n = 3-5 per sampling site) with the colours representing the site types (n = 3) and different degrees of transparency for overlapping dots. The black dots and ranges represent the predicted mean and 95% confidence intervals, respectively. The significance annotations “ * ” and “ ** “ refer to a p-value of < 0.05 and < 0.005, respectively.

4.5 DISCUSSION

4.5.1 Reduced pesticide tolerance and energy reserves of *Gammarus* spp. at agricultural sites

We hypothesised that pesticide exposure sets off local adaptation processes in *Gammarus* spp. observable as increasing pesticide tolerance (hypothesis 1) and decreasing energy reserves (hypothesis 3) from refuge over edge to pesticide-polluted agricultural sites. By contrast, pesticide tolerance was significantly lower (around 2-fold) at agricultural sites and similar in refuge and edge sites. This result may be influenced or partly explained by interspecific differences in physiological sensitivity and body size differences between site types. Regarding interspecific differences, we found a mixture of three *Gammarus* species (*G. fossarum*, *G. pulex* and *G. roeselii)* for most of the downstream agricultural sites, whereas almost all edge and refuge sites were characterised by individuals of *G. fossarum* (Figure 4.1 C), reflecting a turnover in the gammarid community from up- to downstream consistent with the results of a previous study (Schneeweiss et al., 2022). As explained in the method chapter 4.3.2, the examination of pesticide tolerance at the genus level of *Gammarus* spp. permitted rapid handling of the living organisms and avoided stress for organisms through deeper taxonomic identification. We suggest that the results (next paragraph) and main conclusions (Chapter 4.6) hold irrespective of the testing at genus level. This is because, previous laboratory studies indicate higher tolerance of *G. pulex* compared to *G. fossarum* for imidacloprid (Nyman et al., 2014) and another insecticide (Adam et al., 2010), which would rather lead to a higher downstream tolerance. Studies with *G. roeselii* suggest a higher pollution tolerance compared to *G. pulex* (Feiner et al., 2015; Kinzelbach & Claus, 1977) and *G. fossarum* (Arambourou et al., 2019). However, individuals from sites with high relative *G. pulex* and *G. roeselii* abundances exhibited lower imidacloprid tolerance.

Regarding body size, individuals at agricultural sites were significantly heavier (Figure 4.5 C). This may be due to interspecific differences (*G. pulex* and *G. roeselii* are typically larger, consistent with our results see Figure 4.5 D), higher nutrient inputs and a higher temperature at agricultural sites (Schneeweiss et al., 2022; Švara et al., 2021). Again, tolerance rather increases with body size (Baas & Kooijman, 2015), whereas we observed a lower tolerance. Thus, the interspecific differences in physiological sensitivity and differences in body size may rather have partly masked the lower tolerance in the downstream sites. In other words, at downstream sites where only *G. fossarum* occurs, the tolerance might even be lower. Hence, the same ecological explanations and conclusions given below would apply.

The hypothesis 1 of higher tolerance at the agricultural sites is based on previous studies that found generally higher insecticide tolerance in freshwater invertebrate populations in polluted stream sections compared to populations in non-polluted sections (Becker et al., 2020; Becker & Liess, 2017; Shahid et al., 2018). According to a recent meta-analysis, however, field populations of the freshwater amphipod *G. pulex* exhibited particularly high tolerance in tests (1) when sampled populations were more than 3.3 km distant from the nearest refuge because of immigrating sensitive organisms, (2) when sampling occurred outside summer because of additional stress factors such as temperature during that time and (3) when the species diversity at the site of the sample population was low because of stronger inter- then intra-specific competition in more diverse sites (Becker et al., 2020). In the present study, the distance to forest was on average 3.1 km, sampling took place in summer with higher temperatures observed in agricultural sites and multiple closely related *Gammarus* species were present in agricultural sites, potentially contributing to the lower tolerance (more details on environmental conditions and species diversity in Schneeweiss et al., 2022). The underlying explanation is that additional stress factors require energy trade-offs to the benefit of defence processes and detriments of other processes (Sokolova, 2021) that lower the capability of an organism to deal with other stressors such as subsequent pesticide exposure (Ashauer et al., 2007; Russo et al., 2018; Siddique et al., 2021). Also other studies found a lower tolerance of gammarids in pesticide-polluted compared to non-polluted sites (Grethlein et al., 2022; Russo et al., 2018; Švara et al., 2021) potentially related to energy trade-offs in multiple stressor environments. Indeed, concerning energy reserves, we found slightly lower lipid content per mg tissue in gammarids at agricultural sites (Figure 4.5 A), albeit not statistically significant, possibly indicating decreased feeding activities of gammarids in response to pollution (Nyman et al., 2013) or a higher allocation of energy to defence mechanisms including detoxification (Sibly & Calow, 1986, p. 7). However, this decrease in energy reserves was not detected for *G. fossarum* alone (Figure C.4) and may therefore simply reflect a turnover in the gammarid community. Nevertheless, we highlight that *Gammarus* communities with *G. fossarum*, *G. pulex* and *G. roeselii* showed higher sensitivity in the acute toxicity tests than pure *G. fossarum* populations, which might partly be explained by multiple stressor effects in agricultural streams, especially in summer. However, further studies are required to confirm the role of gammarid community composition under multiple stressor effects.

Finally, given that we found no differences in the mortality response between edge and refuge populations which have been similar in the relative abundance of *Gammarus* species (*G. fossarum* +/- 100%), we suggest that acute effects potentially related to a multiple stressor environment have not propagated to the edge of least impacted upstream sections. At agricultural sites, the reduced tolerance may, however, have long-term consequences for the gammarid populations with potential effects on the community and ecosystem functions (Rumschlag et al., 2020; Schäfer et al., 2012).

4.5.2 Pollution gradient is not associated with *Gammarus fossarum* population structure

G. fossarum populations are genetically differentiated at the regional scale partitioned within the riverine network (F_{st}>0.5, Table C.13), but differentiation is much lower at the local scale within the same stream (F_{st}<0.15, Table C.13 and ANOVA, Table C.14), despite the strong gradient in pesticide pollution. This observation contrasts our second hypothesis (2a) that assumed small-scale population differentiation related to local pesticide pollution differences but is in line with the recent results for a single stream (Švara et al., 2021). In the study by *Švara et al.* (2021), no genetic differentiation was found between *G. pulex* populations from sites of the same stream with different levels of pollution, while populations from polluted sites were also more sensitive to imidacloprid. The authors concluded that populations are well-connected and homogeneous. The F_{st} pattern observed in the present study suggests much higher levels of gene flow within the streams than between streams. However, geographically close streams (e.g. Otterbach and Russbach or Hainbach and Modenbach) share some haplotypes (Figure 4.2), suggesting at least a low level of genetic exchange among neighbouring stream systems.

Eleven of the 15 haplotypes occur at two or even all three site types (Figure 4.3), and haplotypes which occur only in one site types were rare (n=4; Table C.9), supporting the view of hardly any local differentiation. In addition, the overall structure of the haplotype network, showing the most abundant and central haplotypes (H10 and H6) to occur in all site types, suggests that gene flow overrules local differentiation.

The pattern of strong population differentiation among rivers (versus low differentiation among river sections) likely reflects historical colonisation events by differentiated subpopulations originating from a diverse source population (river Rhine) or initial invasion of diverse populations but subsequent random genetic drift and bottleneck events resulting in loss of genetic variation over time. As such, 2018, the year preceding this study, was the warmest year on record in the study region and precipitation in the summer of 2018 was more than 50% below average (Ministerium für Klimaschutz, Umwelt, Energie und Mobilität & Rheinland-Pfalz Kompetenzzentrum für Klimawandelfolgen, 2023), resulting in many streams falling sporadically dry (personal observation). Sporadic drought events that might convey population bottlenecks are frequently reported for streams along the palatine forest (Grigoryan et al., 2010).

The application of molecular markers that provide a higher resolution, such as microsatellite DNA or single nucleotide polymorphism (SNP) analyses might have uncovered more information on small-scale local differentiation, however, they would not alter the overall pattern of larger differentiation between streams than among locations within a stream. Furthermore, similar patterns have been found in studies based on microsatellite DNA (e.g. Weiss & Leese, 2016; Westram et al., 2013), which unravelled population genetic structures reflecting the riverine network and colonisation history. On the other hand, some studies found genetic differentiation even at a local scale within the same stream (Alp et al., 2012; Inostroza et al., 2016; Švara et al., 2022; Weiss & Leese, 2016), and a weak correlation with anthropogenic organic pollutants (Švara et al., 2022). A recent study investigating a pollution gradient in one large river catchment including adjacent catchments observed local differences in the genetic structure of *G. fossarum* populations matching differences in insecticide sensitivity (Grethlein et al., 2022). However, these studies differed in terms of their geographic settings, e.g. larger distances and barriers such as weirs or dams between populations compared to *G. fossarum* populations in the present study.

Previous studies found no discrepancies between nuclear and mitochondrial markers (Copilaş-Ciocianu & Petrusek, 2015; Švara et al., 2021; Weiss & Leese, 2016), thus we expect no considerable different population structure if nuclear DNA information had been included. In addition, to the best of our knowledge, there are no known relevant differences in migratory behaviour among males and females of *G. fossarum*.

Given that stream systems are characterised by unidirectional water flow, downstream-biased gene flow in *Gammarus* is most likely (Alp et al., 2012; Morrissey & de Kerckhove, 2009). This may cause gene swamping of the downstream agricultural gene-pool with upstream (potentially maladapted) genotypes and hinder or mask local adaptation to pesticide pollution despite strong selection pressures (Lenormand, 2002; Švara et al., 2021). Notwithstanding, genotypes adapted to local pollution may occur at agricultural sites despite the observation of lower tolerance at these sites. For example, when adaptations are masked by acute pesticide effects or multiple stressors (Becker et al., 2020). Evidence of evolutionary adaptation requires evidence at multiple levels of complexity (Klerks et al., 2011) in addition to tolerance differences between populations. Testing the local occurrence of genotypes adapted to pesticide exposure is relevant, because gene flow may propagate adaptive potential (to pesticide pollution) from agricultural sites to upstream sites, given that our data on population structure suggest bidirectional gene flow (absence of many private haplotypes at either site type). Further, considerable upstream movements for gammarids have been documented (Meijering, 1977; Žganec et al., 2013). Thus, these "refuge" populations even if they never experienced significant pollution levels might be genetically and evolutionarily impacted by pollution (e.g. already pre-adapted). If locally adapted genotypes convey fitness deficiencies, the exchange could result in an overall reduction of fitness of refuge populations. Such processes might have strong implications for nature conservation. For instance, refuge populations, in either natural habitats or managed nature protection areas, cannot be considered pristine or completely pollution naive if gene flow with impacted populations exists.

4.5.3 Equal levels of genetic diversity in refuge, edge and agricultural *Gammarus fossarum* populations

In contrast to our second hypothesis (2b) and a previous study (Švara et al., 2022), we found no gradient of genetic diversity associated with the pollution gradient (high at refuge and low at agricultural sites). This supports our findings based on the population structure (Chapter 4.5.2), suggesting gene flow among site types, which in turn may mask the effects of natural selection (Bach & Dahllöf, 2012; Lenormand, 2002).

The alternative hypothesis compared to a "downstream decrease in genetic diversity" is a "downstream increase in intraspecific (neutral) genetic diversity (DIGD)" hypothesis. DIGD is a general spatial pattern of intraspecific diversity that has been shown for many taxa and was particularly pronounced for aquatic dispersers, due to processes such as downstream-biased dispersal or increases in habitat availability downstream (Alp et al., 2012; Alther et al., 2021; Blanchet et al., 2020; Paz-Vinas et al., 2015). Our data, however, reveal no DIGD pattern. The lack of such a gradient may indicate pesticide effects at the downstream sites, but also other explanations have been suggested such as unstable conditions and associated higher allopatric diversification potential of headwaters (e.g. through drought events; Grethlein et al., 2022; Múrria et al., 2013; Paz-Vinas et al., 2015; Weigand et al., 2020). In addition, the lack of a typical DIGD pattern may be explained by the absence of gene flow from further downstream sites (e.g. confluences) for *G. fossarum*, which can be assumed 1) given that the genetic structure revealed only low exchange among streams and 2) for *G. fossarum* the 200 m isobar is typically the lowest distribution limit in our region (Kinzelbach & Claus, 1977), which matches our observations (Table C.1) and suggests that our agricultural sites are at the very top of the *G. fossarum* population distributional range. Furthermore, DIGD patterns have mainly been shown for larger spatial scales (e.g. 20-40 km, Alp et al., 2012; > 50 km, Alther et al., 2021) than those reported here (3-4 km). Thus, we consider it likely that gene flow between site types at very small scales potentially dilutes or masks the effects of pollution-related local genetic differentiation and adaptation.

4.6 CONCLUSION

We found significantly lower pesticide tolerance of *Gammarus* spp. at agricultural sites compared to edge and refuge sites. Interspecific differences between site types may have partially masked the lower tolerance in the agricultural sites. We suggest that higher environmental stress at agricultural sites related to energy trade-offs partly explains this response. This is in line with our results on energy reserves, indicating slightly lower lipid content per mg gammarid tissue at agricultural sites. We found no differences in pesticide tolerance between edge and refuge populations and thus conclude that the (acute) stress response measured as reduced tolerance at agricultural sites has not propagated to the edge of least impacted upstream sections. Furthermore, we found no population genetic structures among *G. fossarum* subpopulations within a stream, but significant differentiation between streams. We conclude that high gene flow within each stream hindered population differentiation and resulted in similar (pre)adaptations to local stress levels between site types, although they exhibited different levels of pesticide pollution. Our data support the hypothesis, that populations from locally polluted sites are in exchange with populations in least impacted sites. If locally adapted genotypes are associated with fitness deficiencies, the exchange could cause a reduction in fitness of naive populations and thus may undermine the positive effects of refuges.

In the future, studies are required to measure the fitness costs of genotypes adapted to various pollution levels under pristine conditions (either in experiments or field manipulations, i.e. reciprocal translocation). In addition, the propagation of pollutant effects to adjacent least impacted habitats should be tested for taxa with aerial dispersal such as Trichopterans.

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5 GENERAL DISCUSSION

5.1 CROSSING THE BARRIERS BETWEEN BIOLOGICAL LEVELS

The conceptual study of Chapter 2 shows that approaches and tools currently used to predict chemical effects tend to focus on different biological levels (i.e. receptor, cell, organ, organism, population, community, food web, ecosystem) and are associated with distinct scientific concepts and communities. The study accordingly attributes these approaches and tools to three idealised perspectives, the suborganismal, organismal and ecological perspective and suggests potential links between these perspectives. Linking the three perspectives can enhance the mechanistic understanding of the complete phenomenon of chemical effects, which extends from a chemical's uptake into an organism and its transformation, over the triggering of biochemical reactions inside the organism to physiological effects and the propagation to the population, community, food web and ecosystem level (Figure 2.1). The suggestions of Chapter 2 are based on and in line with previous studies that have underscored the importance of increasing mechanistic understanding and more ecological realism in chemical risk assessment (Beketov & Liess, 2012; Clements, 2000; Desforges et al., 2022; Forbes et al., 2017; Forbes & Galic, 2016; Murphy et al., 2018a,b). Previous studies have proposed approaches and concepts to link biological levels, such as quantitative in vitro to in vivo relationships (Bell et al., 2020; Wetmore, 2015) or incorporating adverse outcome pathways (AOPs) in bioenergetic models (Murphy et al., 2018a,b). Generally, mechanistic effect and process-based models are receiving more and more attention in ecotoxicological research and chemical regulation as these enable prediction of effects across different biological levels (Desforges et al., 2022; EFSA et al., 2018; Chapter 2.4). However, these previous proposals have largely focused on parts of the complete phenomenon of chemical effects, whereas ideally a comprehensive framework would capture the entire phenomenon (Chapter 2). Recently, a conceptual framework to predict the effects on prioritised ecosystem services from (sub)organismal responses to chemicals has been suggested (Forbes et al., 2017; Forbes & Galic, 2016), but a unified, comprehensive and mechanistically based framework (and modelling approach) is still missing. The results of Chapter 2 can further serve as a stepping stone towards the development of such overarching frameworks that capture the entire phenomenon of chemical effects, thereby fostering chemical effect prediction in ecosystems and contributing valuable information for chemical risk assessment.

In this context, environmental monitoring data can provide valuable information for the calibration and validation of ecotoxicological models, by ensuring that ecological reality is captured (Desforges et al., 2022). Moreover, measuring multiple metrics (at different biological levels) in field studies can shed light on linkages across levels. In the combined field- and laboratory study of Chapter 3 and 4, this thesis presents results of different metrics on the organism, population and community level along a land use gradient (i.e. site types: refuge, edge and agriculture) in the field. For instance, on the community level, Chapter 3 demonstrates that a rather increasing trend from refuge over edge to agriculture in taxonomic richness contrasted with the decreasing trend in the relative abundance of pesticide-sensitive species ($\mathrm{SPEAR}_{\mathrm{pesticides}}$). Only when considered together do these results suggest a species turnover rather than a species loss at agricultural sites (details in Chapter 3.5.2). Turnovers, if associated with the loss of functional diversity, can affect ecosystem functioning and stability (Cadotte et al., 2011), threatening nature's contribution to human well-being (Díaz et al., 2019). Such a link between taxonomic and functional metrics, is a well-known example used to establish relationships between different biological levels such as biodiversity and ecosystem functioning (Cadotte et al., 2011; Dornelas et al., 2019; Schäfer et al., 2012).

Another attempt to link different biological levels in field studies is the exploration of spatial concordance of patterns in genetic diversity (population level) and species diversity (community level) (Finn & Poff, 2011). Although it must be considered that underlying selective or neutral processes may vary between population and community levels, also concerning spatial and temporal scales (e.g. genetic and ecological drift; see Chapter 5.2 and Finn & Poff, 2011). This thesis presents spatial patterns in genetic (based on mitochondrial DNA, Chapter 4) and species diversity (community metrics, Chapter 3). Both patterns reveal different information concerning the pollution signal and organism flows. The species turnover in Chapter 3 suggests that dispersal rates between site types are insufficient to compensate the pollution effect on the community (Heino et al., 2021). At the population level, the absence of associations between genetic structure and the land use gradient suggests high gene flow between site types within a stream, masking or diluting the pollution signal (Chapter 4). Former studies emphasised that population and community level metrics, when considered independently, can be insufficient to draw representative conclusions (Dornelas et al., 2019; Supp & Ernest, 2014).

In Chapter 4, the simultaneous consideration of a) the imidacloprid tolerance (metric at the organism/population level), b) the lipid content of Gammarids, as an indicator of long-term energy storage (metric at the organism level) and c) the genetic structure (as well as diversity; metrics at population level), indicated an acute response due to energy trade-offs in multiple stressor environments at the agricultural sites (for details see Chapter 4). As discussed in Chapter 2, the lipid content and other analyses of bioenergetics have been repeatedly proposed as common currencies across biological levels, mainly because generic energy flow rules apply universally and because of its fundamental role in regulating structural and functional responses at all levels (Fischer et al., 2013; Forbes et al., 2017; Forbes & Galic, 2016; Segner et al., 2014; Sokolova, 2021). For example, recently an intriguing bioenergetic-AOP framework has been proposed to quantitively link energetic responses across biological levels (Goodchild et al., 2018). Thereby, correlations have been proposed between cellular energy allocation and whole-organism growth, metabolic rate and the scope for growth as well as with the non-traditional response of locomotion, enabling the incorporation of suborganismal insights into bioenergetic models (Goodchild et al., 2018). Such relationships may help to assess if and how lower-level responses (e.g. suborganismal, organismal) manifest themselves in higher-level responses (e.g. population, community, ecosystem). Further relationships, preferably causal and quantitative, between energetic responses from different biological levels could strengthen predictive ecotoxicology (Chapter 2).

With respect to experimental studies, nested designs with experimental subunits from different biological levels may help to establish such relationships (Chapter 2). To illustrate this, multi-species mesocosm experiments may be extended to contain single-species subunits to quantify chemical effects in the absence of interspecific interactions (Figure 2.6). In the future, more complex experiments and field studies (e.g. such as presented in Chapter 3 and 4) are required to simultaneously study responses to chemicals at different biological levels, and test metrics measurable at various biological levels for their potential to integrate across levels (Figure 2.6, details in Chapter 2.7). To sum up, in the words of Clements (2000) "[…] there is no single spatiotemporal scale or level of biological organisation at which ecotoxicological investigations should be conducted." The spatiotemporal scale of ecotoxicological studies is discussed in the next chapter.

5.2 CONSIDERING SPATIOTEMPORAL SCALES

Prediction and measurement of chemical effects can encompass various scopes, which include biological levels but also spatial and temporal scales. The three idealised perspectives emphasise distinct biological levels, which usually imply particular spatial and temporal scales (Chapter 2, Figure 2.5).

Higher biological levels typically cover larger and longer scales than lower biological levels (details in Chapter 2.6; Figure 2.5). Traditional ecotoxicological studies at the (sub)organism level but also single- or multi-species micro-/mesocosm studies are usually of short duration (few hours to days) and spatially restrictive thereby largely ignoring ecological processes that underly community dynamics (e.g. environmental or biotic selection, dispersal, ecological drift, speciation) or evolutionary processes at the population level (e.g. natural selection, gene flow, genetic drift and mutation) (Govaert et al., 2021; Orr et al., 2021; Schäfer et al., 2023; Schiesari et al., 2018; Streib et al., 2022). In line with a former study (Govaert et al., 2021), Chapter 2 underlines the importance of considering eco-evolutionary dynamics at appropriate spatiotemporal scales in theory, experiments and prediction, as these processes can considerably moderate biological responses. The incorporation may enable to shift from a static to a dynamic prediction framework (Chapter 2). Which spatiotemporal scale is appropriate for prediction or measurement could be determined in collaboration with experts from different backgrounds by prioritising the most critical processes in terms of the biological level, but also in terms of the organisms, chemicals and potential characteristics of the (eco)system under investigation (Chapter 2). This is because the spatiotemporal scale of a biological response also depends on the type of organism and chemical, adding to the challenge of predicting chemical effects (discussed in Chapter 5.3). Currently, ecotoxicological research is shifting towards increasingly advanced and data-rich methods also with respect to resolution in time and space. Promising study designs will increasingly enable measurements of effects by taking spatial and temporal dynamics into account (Altermatt et al., 2015; Fronhofer et al., 2018; Gilarranz et al., 2017; Govaert et al., 2021; Harvey et al., 2020), of which a few are illustrated in Chapter 2. The progress can improve the calibration and parametrisation of models used for predictions (Schneider, 2001). Finally, spatial and temporal dynamics can be further assessed retrospectively in the field, as realised in Chapters 3 and 4 of this thesis and discussed in Chapters 5.2.1 and 5.2.2.

5.2.1 The edge effect of pesticides: limitations and future challenges

In Chapters 3 and 4, we examined the edge effect of pesticides, thereby considering the importance of spatial (i.e. species dispersal and gene flow) and partly of temporal dynamics (i.e. tolerance as a prerequisite of adaptation) for the trajectory of populations and communities in ecosystems. In detail, we assessed the potential propagation of pesticides (exposure perspective) and their effects (effect perspective) from agricultural stream sections to the edge of upstream least impacted sites (Figure 3.1).

Concerning the exposure perspective, we found significantly higher pesticide concentrations and toxicity at agricultural compared to upstream least impacted sites (i.e. edge and refuge sites; Chapter 3). Notwithstanding, we detected occasionally ecologically relevant pesticide exposure at the least impacted sites. The results of Chapter 3 are in line with previous studies that reported pesticide exposure in least impacted (including protected) areas in Germany (Kruse-Plaß et al., 2020; Wolfram et al., 2023), and globally (e.g. Guida et al., 2018; Wang et al., 2019; Zhan et al., 2021). Pesticide exposure in least impacted habitats compromises the ecological quality of these habitats, which can serve as refuges for various species and buffer against pesticide effects in agricultural areas (Knillmann et al., 2018; Liess & Schulz, 1999; Orlinskiy et al., 2015).

Past or forest-related use was excluded as a source of contamination for the least impacted upstream sites (see discussion in Chapter 3.5.1). Given that we found a subset of downstream compounds at the upstream sites, we assumed that downstream agricultural land use was probably also the main source of contamination and concluded that regional atmospheric transport of pesticides may lead to this exposure in forested sections within a few kilometres upstream of agricultural areas (Figure 3.2). This would raise questions about the safety of pesticide application methods and the need for more effective risk mitigation methods during application. To further scrutinise sources of pesticide contamination in least impacted habitats, future studies should ideally focus on catchments with known agricultural and non-agricultural pesticide use and examine potential transport paths, which remained speculative in Chapter 3. For example, recent studies suggest that very low proportions of urban land use (<5%; Sandstrom et al., 2022) or agriculture (7%; Schreiner et al., 2021) in the catchment can already lead to pesticide contamination of streams.

Concerning the effect perspective, in Chapter 3 the tested indicators of community responses (Jaccard Index, taxonomic richness, total abundance, $\text{SPEAR}_{\text{pesticides}}$) together suggested a species turnover from upstream forested to downstream agricultural sites and a potential influence of adjacent agriculture on the edge sites. In Chapter 4, no particular edge effects, discriminating edge organisms and populations from those in more upstream sites, were found. For example, *Gammarus fossarum* populations at edges showed equal levels of imidacloprid tolerance, energy reserves as well as genetic diversity and differentiation to populations further upstream. The reduced imidacloprid tolerance at agricultural sites, interpreted as an acute stress response in multiple stressor environments, had not propagated to the edge of least impacted sections (Chapter 4). Nevertheless, the genetic population structure indicated bidirectional gene flow, thereby supporting the hypothesis that well-adapted genotypes - if present at locally polluted sites - may spread into populations at least impacted sites (details in Chapter 4.5.2). Taken together, Chapters 3 and 4 illustrate empirically for non-target species, that pesticide effects may potentially propagate from agricultural to least impacted upstream sections, for the first time to our knowledge. Propagated effects that are known from modelling studies (Schäfer et al., 2017; Spromberg et al., 1998; Willson & Hopkins, 2013), may consequently be of ecological relevance in real-world ecosystems. Similarly, edge effects that are known for terrestrial ecosystems (Magura et al., 2017; Ries et al., 2004; Wimp & Murphy, 2021), may need stronger consideration in freshwaters. For edge and refuge populations, the propagation of downstream pesticide effects may (genetically) be unfavourable and be related to reductions in fitness, potentially undermining the positive effect of edges and refuges. The results of Chapters 3 and 4 may help in predicting or explaining population and community dynamics in least impacted habitats and may ultimately inform pesticide management as well as the planning of freshwater restoration measures, thereby contributing to the conservation and protection of biodiversity. It is important to conduct further research on the propagation of pesticide effects to determine whether such effects may contribute to the insect and arthropod declines in least impacted habitats (Hallmann et al., 2017; Seibold et al., 2019).

Above all, the broader extent of edge effects needs to be tested and drivers disentangled. For example, the degree of connectivity between habitats affects the propagation of pesticides and their effects (Heino et al., 2021; Schäfer et al., 2023). Habitat connectivity is in turn determined by the dispersal capacity of organisms, the distance between habitats and the impact of the landscape structure on dispersal (Amarasekare, 2004; Schäfer et al., 2023; Streib et al., 2022). Regarding the distance between habitats, a metapopulation model demonstrated that depending on the distance between patches of toad populations the contamination of one patch can influence the extinction risk of populations in non-contaminated patches (Willson & Hopkins, 2013). Furthermore, the distance to and the length of least impacted habitats can affect recovery (Orlinskiy et al., 2015; Trekels et al., 2011), and may therefore also influence the propagation of pesticide effects. In Chapters 3 and 4, the distances between refuge, edge and agricultural sites varied slightly for the six streams and refuges were large in proportion to the agricultural land involved within the catchments (Figure 3.1; Table B1). Regarding the dispersal capacity of organisms, a metapopulation model focussing specifically on a merolimnic (aquatic and terrestrial life stages) insect with aerial dispersal, showed a significant carryover of pesticide effects from polluted to non-polluted upstream patches (Schäfer et al., 2017). Chapter 4 focuses on *Gammarus* spp., an aquatic disperser group constrained to movements in the riverine network. The propagation of effects may differ considerably for taxa with aerial dispersal. To sum up, future studies should investigate the influence of spatial relationships and species traits on the propagation of pesticide effects. The temporal scale should thereby receive more attention (Chapter 5.2.2).

5.2.2 The temporal scale of pesticide edge effects: limitations and future challenges

The temporal scope of studies can influence conclusions such as on the extent of propagated pesticides and their effects.

For instance, concerning the exposure perspective, in Chapter 3 of this thesis, we conclude that the applied weather independent sampling may underestimate the pesticide exposure in agricultural stream sections, but not in the least impacted sites, as runoff from pesticide-treated surfaces is a major route of rain-driven pesticide input (Leu et al., 2004; Liess et al., 1999; Szöcs et al., 2017), which probably did not contribute to the contamination of the least impacted upstream sites (for details see 3.4.1). Conversely, in a recent study with upstream agricultural activities, fluvial inputs mainly contributed to the contamination of several nature conservation areas in Germany (Wolfram et al., 2023). Under such topographic settings, rain-driven pesticide pulses would need to be captured in the least impacted areas through automated rain-driven sampling methods (Xing et al., 2013).

Concerning the effect perspective, long-term consequences of pesticide effects such as on the gene pool of populations potentially compromising population viability are still insufficiently understood in real-world ecosystems, though the field of evolutionary toxicology has been growing in previous years with promising tools (e.g. omics, eDNA, more details in Chapter 2) (Brady et al., 2017; Oziolor et al., 2017; Rusconi et al., 2018). An example of a process with potential long-term consequences that has rarely been considered within any of the perspectives outlined in Chapter 2, but can be relevant at all biological levels and may provide links between them, is evolutionary adaptation. In Chapter 4, we studied adaptive processes for *Gammarus* spp., and the potential of these to propagate from pesticide affected to adjacent least impacted populations. To this end, we assessed in the laboratory the tolerance (i.e. mortality) towards a single pesticide frequently occurring in the study region, namely imidacloprid. The approach has proven useful in numerous studies to investigate site-specific pesticide tolerance differences between populations (Becker & Liess, 2017; Grethlein et al., 2022; Shahid et al., 2018a; Švara et al., 2021). However, a meta-analysis recently examined under which environmental contexts gammarids adapt to pesticides (i.e. show increased tolerance; Becker et al., 2020). The analysis revealed that tolerance differences between gammarids from differently polluted sites were most pronounced in autumn or winter, at least for regions in Central Europe (Becker et al., 2020). This was due to organisms being relieved of the acute effects of the multiple stresses that occurred in summer. As shown by Becker et al. (2020), the time point of organism sampling may thus determine whether propagated pesticide effects, such as increased tolerance, can be detected. In Chapter 4, Gammarid sampling for tolerance tests was conducted in summer (June/July), with higher temperatures observed in agricultural sites, potentially representing an additional stress and explaining, at least partly, the decreased imidacloprid tolerance at agricultural sites (details in Chapter 4.5.1).

With respect to the general investigation of adaptation processes, assessing tolerance differences between populations remains only one of the presuppositions for local adaptation. Evidence of evolutionary adaptation mediated by natural selection requires evidence at multiple levels of complexity (Klerks et al., 2011). For instance, further studies on target genes (e.g. conferring pesticide tolerance), population structure and fitness of different phenotypes in particular among non-target organisms (e.g. in reciprocal translocation or common garden experiments; Piola & Johnston, 2006; Taddei et al., 2021) are needed for least impacted ecosystems to scrutinise the potential of (long-term) pesticide effects to propagate and understand the underlying mechanisms. As discussed in Chapter 2, advanced genomic techniques, such as restriction site-associated DNA sequencing (RADseq), shotgun population variation profiling (PoolSeq), transcriptome sequencing (RNAseq) or whole-genome resequencing, may help in the future to locate genetic sites of adaptation, even in non-model species where no prior genomic information exists (Weigand & Leese, 2018).

5.3 SPECIES TRAITS AND CHEMICAL PROPERTIES AS RELEVANT PREDICTORS

It has been previously emphasised that data on organism traits, which represent measurable characteristics of an individual such as dispersal ability or life history (cf. Violle et al., 2007), and chemical properties are relevant predictors for biological responses and can be used to aggregate effect results among groups of organisms or chemicals (Rumschlag et al., 2020).

Concerning different biological levels and traits, Chapter 2 emphasises that traits have been typically related to responses at the level of organisms or populations (van den Berg et al., 2021; Wiberg-Larsen et al., 2016), while suborganismal processes may also relate to organism traits (Gergs et al., 2015, 2019) and testing such relationships may enhance prediction (Chapter 2). Of particular interest are relationships with traits with a strong mechanistic link to chemical effects, such as the capacity for production of heat shock proteins or the content of energy storage molecules (e.g. lipid content in Chapter 4), average biomass or body size (Gergs et al., 2015). Such traits have rarely been measured for many organism groups that are particularly at risk from chemical effects such as invertebrates or fungi (Rubach et al., 2011).

Concerning spatiotemporal scales and traits, the trait-based approach may need to be recognized more flexibly in the future (Chapter 2). Today, traits are largely considered static, but traits can vary throughout a species' lifetime (Lancaster & Downes, 2010), the average trait of a population can change with environmental conditions (e.g. adaptation; Dinh et al., 2016; Shahid et al., 2018b) and the biotic selection and dispersal are usually not fully accounted for (Cadotte & Tucker, 2017). With respect to the edge effect of pesticides, Chapter 5.2.1 highlights the potential differences for species with aquatic or aerial dispersal and the need to study these in the field.

Ultimately, the challenge of future ecotoxicology is to reduce the uncertainty in measuring and predicting chemical effects in ecosystems while increasing their ecological relevance, taking into account the feasibility of data collection and the complexity of models (Chapter 2). The aggregation of effect results among groups of organisms or chemicals can help to keep the effort in data acquisition manageable, reduce the complexity of the prediction process and generalise results (Rumschlag et al., 2020; Chapter 2). To improve prediction, effects need therefore be increasingly tested for relationships with chemical properties and traits of organisms (Figure 2.6). For example, within the subunits of the nested systems mentioned in Chapter 5.1, species could be selected to cover a wide range of traits, fostering cross-species extrapolation based on traits (van den Berg et al., 2021; Chapter 2).

5.4 OUTLOOK: DIVERSE APPROACHES FOR DIVERSE STRESSORS

The present thesis incorporates approaches at different levels of biological organisation and considers spatiotemporal scales concerning both the prediction and measurement of effects of chemicals. Uniting researchers from different disciplines such as ecotoxicology and evolutionary biology enabled a more holistic view of the effects of chemicals in ecosystems regarding both the prediction and measurement chapters (e.g. genetics in landscape ecotoxicology). This is a promising first step, but more needs to be done to really understand and ultimately predict the effects of chemicals in real-world ecosystems.

To further develop the prediction and measurement of chemical effects in terms of relevance to real-world ecosystems, it is necessary to further understand the diversity of approaches and tools available in different research communities or disciplines to ultimately identify the potential for integration. This may include examining and sharing each other's research paradigms, topics, journals or conferences and work across disciplines (Forbes et al., 2020; Orr et al., 2020; Schäfer et al., 2016, 2023). Such overarching cross-disciplinary work is particularly relevant with respect to one of the biggest future challenges for ecotoxicological and ecological research, namely that multiple anthropogenic stressors, including climate change, are the new norm in real-world ecosystems (Pirotta et al., 2022; Schäfer et al., 2023). Multiple stressors are also suggested to drive the decline in arthropod biodiversity (Sánchez-Bayo & Wyckhuys, 2019), and disentangling their influence and potential interactions is crucial to enable effective mitigation measures (Schäfer et al., 2023). Notwithstanding, as recently stated "the response need not wait for full resolution" (Forister et al., 2019).

Today it is certain that chemicals, and pesticides in particular, play their part in the widespread decline of arthropod diversity (Beketov et al., 2013; Hallmann et al., 2017; Liess et al., 2021; Sánchez-Bayo & Wyckhuys, 2019). In other words, if we aim at protecting biodiversity and ecosystem integrity, the chemical crisis - as set out in the introduction of this thesis - requires urgently effective action at various geographical and political levels, even if the scientific evidence about ecosystem effects of a large number of chemicals is not definitive (Brack et al., 2022; Cousins et al., 2016; Forister et al., 2019; Persson et al., 2022; Schäfer et al., 2019).

As a first step, chemical risk assessment procedures need refinement to better assess chemical effects at the ecosystem level, including exposure to multiple chemicals as well as potential interactions with climate change and other stressors and reduce the threat of regrettable substitutions (EEA, 2023a; Schäfer et al., 2019). Furthermore, an improved public access to chemical use and application data is essential to understand and manage risks (EEA, 2023a).

Regarding pesticides, binding rules at European and national level for measures to reduce uses and to apply integrated pest management are a key to mitigate risks for non-target ecosystems (EEA, 2023a). This requires support, such as training and incentives for farmers to use integrated pest management, high-precision pesticide application technologies, appropriately sized and intact buffer strips, crop rotations and features on arable land (EEA, 2023a, 2023b; Kreuger & Nilsson, 2001; Stehle et al., 2016; Verheggen et al., 2022). Another instrument to reduce the use of pesticides can be the introduction of high tax rates for environmentally more harmful pesticides (Nielsen et al., 2023). A study analysing data from almost 1000 non-organic arable commercial farms showed that the application quantity of pesticides can be reduced by approximately half while preserving crop yields for the majority of farms (Lechenet et al., 2017).

To date, reconciling the use of chemicals while meeting the needs of the growing world population with environmental protection remains one of the most ambitious challenges for humanity to secure the lives of current and future generations.

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APPENDIX

A - SUPPLEMENTARY INFORMATION FOR THE MANUSCRIPT "THREE PERSPECTIVES ON THE PREDICTION OF CHEMICAL EFFECTS IN ECOSYSTEMS"

Text A.1: Scenario-based guide to prediction

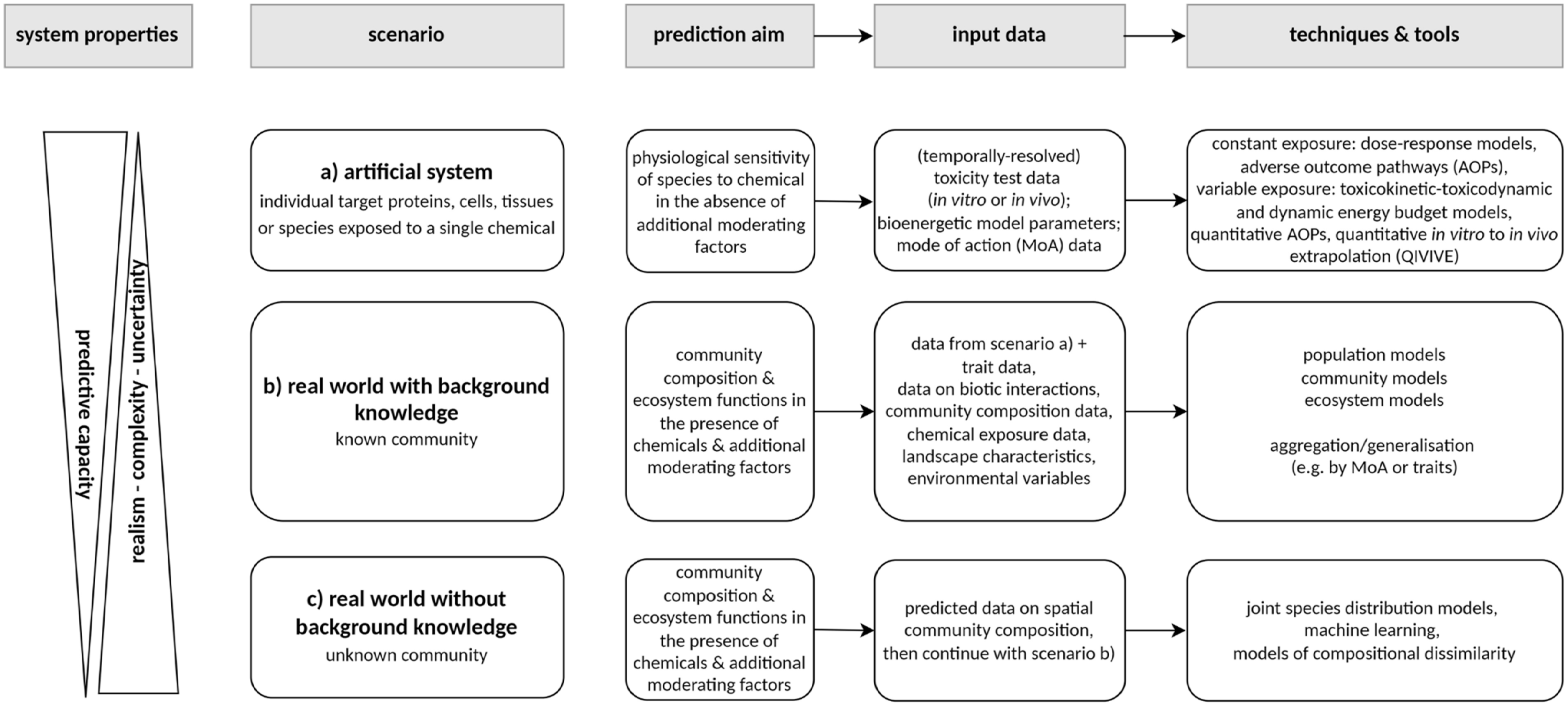
Here, we define three exemplary scenarios with associated prediction aims and outline required input data as well as tools and techniques for prediction (Figure A.1).

The first scenario describes the exposure of a single species to single chemicals in the laboratory typically in the absence of additional stressors (e.g. sufficient food, optimal temperature). This scenario is common in single species tests for chemical risk assessment and for determining the tolerance of a species in isolation (Cadotte & Tucker, 2017). If exposure concentrations fluctuate, the experimental data can be used to fit bioenergetic models. If correctly parameterised, such a model can subsequently be used to predict the effect of a range of exposure conditions. In the case of constant exposure, also concentration-response models can be used to predict the same response. Concerning data from suborganismal bioassays, sound *in vitro* to *in vivo* extrapolation methods that are rooted in quantitative adverse outcome pathways (AOPs) can be used to translate *in vitro* concentration-response relationships to equivalent *in vivo* effects (Villeneuve et al., 2019). Such models exist so far mainly for extrapolation of effects measured in human cell lines (Wetmore, 2015) but the concept can be principally expanded to environmental endpoints. This is the least complex scenario, having high predictive capacity, however coupled with a high degree of uncertainty when the aim is to estimate ecosystem effects of chemicals (Figure A.1).

The second scenario describes the response of a community and associated ecosystem functions under real world environmental conditions with a known community (Figure A.1). Such a scenario captures an accidental chemical spill in ecosystems with or without prior exposure as well as mesocosm experiments. Here data on the organism response (e.g. resulting from first scenario) needs to be extended in time (e.g. data from life cycle tests) or by information on life-history traits and intraspecific interactions (e.g. competition) to allow for prediction on the population level. Effects of chemicals on populations can then be estimated through population models such as ordinary differential equation models, structured demographic (life-history) models or individual-based models (Kramer et al., 2011; Martin et al., 2014; Murphy et al., 2018a,b). Population models can also partially address community-level effects (see Kramer et al., 2011). Community models need to incorporate biotic interactions (Ovaskainen & Abrego, 2020), trait information, environmental variables and physiological sensitivity to predict effects on the ecosystem (examples in Chapter 2.4). Theoretically, a community model becomes an ecosystem model if biogeochemical dynamics are explicitly modelled (e.g. Leroux & Loreau, 2012). However, for the prediction of the fate and effects of chemicals in ecosystems, many other environmental factors (e.g. climatic, geological) and their dynamics can be relevant that are typically ignored in community models. Moreover, the spatial dimension of an ecosystem influences the fate and effects of chemicals, for example through metacommunity dynamics (Schiesari et al., 2018). The compartments of ecosystem models often aggregate taxa into functional groups (e.g. primary consumers, secondary consumers), which reduces model complexity. However, this may mask community turnover by selection processes in response to chemicals (e.g. pollution induced community tolerance) because functional groups are usually less sensitive than the most sensitive species in the community (Blanck et al., 1988; Tlili et al., 2016). Besides, trait and MoA data can be used to aggregate results among groups of organisms or chemicals, helping to reduce the complexity of the prediction process and to generalise results (Rumschlag et al., 2020). To be useful, ecosystem models need to prioritise and incorporate the processes that are most critical in light of a prediction aim, which should include a definition of biological level and spatiotemporal scale. The prediction aim will determine how much data is required and both, the prediction aim and data availability, will determine the choice of the model (Forbes et al., 2017; Forbes & Galic, 2016). For example, ecological risk assessment may focus on the prediction of effects on prioritised ecosystem services, which contributes to model selection (Forbes et al., 2017). An example of a model that evaluates the effects of chemicals and other stressors on the aquatic ecosystem and allows for different levels of complexity is the AQUATOX model (Park et al., 2008).

The third scenario reflects probably the most frequent risk assessment situation involving chemical contamination of an ecosystem where the community is unknown (Figure A.1). Here, the first step is to predict the (typical) community composition by using methods, such as joint species distribution models, machine learning or models of compositional dissimilarity (D'Amen et al., 2017; Ferrier & Guisan, 2006). Subsequently, community and ecosystem effects can be predicted using approaches of the second scenario.

Figure A.1 : Flowchart linking environmental scenarios and associated prediction aims to tools and techniques based on input data.



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B - SUPPLEMENTARY INFORMATION FOR THE MANUSCRIPT "POTENTIAL PROPAGATION OF AGRICULTURAL PESTICIDE EXPOSURE AND EFFECTS TO UPSTREAM SECTIONS IN A BIOSPHERE RESERVE"

Table B.1.: Distances and elevation differences in meter between sampling sites.

Stream	**Agriculture to Edge**		**Edge to Refuge**	
	Distance [m]	**Elevation [m]**	**Distance [m]**	**Elevation [m]**
Hainbach	1550	63	1170	44
Kropsbach	4840	145	1180	79
Modenbach	4080	60	1460	43
Otterbach	3620	77	1100	14
Russbach	3210	88	640	11
Triefenbach	1440	31	1380	67

Table B.2: Keys used to morphologically identify macroinvertebrates.

Taxonomic group	**Key**
All	(Schmedtje and Kohmann, 1992)
Amphipoda	(Eggers and Martens, 2001)
Diptera	(Sundermann and Lohse, 2006)
Ephemeroptera	(Bauernfeind et al., 2001)
Mollusca	(Glöer, 2017)
Odonata	(Heidemann and Seidenbusch, 2002)
Plecoptera	(Zwick, 2003)
Trichoptera	(Waringer and Graf, 2011)

Table B.3.: Proportion [%] of Surber kick-samples taken during macroinvertebrate sampling per substrate type within a stream section of 25 m per site type (A-Agriculture, E-Edge, R-Refuge).

Substrate type	**Hainbach**			**Kropsbach**			**Modenbach**			**Otterbach**			**Russbach**			**Triefenbach**		
	A	**E**	**R**	**A**	**E**	**R**	**A**	**E**	**R**	**A**	**E**	**R**	**A**	**E**	**R**	**A**	**E**	**R**
Akal	20	20	0	0	20	0	30	0	0	30	0	20	0	0	40	10	20	20
Argyllal	20	20	0	0	0	0	0	0	0	20	40	0	20	40	20	30	0	0
CPOM	0	0	20	10	20	0	10	20	10	10	40	0	20	20	20	0	0	20
FPOM	0	0	0	0	0	0	0	0	0	0	0	0	0	20	20	0	0	0
Living parts of terrestrial plants	0	0	0	20	0	0	10	0	0	10	0	20	0	0	0	20	20	0
Makrolithal	0	0	0	0	0	40	0	0	0	0	20	0	10	0	0	0	0	0
Megalithal	10	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mesolithal	0	0	0	40	20	40	0	0	10	0	0	20	0	0	0	0	0	0
Mikrolithal	0	0	0	20	0	0	0	0	20	0	0	0	0	0	0	0	0	20
Psammal	30	60	60	0	40	20	50	80	50	30	0	40	50	20	0	40	60	40
Submersed Makrophytes	10	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0
Technolithal 1	10	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table B.4.: Information on analysed pesticides including CAS, minimum and maximum detected concentration in µg/L per site type for the June sampling, EC50 values with corresponding references and maxTU per site type for the June sampling. See supporting information excel file (available at https://doi.org/10.1016/j.scitotenv.2022.155688).

Table B.5.: Information on measured physicochemical habitat properties per site type, including stream width, stream depth, flow velocity, water temperature, dissolved oxygen, conductivity, pH and nutrient concentrations. See supporting information excel file (available at https://doi.org/10.1016/j.scitotenv.2022.155688).

Table B.6.: Information on site-specific pesticide exposure, including number of detected pesticides, total concentration as well as sumTU, and community metrics, including taxonomic richness, total abundance and $\text{SPEAR}_{\text{pesticides}}$ indicator. See supporting information excel file (available at https://doi.org/10.1016/j.scitotenv.2022.155688).

Table B.7.: Information on the site-specific community metric Jaccard index. See supporting information excel file (available at https://doi.org/10.1016/j.scitotenv.2022.155688).

Table B.8.: Descriptive statistics for number of detected pesticides, total concentration, sumTU, maxTU and for the different community metrics per site type (only June samples included).

Variable [unit]	Site type	Minimum	Maximum	Median	Mean	SD
Number of detected pesticides	Refuge	3	9	5.50	5.50	2.07
	Edge	2	11	6.50	6.33	2.94
	Agriculture	11	38	19.00	22.33	11.06
Total concentration [ng/L]	Refuge	0.53	3.02	1.47	1.64	0.97
	Edge	0.39	6.34	2.28	2.73	2.12
	Agriculture	24.21	1465.57	93.31	391.42	571.66
sumTU freshwater invertebrates	Refuge	-6.97	-2.79	-4.78	-4.80	1.77
	Edge	-7.04	-2.74	-5.69	-5.07	1.76
	Agriculture	-3.73	-0.88	-1.93	-2.07	1.10
maxTU freshwater invertebrates	Refuge	-7.04	-2.79	-4.80	-4.83	1.80
	Edge	-7.09	-2.74	-5.85	-5.18	1.83
	Agriculture	-3.79	-1.00	-2.08	-2.19	1.07
Taxonomic richness [nr of taxa]	Refuge	26	53	32.00	34.83	10.11
	Edge	36	49	36.50	38.67	5.13
	Agriculture	30	47	41.50	40.50	5.61
Total abundance [individuals/m²]	Refuge	1190	12715	3647	5807	5201
	Edge	1483	23635	5458	7623	8005
	Agriculture	1121	41117	5961	12261	14961
Jaccard index	Agriculture_Edge	0.26	0.65	0.36	0.40	0.13
	Agriculture_Refuge	0.26	0.42	0.33	0.33	0.05
	Edge_Refuge	0.40	0.59	0.48	0.49	0.07
$\text{SPEAR}_{\text{pesticides}}$	Refuge	0.29	0.92	0.72	0.64	0.24
	Edge	0.45	0.69	0.62	0.59	0.09
	Agriculture	0.23	0.51	0.33	0.34	0.10

Table B.9.: Model outputs of separate full models for sumTU, taxonomic richness, total abundance, Jaccard index and $\text{SPEAR}_{\text{pesticides}}$ as response to site type as well as the model output for $\text{SPEAR}_{\text{pesticides}}$ as response to sumTU and site type (without interaction term). Linear mixed models (LMM) were applied including stream as random factor. For the table outputs of LMM summaries, the sjPlot package 2.8.10 was used (Lüdecke, 2021), which calculates marginal and conditional R-squared statistics based on Nakagawa et al. (2017).

	sumTU			**Taxonomic richness**			**Log_{10}(Total abundance)**			**Jaccard index**			**$\text{SPEAR}_{\text{pesticides}}$**			**$\text{SPEAR}_{\text{pesticides}}$**		
Predictors	*Estimates*	*SE*	*p*	*Estimates*	*SE*	*p*	*Estimates*	*SE*	*p*	*Estimates*	*SE*	*p*	*Estimates*	*SE*	*p*	*Estimates*	*SE*	*p*
(Intercept)	-2.07	0.64	**0.006**	40.50	2.98	**<0.001**	3.83	0.19	**<0.001**	0.40	0.04	**<0.001**	0.34	0.07	**<0.001**	0.35	0.09	**0.001**
Edge	-3.00	0.91	**0.008**	-1.83	3.77	0.637	-0.10	0.25	0.693				0.25	0.07	**0.006**	0.27	0.11	**0.035**
Refuge	-2.73	0.91	**0.013**	-5.67	3.77	0.164	-0.23	0.25	0.371				0.29	0.07	**0.002**	0.31	0.11	**0.015**
Agric-refuge										-0.07	0.05	0.180						
Edge-refuge										0.09	0.05	0.095						
sumTU																0.00	0.03	0.856
Random Effects																		
σ^2	2.48			42.63			0.19			0.01			0.02			0.02		
τ_{00}	0.00_{stream}			10.68_{stream}			0.02_{stream}			0.00_{stream}			0.01_{stream}			0.01_{stream}		
ICC	0.00			0.20			0.09			0.23			0.38			0.35		
N	6_{stream}			6_{stream}			6_{stream}			6_{stream}			6_{stream}			6_{stream}		
Observations	18			18			18			18			18			18		
Marginal R^2 / Conditional R^2	0.439 / NA			0.100 / 0.280			0.045/ 0.131			0.331 / 0.482			0.409 / 0.632			0.398 / 0.611		

Table B.10.: Type III analysis of variance table with Kenward-Roger's method for linear mixed effect models listed in B.9 and for one additional linear model. LM stands for linear model, LMM for linear mixed model and REML for restricted maximum likelihood. The p-values in bold are significant at <0.05.

Model type	**Response variable**	**Fixed predictor**	**SumSq**	**MeanSq**	**NumDF**	**DenDF**	**F value**	**Pr(>F)**
LMM (fit by REML)	sumTU	Site type	33.04	16.52	2	10	6.66	**0.015**
	Taxonomic richness	Site type	100.33	50.17	2	10	1.18	0.348
	Log_{10}(Total abundance)	Site type	0.17	0.08	2	10	0.44	0.656
	Jaccard index	Site type	0.07	0.03	2	10	5.43	**0.025**
	$SPEAR_{pesticides}$	Site type	0.30	0.15	2	10	9.47	**0.005**
	$SPEAR_{pesticides}$	sumTU	0.0006	0.0006	1	12	0.03	0.856
		Site type	0.15	0.08	2	9.8	4.36	**0.044**
LM	sumTU	Sampling date	1.36	0.34	4		0.14	0.968

Figure B.1: sumTU over the entire sampling period in 2019 at the subset of KgM sites (six agricultural sites and the Modenbach refuge site) and for the complete June sampling. Each coloured dot/triangle represents a single sample taken in each of the six streams, with the colours representing the site types. The red and orange line represent the thresholds for potential effects on invertebrates (-3) and reference sites (-4), respectively (for details see Chapter 3.3.5.1.).

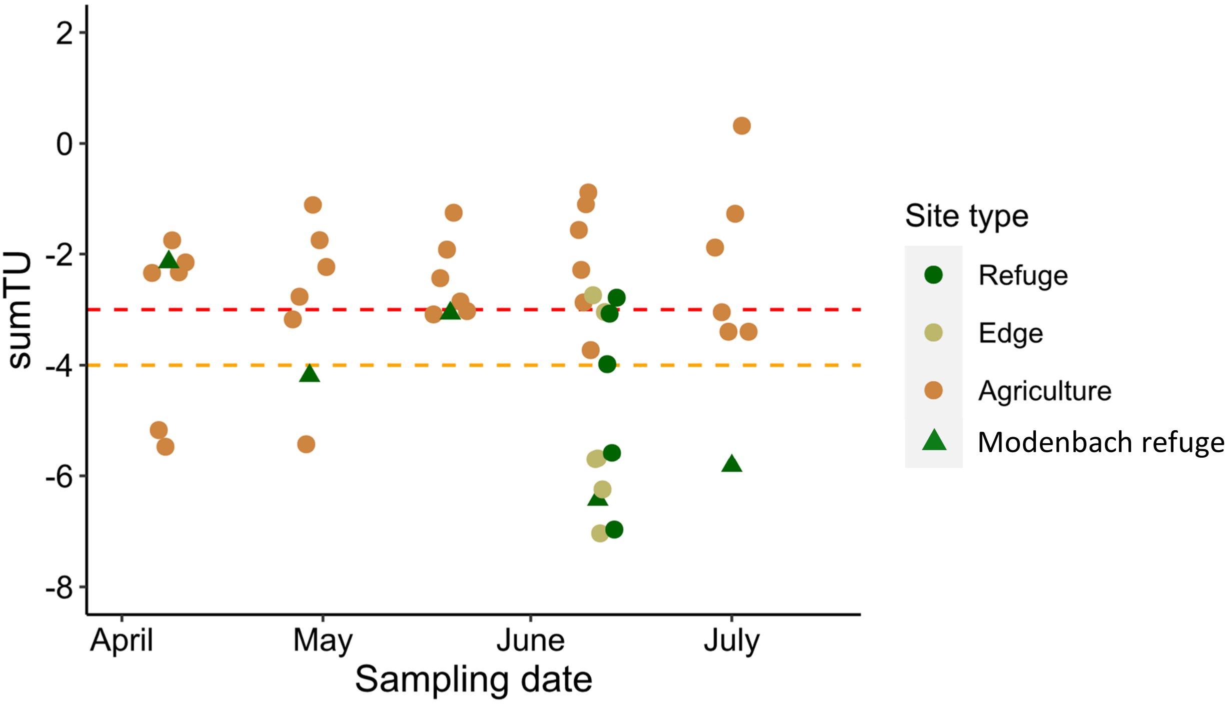


Figure B.2: The number of detected compounds (A), the total concentration in µg/L (B, on a logarithmic scale) and the estimated invertebrate toxicity (logarithmic sum toxic unit, sumTU) (C) per pesticide type analysed in surface water grab samples from refuge, edge and agricultural sites. Each coloured dot represents a single sample taken in each of the six streams, with the colours representing the site types. The red and orange line represent the thresholds for potential effects on invertebrates (-3) and reference sites (-4), respectively (for details see Chapter 3.3.5.1.).

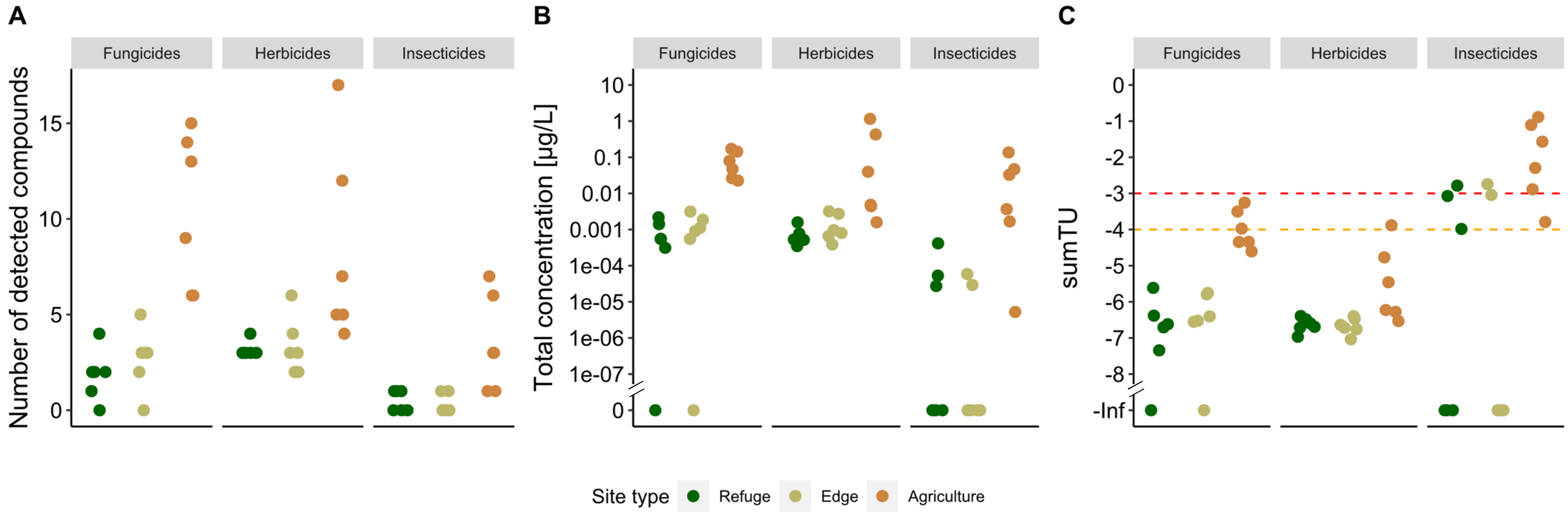


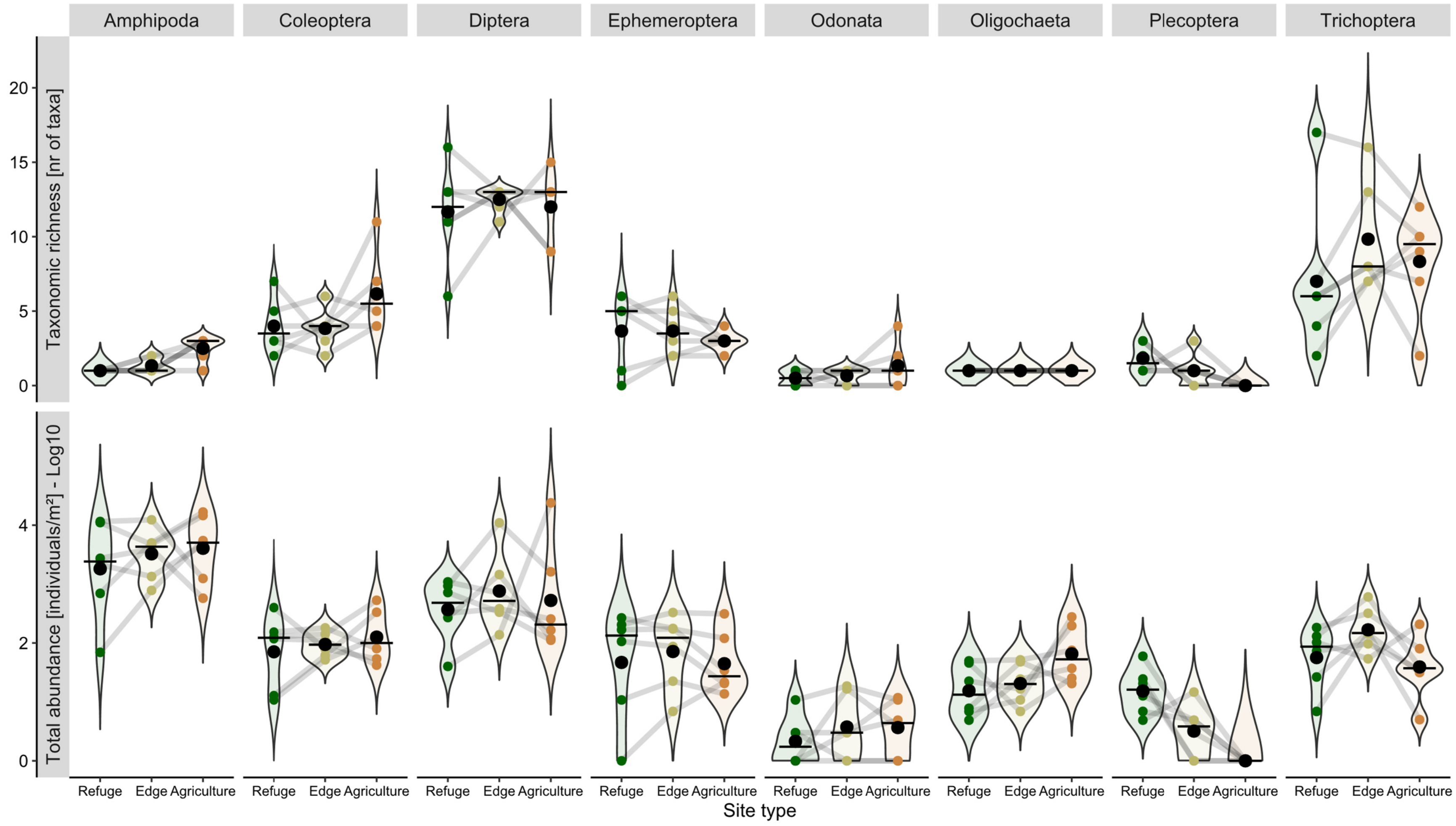
Table B.11.: Pesticides that contributed considerably (more than 10%) to relevant pesticide toxicity (sumTU ≥ -4) at refuge, edge and agricultural sites and their respective number of considerable contributions, separately for the June sample (all sites monitored) and the remaining sampling period (subset of KgM sites monitored). The brackets after each site type indicate the number of grab samples where sumTU ≥ -4 / total number of grab samples taken.

sumTU-driving pesticide	**Pesticide type**	**Number of contributions**				
		June			**April, May, July**	
		Refuge (3/6)	**Edge (2/6)**	**Agriculture (6/6)**	**Refuge (2/4)**	**Agriculture (21/24)**
Acetamiprid	Insecticide	0	0	0	0	1
Chlorantraniliprole	Insecticide	1	0	0	1	0
Clothianidin	Insecticide	0	0	0	1	3
Dimethoat	Insecticide	0	0	4	0	3
Fipronil	Insecticide	2	2	4	0	5
Imidacloprid	Insecticide	0	0	1	2	15
Methiocarb	Insecticide	0	0	0	1	4
Thiacloprid	Insecticide	0	0	0	0	6

Table B.12.: Pesticides detected at refuge, edge and agricultural sites and their detection frequency [%] for the June sample (six streams monitored).

Detected pesticide	Pesticide type	Detection frequency		
		Refuge	Edge	Agriculture
2_4_d	Herbicide	0	0	33
Acetamiprid	Insecticide	0	0	33
Azoxystrobin	Fungicide	0	0	50
Bentazon	Herbicide	0	0	33
Bixafen	Fungicide	0	0	17
Boscalid	Fungicide	17	33	100
Bromoxynil	Herbicide	0	0	17
Chlorantraniliprole	Insecticide	17	0	67
Chloridazon	Herbicide	0	17	0
Cyprodinil	Fungicide	0	0	17
Dichlorprop_p	Herbicide	0	0	17
Difenoconazol	Fungicide	0	0	100
Diflufenican	Herbicide	0	0	17
Dimethenamid	Herbicide	0	0	33
Dimethoat	Insecticide	0	0	67
Dimethomorph	Fungicide	67	83	100
Epoxiconazol	Fungicide	0	0	50
Fipronil	Insecticide	33	33	83
Fludioxonil	Fungicide	0	0	50
Flufenacet	Herbicide	17	33	100
Fluxapyroxad	Fungicide	17	33	100
Imidacloprid	Insecticide	0	0	50
Isoproturon	Herbicide	0	0	50
Mcpa	Herbicide	0	0	50
Mecoprop_p	Herbicide	0	0	50
Mesotrione	Herbicide	0	0	17
Metamitron	Herbicide	0	0	17
Metazachlor	Herbicide	0	0	17
Metrafenone	Fungicide	50	67	100
Nicosulfuron	Herbicide	0	0	33
Pirimicarb	Insecticide	0	0	33
Propamocarb	Fungicide	0	0	50
Propiconazol	Fungicide	17	33	83
Propyzamid	Herbicide	17	17	17
Prosulfocarb	Herbicide	100	100	100
Prosulfuron	Herbicide	17	0	0
Pyraclostrobin	Fungicide	0	0	33
Quinmerac	Herbicide	0	0	17
S_metolachlor	Herbicide	67	67	100
Spiroxamine	Fungicide	17	17	67
Tebuconazol	Fungicide	0	0	83
Terbuthylazin	Herbicide	100	100	100
Thiacloprid	Insecticide	0	0	17
Trifloxystrobin	Fungicide	0	0	50
Tritosulfuron	Herbicide	0	0	17

Figure B.3: Taxonomic richness and total abundance for selected orders for each site type. Note that the y-axis of the total abundance plot represents a logarithmic scale. Each coloured dot represents a single sample taken in each of the six streams, with the colours representing the site types and grey lines connecting site types in the same stream. The black dot and line represent the mean and the median, respectively, for the six streams per site type.



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C - SUPPLEMENTARY INFORMATION FOR THE MANUSCRIPT "POPULATION STRUCTURE AND INSECTICIDE RESPONSE OF *GAMMARUS* SPP. IN AGRICULTURAL AND UPSTREAM FORESTED SITES OF SMALL STREAMS"

Table C.1.: Coordinates in decimal degrees and elevation in meter above sea level of the sampling sites (Schneeweiss et al., 2022).

Stream	**Site type**	**Latitude**	**Longitude**	**Elevation in m above sea level**
Hainbach	Refuge	49.24338	8.044861	327
Hainbach	Edge	49.23622	8.052707	283
Hainbach	Agriculture	49.23616	8.075042	220
Kropsbach	Refuge	49.30566	8.070927	369
Kropsbach	Edge	49.30188	8.086755	290
Kropsbach	Agriculture	49.30245	8.147199	145
Modenbach	Refuge	49.26915	8.052505	268
Modenbach	Edge	49.26052	8.065709	225
Modenbach	Agriculture	49.25873	8.118499	165
Otterbach	Refuge	49.07434	7.946536	257
Otterbach	Edge	49.07007	7.958075	243
Otterbach	Agriculture	49.06871	8.005802	166
Russbach	Refuge	49.06414	7.943377	265
Russbach	Edge	49.06038	7.949459	254
Russbach	Agriculture	49.04677	7.986717	166
Triefenbach	Refuge	49.28506	8.076674	277
Triefenbach	Edge	49.2818	8.093535	210
Triefenbach	Agriculture	49.28122	8.113479	179

Table C.2.: Relative abundance [%] of *Gammarus fossarum*, *Gammarus pulex* and *Gammarus roeselii* at the sampling sites.

Sampling site	***Gammarus fossarum***	***Gammarus pulex***	***Gammarus roeselii***
Hainbach-agriculture	100.00	0.00	0.00
Hainbach-edge	100.00	0.00	0.00
Hainbach-refuge	100.00	0.00	0.00
Kropsbach-agriculture	0.00	68.75	31.25
Kropsbach-edge	80.00	20.00	0.00
Kropsbach-refuge	100.00	0.00	0.00
Modenbach-agriculture	100.00	0.00	0.00
Modenbach-edge	95.00	5.00	0.00
Modenbach-refuge	100.00	0.00	0.00
Otterbach-agriculture	19.64	5.36	75.00
Otterbach-edge	100.00	0.00	0.00
Otterbach-refuge	100.00	0.00	0.00
Russbach-agriculture	5.56	8.33	86.11
Russbach-edge	100.00	0.00	0.00
Russbach-refuge	100.00	0.00	0.00
Triefenbach-agriculture	50.00	50.00	0.00
Triefenbach-edge	95.00	5.00	0.00
Triefenbach-refuge	100.00	0.00	0.00

Table C.3.: Physicochemical parameters of test medium at test start (mean ± sd per stream, if multiple measurements were taken).

Stream	**Date**	**Temperature [°C]**	**pH**	**Conductivity [µS/cm]**
Hainbach	2019-06-11	NA	9.06 ± 0.04	633.25 ± 1.71
Kropsbach	2019-07-02	17.60 ± NA	7.08 ± 0.16	642.00 ± NA
Modenbach	2019-06-18	17.65 ± 0.92	7.98 ± 0.03	662.00 ± 2.83
Otterbach	2019-07-22	16.00 ± NA	7.87 ± NA	646.00 ± NA
Russbach	2019-07-09	16.40 ± 0.28	7.63 ± 0.10	614.00 ± 15.56
Triefenbach	2019-06-25	22.50 ± NA	8.64 ± NA	625.00 ± NA

Table C.4.: Physicochemical parameters of test medium at test end (mean ± sd per stream, at least one replicate per concentration was measured).

Stream	Temperature [°C]	pH	Conductivity [µS/cm]	Dissolved oxygen [%]	Dissolved oxygen [mg/L]
Hainbach	21.97 ± 0.28 *	7.62 ± 0.12	695.30 ± 18.50	71.57 ± 22.02	6.13 ± 1.86
Kropsbach	21.66 ± 0.92	7.72 ± 0.09	685.06 ± 15.79	95.51 ± 2.63	8.27 ± 0.30
Modenbach	19.90 ± 1.62	7.81 ± 0.12	711.46 ± 16.75	93.94 ± 3.30	8.47 ± 0.45
Otterbach	21.99 ± 0.84	7.55 ± 0.06	664.82 ± 9.87	96.73 ± 2.28	8.29 ± 0.21
Russbach	19.39 ± 0.14	7.60 ± 0.12	667.70 ± 27.92	94.54 ± 1.44	8.75 ± 0.17
Triefenbach	22.06 ± 0.97	7.51 ± 0.17	673.92 ± 14.92	83.60 ± 16.94	7.24 ± 1.50

* the tests were carried out in climatic chambers at 16 °C, mimicking average water temperature of the streams; the temperatures given here may deviate from this, as temperature was measured shortly after test end

Text C.1: Chemical analyses in acute toxicity tests.

Detailed information on the applied method (e.g. mobile phase gradient) are provided in (Fernández et al., 2016). In brief, we thawed the samples, vortexed them and took subsamples of 1 mL, which were stored at 4°C overnight. We centrifuged subsamples at 4000 rpm for 30 min and used the supernatants (180 µL) for chemical analysis. In slight modification to (Fernández et al., 2016), we used H_2O/MeOH (LC-MS grade; Merck, Darmstadt, Germany) with 0.1 % formic acid as mobile phase. Prior to analysis, we diluted the samples with high nominal concentrations using ultrapure water (1:10). We measured imidacloprid concentrations via an Exactive (LC-HRMS) Orbitrap system (Thermo Fisher Scientific Corporation, Waltham, USA) using a 50 x 2.1 mm Thermo Hypersil GOLD™ column (1.9 mm particle size) and injected 20 µL. A calibration curve created with PESTANAL® analytical standard (LOT#BCBZ3018, Sigma-Aldrich, Buchs, Switzerland) showed a linear range between 0.1 and 200 µg/L. To take into account a possible matrix effect, the calibration curve was created with the medium used in the experiment, including the dilution step mentioned above.

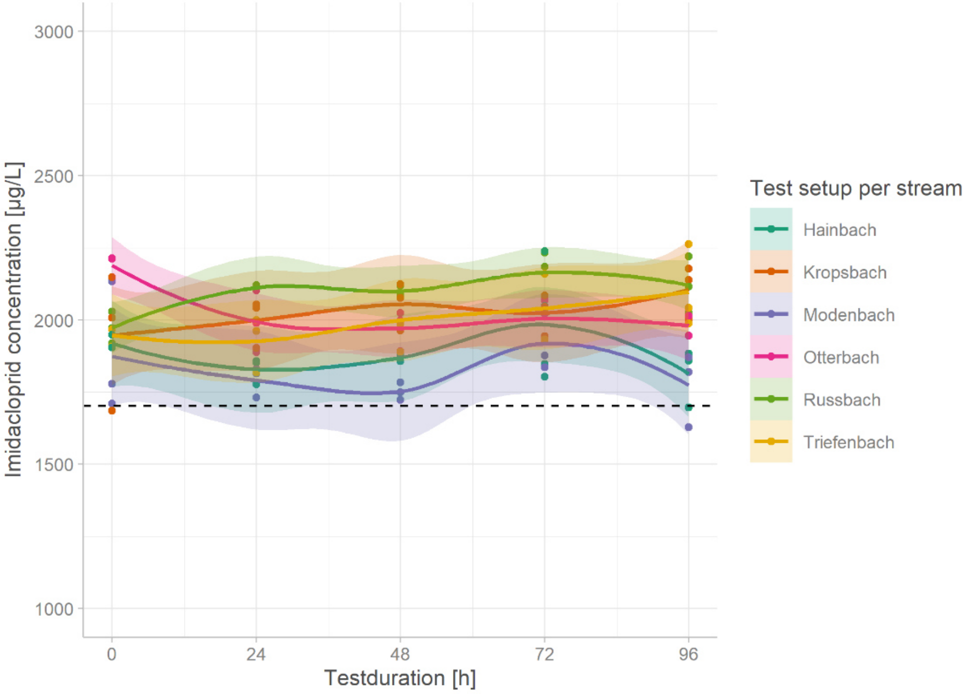
Table C.5.: Nominal and measured concentrations (in µg/L; mean ± sd per stream, if multiple measurements were taken) applied in acute toxicity test setup at test start (0h). The limit of quantification (LOQ) was 0.3 µg/L.

Nominal Concentration	Hainbach	Kropsbach	Modenbach	Otterbach	Russbach	Triefenbach
0.0	< LOQ	< LOQ	< LOQ	< LOQ	1.01 ± 0.54 °	< LOQ
7.0	6.47	6.58	6.17	5.73	6.59	7.29
21.0	19.57	20.35	17.10	19.16	21.11	21.18
63.0	57.07	66.09	55.70	65.56	101.86	62.11
189.0	171.25	151.71	141.44	165.26	173.22	174.99
567.0	626.00	713.00	757.00	841.00	770.00	775.00
1134.0$^{\$}$	NA	1373.00	NA	1326.00	1400.00	NA
1701.0	1899.00 ± 44.47	1778.00 ± 308.41	1870.33 ± 230.80	2188.33 ± 42.74	1972.67 ± 54.52	1885.50 ± 126.76

° Likely cross-contamination from the introduction of organisms collected in the field into the test vessels, because a water sample collected six days earlier at the sampling site had a high imidacloprid concentration (0.28 µg/L) (Schneeweiss et al., 2022). Since no mortality was recorded within these controls, we kept them for further analysis.

$ This exposure concentration was added for the *Gammarus* spp. tests in three of the six analysed streams to strengthen the concentration-response relationship within this concentration range.

Figure C.1: Stability of the highest toxicity test concentration (nominal test concentration of 1701 µg/L visualised as black dotted line) from start to the end of the test (0 - 96 h). The colours refer to the different test setups that were realised per stream.



Text C.2: Slightly modified salt-extraction protocol after Aljanabi (1997) for the extraction of genomic DNA.

We transferred detached tissue into sterile (autoclaved) 2 mL Eppendorf tubes and placed them in a Thermoblock (Labnet international Inc, Edison, USA) for drying at 60 °C until total evaporation of ethanol (approximately 20 min). Subsequently, we added two 2-3 mm sterile stainless-steel beads to each tube and homogenised the tissue in a TissueLyser II bead mill (Qiagen, Hilden, Germany) for 3 min at 30 Hz. Afterwards, we added 450 µL salt extraction buffer (2 mM EDTA pH 8; 10 mM Tris-HCl pH 8; 0.4 M NaCl), 100 µL SDS (10 %) and 5 µL Proteinase K (10 mg/mL), all provided by Roth (Karlsruhe, Germany) to each tube. We vortexed each tube for at least 30 s and incubated them at 60 °C in a Thermomixer (400 rpm, Eppendorf, Hamburg, Germany). After 30 min of incubation, we vortexed all tubes again. After another 30 min of incubation, we added 350 µL 5 M NaCl (Carl Roth, Karlsruhe, Germany) solution to each tube, vortexed thoroughly and centrifuged them for 30 min (16.200 x g). Subsequently, we transferred 600 µL supernatant into new sterile 1.5 mL Eppendorf tubes, added 600 µL of ice-cold isopropanol (≥ 99.8 % p.a.; Carl Roth, Karlsruhe, Germany) and mixed the solutions carefully. We stored tubes overnight at -20 °C (or for 30 min at -80 °C). We then centrifuged all tubes at 4 °C for 20 min (16.200 x g), discarded the supernatant carefully and added 200 µL of ice-cold 70 % ethanol. We centrifuged all tubes again at 4 °C for 10 min (16.200 x g) and carefully pipetted off the supernatant. Finally, we dried the pellet for approximately 20 min at 60 °C until all ethanol was evaporated and dissolved it in 25 µL TE-Buffer (10x; 10 mM Tris pH 8; 1 mM EDTA; Carl Roth, Karlsruhe, Germany).

Text C.3: Slightly modified lipid-quantification protocol after Van Handel (1985).

We stored each individual for 72 h in 0.5 mL of a 1:1 chloroform:methanol (GC and HPLC grade; Merck, Darmstadt, Germany and Carl Roth, Karlsruhe, Germany) solution. Subsequently, we mortared each individual until the tissue was completely dissolved, filled them into individual clean culture tubes, equalised the fill level with the 1:1 chloroform:methanol solution and centrifuged them at 2000 rpm for 1 min. The supernatant was then transferred to new culture tubes. In parallel, calibration concentrations including three blanks were prepared separately on each day of analysis with soya oil (Sojola Soja-Öl, Vandemoortele, Ghent, Belgium) (25, 50, 100, 200, 400, 800, 1600 µL of stock solution: 50 mg soy oil in 50 mL chloroform) and processed in the same way as the sample replicates. Then all sample replicates and calibration tubes were placed in a water bath at 95 °C for 15-20 min until the solvent solution had completely evaporated. We now added 0.2 mL of sulphuric acid (≥ 95 % p.a.; Carl Roth, Karlsruhe, Germany) to each vial, sealed them and heated them again in the water bath at 95 °C for 10 min. After cooling to room temperature, we added 5 mL of a vanillin phosphoric acid (600 mg vanillin in 400 mL phosphoric acid 80 % p.a.; Carl Roth, Karlsruhe, Germany), vortexed for 30 s and waited for about 5 min reaction time. We then added 80 µL of each sample to a 96-well plate (microplate BRANDplates, pureGradeS, BRAND, Wertheim, Germany) and measured the absorbance at 490 nm in a microplate reader (Tecan Infinite M200, Tecan Group, Männedorf, Switzerland) within the subsequent 30 min. We converted the absorbance to the lipid content in mg/gammarid using the day-specific calibration curves.

Text C.4: CO1 sequence data preparation, quality control and taxonomy assignment.

We firstly computed a multiple sequence alignment with high accuracy and high throughput (MUSCLE; version 3.8.425; Edgar, 2004) including all sequences to realise batch editing. We removed low-quality margins of each chromatogram (i.e. threshold: > 5 % error probability). We then manually inspected each trace within the overall batch to verify its quality and to solve ambiguities. For quality control, we translated all nucleotides of the DNA sequence into amino acids of a protein sequence and checked for unexpected stop codons and diverging amino acids. We exported the DNA sequences as FASTA files and continued assigning taxonomy to each sequence as well as computing and visualising population genetic metrics using the software R (version 4.2.0.; R Core Team, 2022). We assigned the taxonomy with the Basic Local Alignment Search Tool (BLAST) using BLAST+ (version 2.13.0; Camacho et al., 2009) and the rBLAST package (version 0.99.2; Hahsler & Nagar, 2019). For further population structural analysis, we filtered the sequences for *G. fossarum* and high quality (threshold: percentage of untrimmed bases in a sequence that are of high quality > 50%). For the filtered dataset, we aligned the sequences (MUSCLE; version 3.8.425; Edgar, 2004) and checked the alignment for gaps. To achieve a full alignment without gaps, we removed short sequences that would have been too length-limiting. For control, we also ran the whole analysis with the short alignment containing all sequences, which indicated the same main conclusion concerning our hypotheses.

Table C.6.: Model outputs of separate full models for *Gammarus* spp. mortality or immobility (includes dead organisms) after 96 h exposure to imidacloprid. Generalized linear mixed-effects models (GLMM) with binomial distribution and logit link were applied. For the table outputs of GLMM summaries, the sjPlot package (version 2.8.10; (Lüdecke, 2021) was used, which calculates marginal and conditional R-squared statistics based on (Nakagawa et al., 2017).

	Proportion dead 96			**Proportion dead 96**			**Proportion immobile 96**		
Predictors	*Odds Ratios*	*std. Error*	*p*	*Odds Ratios*	*std. Error*	*p*	*Odds Ratios*	*std. Error*	*p*
(Intercept)	0.02	0.01	**<0.001**	0.02	0.00	**<0.001**	0.00	0.00	**<0.001**
LogConcentration	4.87	0.50	**<0.001**	4.84	0.49	**<0.001**	24.39	4.74	**<0.001**
fSiteType [edge]	0.63	0.13	**0.027**				0.76	0.28	0.462
fSiteType [refuge]	0.58	0.12	**0.010**				0.90	0.33	0.777
fGFossarumAbundance [low]				1.80	0.38	**0.005**			
Random Effects									
σ^2	3.29			3.29			3.29		
τ_{00}	0.59 $_{\text{fSubunit}}$			0.59 $_{\text{fSubunit}}$			2.04 $_{\text{fSubunit}}$		
	0.06 $_{\text{fStream}}$			0.06 $_{\text{fStream}}$			0.00 $_{\text{fStream}}$		
ICC	0.17			0.17					
N	6 $_{\text{fStream}}$			6 $_{\text{fStream}}$			6 $_{\text{fStream}}$		
	135 $_{\text{fSubunit}}$			135 $_{\text{fSubunit}}$			135 $_{\text{fSubunit}}$		
Observations	540			540			540		
Marginal R^2 / Conditional R^2	0.350 / 0.458			0.351 / 0.458			0.720 / NA		

Table C.7.: Analysis of deviance table for the generalised linear mixed-effects models (GLMM) listed in Table C.6 using type II Wald Chi squared tests. The p-values in bold are significant at <0.05.

Model type	**Response variable**	**Fixed predictor**	**Chisq**	**Df**	**Pr(>Chisq)**
GLMM	Mortality – *Gammarus* spp.	LogConcentration	242.1	1	**<2e-16**
		Site type	7.9	2	**0.0194**
	Mortality – *Gammarus* spp.	LogConcentration	242.2	1	**<2e-16**
		Abundance *G. fossarum*	7.9	1	**0.0053**
	Immobility – *Gammarus* spp.	LogConcentration	269.5	1	**<2e-16**
		Site type	0.5	2	0.7602

Table C.8.: Estimated lethal and effect concentrations in µg/L after 96 h exposure to imidacloprid where 50 % of test organisms (*Gammarus* spp.) have been affected for each site type.

Site type	**LC_{50} [µg/L] *Gammarus* spp. mortality**	**EC_{50} [µg/L] *Gammarus* spp. immobility**
Refuge	492	72
Edge	439	87
Agriculture	224	77
High *G. fossarum* abundance	443	-
Low *G. fossarum* abundance	189	-

Figure C.2: Concentration-response relationship visualised for the immobility of *Gammarus* spp. individuals after 96 h of exposure to imidacloprid. Each coloured dot represents a single test replicate, with colours representing the site types. The lines represent the average estimate of immobility per concentration and site type for a typical stream with the shadowed areas indicating the 95% confidence intervals.

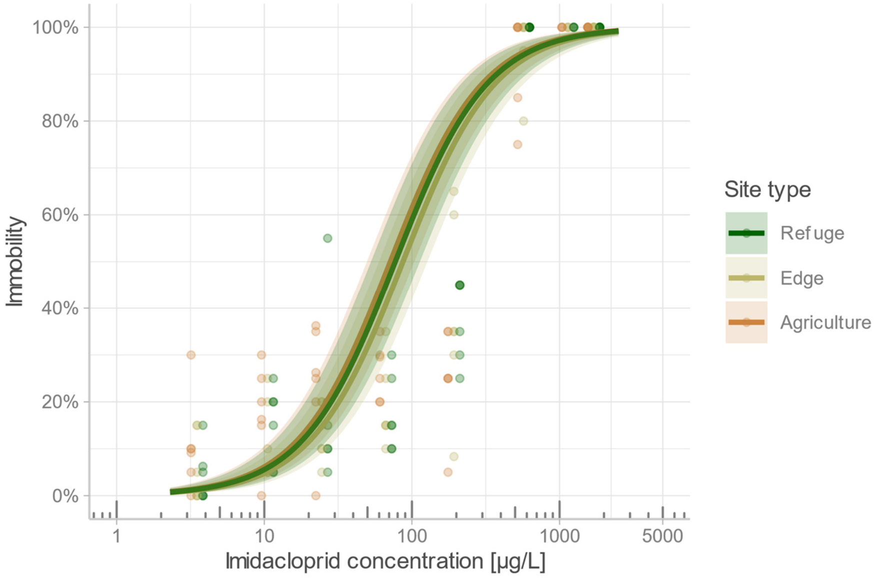


Table C.9.: Overview of the absolute and relative haplotype frequency as well as variable sites sampled per haplotypes in a fragment of the mitochondrial cytochrome c oxidase subunit one gene (CO1) of *Gammarus fossarum.*

Haplotype	**Frequency [absolute]**	**Frequency [%]**	**Variable sites (non-contiguous fragment)**
H1	59	20.14	ACTCCAAATCATCGATAT
H2	2	0.68	....T.G.C...TAG.G.
H3	51	17.41	....T.G.....TAG.G.
H4	26	8.87	.....G.......A..G.
H5	7	2.39	..C..G....G..A..G.
H6	72	24.57	....T.G.....TA..G.
H7	22	7.51	G...T.G.....TA..G.
H8	16	5.46	.........T...A..G.
H9	1	0.34	.............A..GC
H10	21	7.17	.............A..G.
H11	7	2.39	.........T.C.A..G.
H12	6	2.05	.........T...AGCG.
H13	1	0.34	.......G.....A..G.
H14	1	0.34	...T.........A..G.
H15	1	0.34	.T...........A..G.

Table C.10.: Overview table of population genetic metrics for *Gammarus fossarum* per sampling site. “N” refers to total number of individual sequences. “H” refers to haplotype. H1 - H15 illustrate the relative occurrence [%] of each haplotype per sampling site.

Stream	Site type	N	total H	private H	H diversity	Nucleotide diversity	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15
Hainbach	agriculture	18	3	0	0.451	0.005	22	6	72	0	0	0	0	0	0	0	0	0	0	0	0
Hainbach	edge	20	3	0	0.353	0.003	15	5	80	0	0	0	0	0	0	0	0	0	0	0	0
Hainbach	refuge	20	2	0	0.395	0.005	25	0	75	0	0	0	0	0	0	0	0	0	0	0	0
Kropsbach	edge	15	2	0	0.133	0.001	0	0	0	93	7	0	0	0	0	0	0	0	0	0	0
Kropsbach	refuge	18	2	0	0.471	0.002	0	0	0	67	33	0	0	0	0	0	0	0	0	0	0
Modenbach	agriculture	18	1	0	0.000	0.000	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Modenbach	edge	18	2	0	0.294	0.003	83	0	17	0	0	0	0	0	0	0	0	0	0	0	0
Modenbach	refuge	15	2	0	0.248	0.003	87	0	13	0	0	0	0	0	0	0	0	0	0	0	0
Otterbach	agriculture	12	1	0	0.000	0.000	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0
Otterbach	edge	17	1	0	0.000	0.000	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0
Otterbach	refuge	19	1	0	0.000	0.000	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0
Russbach	agriculture	8	2	0	0.536	0.001	0	0	0	0	0	63	38	0	0	0	0	0	0	0	0
Russbach	edge	20	2	0	0.521	0.001	0	0	0	0	0	55	45	0	0	0	0	0	0	0	0
Russbach	refuge	18	2	0	0.523	0.001	0	0	0	0	0	44	56	0	0	0	0	0	0	0	0
Triefenbach	agriculture	20	7	2	0.800	0.004	0	0	10	0	0	0	0	35	5	30	5	10	0	0	5
Triefenbach	edge	18	4	0	0.660	0.002	0	0	0	0	0	0	0	33	0	50	11	6	0	0	0
Triefenbach	refuge	19	7	2	0.842	0.004	5	0	0	0	0	0	0	16	0	32	21	16	5	5	0

Table C.11.: Model outputs of separate full models for the haplotype- and nucleotide diversity in a fragment of the mitochondrial cytochrome c oxidase subunit one gene (CO1) of *Gammarus fossarum* individuals as response to site type. Linear mixed-effects models (LMM) were applied including stream as random factor. For the table outputs of LMM summaries, the sjPlot package (version 2.8.10; Lüdecke, 2021) was used, which calculates marginal and conditional R-squared statistics based on (Nakagawa et al., 2017).

	Haplotype diversity			**Nucleotide diversity**		
Predictors	*Estimates*	*std. Error*	*p*	*Estimates*	*std. Error*	*p*
(Intercept)	0.34	0.12	**0.024**	0.00	0.00	**0.046**
site type [edge]	-0.02	0.07	0.795	0.00	0.00	0.966
site type [refuge]	0.07	0.07	0.335	0.00	0.00	0.359
Random Effects						
σ^2	0.01			0.00		
τ_{00}	0.07_{stream}			0.00_{stream}		
ICC	0.85			0.66		
N	6_{stream}			6_{stream}		
Observations	17			17		
Marginal R^2 / Conditional R^2	0.019 / 0.857			0.030 / 0.667		

Table C.12.: Type III analysis of variance table with Kenward-Roger's method for linear mixed effect models listed in Table C.11. LMM for linear mixed model and REML for restricted maximum likelihood. The p-values in bold are significant at <0.05.

Model type	**Response variable**	**Fixed predictor**	**SumSq**	**MeanSq**	**NumDF**	**DenDF**	**F value**	**Pr(>F)**
LMM fit by REML	Haplotype diversity	Site type	0.024	0.012	2	9.04	1.04	0.392
	Nucleotide diversity	Site type	1.5e-06	7.8e-07	2	9.1	0.721	0.512

Figure C.3: Heatmap visualising the genetic differentiation between *Gammarus fossarum* populations from different sampling sites measured as pairwise CO1 fixation index (F_{ST}) values calculated using the method from Nei (1987) as implemented in the hierfstat package (version 0.5-11; Goudet & Jombart, 2022).

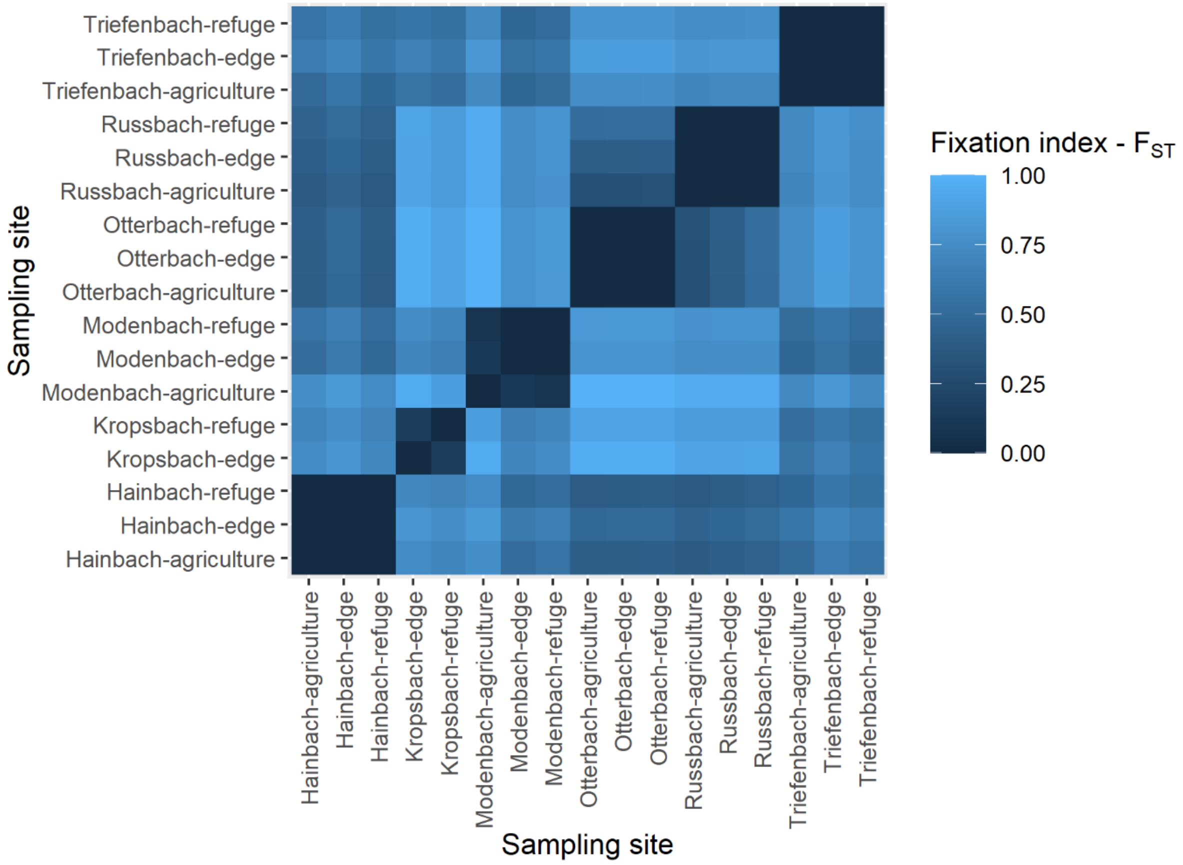


Table C.13.: Pairwise CO1 fixation index (F_{ST}) values between all sampling sites calculated using the method from Nei, (1987) as implemented in the hierfstat package 0.5-11 (Goudet & Jombart, 2022). A, E and R are abbreviations for the three site types agriculture, edge and refuge, respectively. Red values indicate F_{ST} values between site types within the same stream.

Stream		Hainbach			Kropsbach		Modenbach			Otterbach			Russbach			Triefenbach		
	Site type	*A*	*E*	*R*	*E*	*R*	*A*	*E*	*R*	*A*	*E*	*R*	*A*	*E*	*R*	*A*	*E*	*R*
Hainbach	*A*	NA																
	E	0	NA															
	R	0	0	NA														
Kropsbach	*E*	0.74	0.80	0.71	NA													
	R	0.70	0.76	0.68	0.15	NA												
Modenbach	*A*	0.76	0.84	0.74	0.96	0.87	NA											
	E	0.52	0.62	0.48	0.71	0.66	0.12	NA										
	R	0.56	0.66	0.53	0.74	0.69	0.08	0	NA									
Otterbach	*A*	0.41	0.49	0.40	0.97	0.90	1.00	0.79	0.83	NA								
	E	0.42	0.49	0.41	0.97	0.90	1.00	0.80	0.83	0	NA							
	R	0.42	0.50	0.41	0.97	0.90	1.00	0.80	0.83	0	0	NA						
Russbach	*A*	0.38	0.45	0.37	0.91	0.85	0.95	0.75	0.79	0.30	0.31	0.31	NA					
	E	0.42	0.48	0.41	0.91	0.86	0.95	0.76	0.80	0.41	0.42	0.42	0	NA				
	R	0.44	0.50	0.43	0.92	0.86	0.95	0.77	0.80	0.52	0.53	0.53	0	0	NA			
Triefenbach	*A*	0.50	0.58	0.48	0.56	0.53	0.73	0.47	0.51	0.75	0.76	0.76	0.71	0.72	0.73	NA		
	E	0.62	0.70	0.60	0.67	0.60	0.82	0.55	0.59	0.87	0.87	0.87	0.82	0.82	0.83	0	NA	
	R	0.57	0.64	0.54	0.58	0.54	0.72	0.48	0.51	0.80	0.80	0.80	0.75	0.76	0.77	0	0	NA

Table C.14.: Analysis of molecular variance (AMOVA) results for mtDNA CO1 data of *Gammarus fossarum* individuals from 17 sampling sites (=populations) modelled separately for two different groups (i.e. 6 streams and 3 site types). The table lists sums of square deviations (SSD), mean square deviations (MSD) and the number of degrees of freedom (df). The p-values in bold are significant at <0.05. A hamming distance matrix was used as a measure of genetic distance.

	Groups = streams							**Groups = site types**						
Source of variation	SSD	MSD	df	sigma2	p-value	Variance coefficients	Phi	SSD	MSD	df	sigma2	p-value	Variance coefficients	Phi
Between groups	1502.46	300.49	5	6.15	**<0.0001**	17.01	0.73	16.85	8.43	2	-1.06	0.99	17.14	-0.01
Between populations within groups	19.48	1.77	11	-0.03	0.58	17.61	-0.01	1505.09	107.51	14	6.13	**<0.0001**	17.60	0.72
Within populations	648.85	2.35	276	2.35		48.55	0.72	648.85	2.35	276	2.35		96.46	0.68
Total	2170.78	7.43	292					2170.78	7.43	292				

Table C.15.: Overview table of population genetic metrics for *Gammarus pulex* per sampling site. "N" refers to total number of individual sequences. "H" refers to haplotype.

Sampling site	**N**	**H**	**Private H**	**H diversity**	**Nucleotide diversity**	**H1**
Kropsbach-agriculture	19	1	0	0	0	19
Kropsbach-edge	4	1	0	0	0	4
Modenbach-edge	1	1	0	0	0	1
Otterbach-agriculture	3	1	0	0	0	3
Russbach-agriculture	15	1	0	0	0	15
Triefenbach-agriculture	20	1	0	0	0	20
Triefenbach-edge	1	1	0	0	0	1

Text C.5: Lower between stream population differentiation for *Gammarus pulex* compared to *Gammarus fossarum* – extended discussion.

At the same regional scale at which *G. fossarum* showed differentiation into riverine networks and some haplotype diversity, *G. pulex* populations showed no differentiation and no haplotype diversity (Table C.15). This is in line with the findings of several former studies that found higher local genetic diversity for *G. fossarum* compared to *G. pulex* (Wattier et al., 2020; Weigand et al., 2020) and suggested that only a high sample size may reveal the local diversity of *G. pulex*. For example, within 13 samples of *G. pulex* clade D also no diversity was found (Weigand et al., 2020). Our sampling size for *G. pulex* was also relatively low for most sites (6 sites, 3 of them < 10 individuals; Table C.15) and thus potentially did not capture the complete local diversity. The lack of any genetic differentiation, however, suggests that *G. pulex* individuals may be in continuous exchange with the common source (e.g. Rhine River; "High gene flow scenario"). Besides, for *G. pulex* the 200 m isobar is typically the highest distribution limit in our region (Kinzelbach & Claus, 1977), which matches our observations and suggests that our agricultural sites are at the very top of the *G. pulex* population distribution. Due to asymmetric gene flow, the genetic diversity may be depleted at these sites and enriched at the rhine confluence ("DIGD scenario"). However, other explanations are conceivable, such as *G. pulex* individuals recently immigrated from the same main source into the six streams (from Rhine River), and have not diverged since then ("Recent migration scenario"). If the presence of *G. pulex* populations in the six streams would be due to an older immigration event and genetic exchange with downstream populations would be absent, then the populations are likely to be under strong selection ("Directional selection scenario").

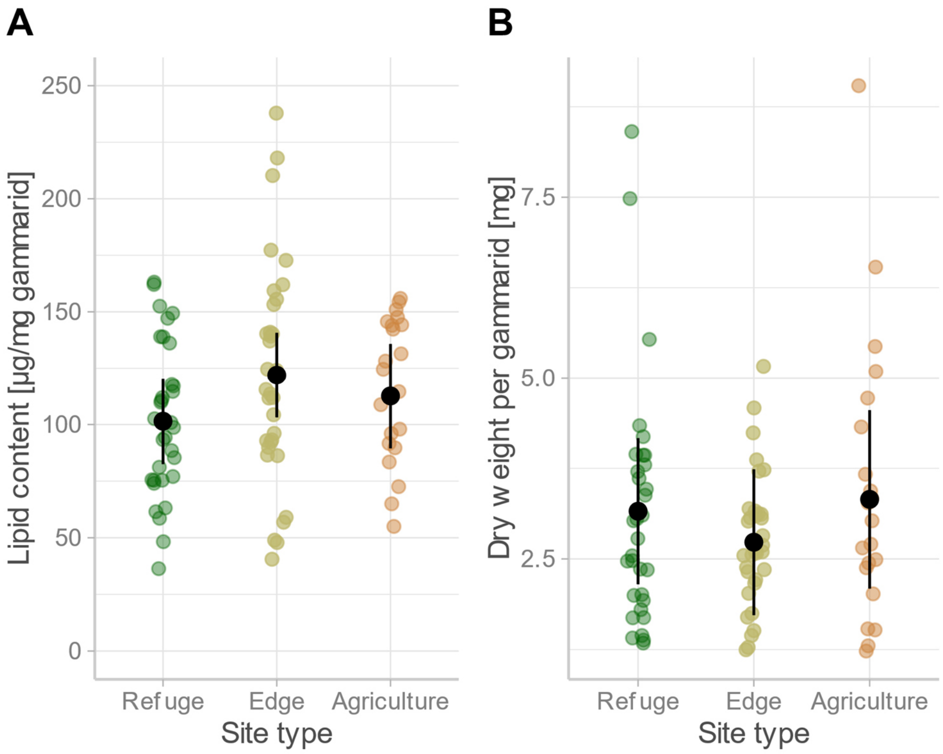
Table C.16.: Model outputs of separate full models for the lipid content in µg/mg gammarid or the dry weight in mg as response to site type or the abundance of *Gammarus fossarum*. Linear mixed-effects models (LMM) were applied including stream and the replication subunit as random factor. For the table outputs of LMM summaries, the sjPlot package 2.8.10 was used (Lüdecke, 2021), which calculates marginal and conditional R-squared statistics based on (Nakagawa et al., 2017).

	***Gammarus* spp.**												***Gammarus fossarum***					
	Dry weight			**Dry weight**			**Lipid content**			**Lipid content**			**Dry weight**			**Lipid content**		
Predictors	*Estimates*	*std. Error*	*p*	*Estimates*	*std. Error*	*p*	*Estimates*	*std. Error*	*p*	*Estimates*	*std. Error*	*p*	*Estimates*	*std. Error*	*p*	*Estimates*	*std. Error*	*p*
(Intercept)	4.14	0.34	**<0.001**	6.19	0.53	**<0.001**	106.41	7.23	**<0.001**	87.86	11.16	**<0.001**	3.32	0.65	**<0.001**	112.63	12.20	**<0.001**
fGFossarumAbundance [low]	2.72	0.74	**0.003**				-24.83	15.97	0.143									
site type [edge]				-2.03	0.75	**0.022**				13.44	15.21	0.397	-0.59	0.78	0.465	9.31	14.47	0.536
site type [refuge]				-2.21	0.75	**0.014**				25.23	15.21	0.128	-0.16	0.78	0.838	-11.19	14.52	0.460
Random Effects																		
σ^2	6.98			7.40			1511.37			1517.30			1.12			1353.70		
τ_{00}	$0.00_{\text{replicate}}$			$0.00_{\text{replicate}}$			$364.42_{\text{replicate}}$			$349.95_{\text{replicate}}$			$1.11_{\text{replicate}}$			$206.05_{\text{replicate}}$		
	0.00_{stream}			0.00_{stream}			0.00_{stream}			50.86_{stream}			0.22_{stream}			83.97_{stream}		
ICC										0.21			0.54			0.18		
N	6_{stream}			6_{stream}			6_{stream}			6_{stream}			6_{stream}			6_{stream}		
	$18_{\text{replicate}}$			$18_{\text{replicate}}$			$18_{\text{replicate}}$			$18_{\text{replicate}}$			$16_{\text{replicate}}$			$16_{\text{replicate}}$		
Observations	81			81			81			81			86			86		
Marginal R^2 / Conditional R^2	0.161 / NA			0.121 / NA			0.069 / NA			0.053 / 0.251			0.025 / 0.554			0.047 / 0.215		

Table C.17.: Type III analysis of variance table with Kenward-Roger's method for linear mixed effect models listed in Table C.16. LMM for linear mixed model and REML for restricted maximum likelihood. The p-values in bold are significant at <0.05.

Model type	Response variable	Fixed predictor	SumSq	MeanSq	NumDF	DenDF	F value	Pr(>F)
LMM (fit by REML)	Dry weight - *Gammarus* spp.	Abundance *G.fossarum*	93.323	93.323	1	12.89	13.37	**0.0029**
	Dry weight - *Gammarus* spp.	Site type	80.264	40.132	2	9.81	5.42	**0.0259**
	Dry weight - *G. fossarum*	Site type	0.792	0.396	2	8.69	0.35	0.712
	Lipid content - *Gammarus* spp.	Abundance *G.fossarum*	3656.4	3656.4	1	13.51	2.42	0.143
	Lipid content - *Gammarus* spp.	Site type	4182.5	2091.2	2	9.95	1.38	0.296
	Lipid content - *G. fossarum*	Site type	3735.6	1867.8	2	8.62	1.38	0.302

Figure C.4: Dot plots (Wickham, 2016) visualising the total lipid content [µg] normalised by the dry weight per gammarid [mg] (A) and the dry weight per gammarid (B) for the second experimental run for *Gammarus fossarum*. Each coloured dot represents a replicate on the measurement subunit (n = 4-5 per sampling site) with the colours representing the site types (n = 3) and different degrees of transparency for overlapping dots. The black dots and ranges represent the predicted mean and 95 % confidence intervals, respectively.



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DECLARATION OF AUTHORSHIP

I, the author of this work, certify that this dissertation has been prepared independently. All aids and sources are clearly indicated and the contributions of other authors documented. The dissertation has neither been submitted at any other university nor been part of any other scientific examination.

Place, date

Signature

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ARTICLE 1

TITLE: Three perspectives on the prediction of chemical effects in ecosystems.

AUTHORS: **Schneeweiss, A**., Juvigny-Khenafou, N. P. D., Osakpolor, S., Scharmüller, A., Scheu, S., Schreiner, V. C., Ashauer, R., Escher, B. I., Leese, F., & Schäfer, R. B.

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CONTRIBUTIONS: Schneeweiss wrote in collaboration with Schäfer the main part of the original draft, and incorporated the reviewers' suggestions. All authors contributed to the general conceptual development, visualisation, writing of selected parts and reviewing of the manuscript.

ARTICLE 2

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STATUS: Published in 2022 in *Science of The Total Environment*, *836*, 155688. https://doi.org/10.1016/j.scitotenv.2022.155688

CONTRIBUTIONS: Schneeweiss designed the study, performed the field and laboratory work, conducted the data analyses, wrote the manuscript; Schreiner supported field and laboratory work and provided critical feedback on the R-script; Reemtsma conducted the chemical analyses; Liess designed the study; Schäfer designed the study, provided critical feedback on the statistical analysis, discussed results and edited the manuscript. All authors provided critical feedback on the manuscript.

ARTICLE 3

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STATUS: Published in 2023 in *Environmental Sciences Europe*, 35(1), 41. https://doi.org/10.1186/s12302-023-00747-y

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