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Living on the edge: Adaptation to pesticides and associated fitness costs

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Living on the edge: Adaptation to pesticides and associated fitness costs

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Ayesha Siddique

The thesis is based on the following publications

1. **Siddique, A**., Liess, M., Shahid, N., & Becker, J. M. (2020). Insecticides in agricultural streams exert pressure for adaptation but impair performance in *Gammarus pulex* at regulatory acceptable concentrations. Science of the Total Environment, 722, 137750. doi: https://doi.org/10.1016/j.scitotenv.2020.137750

2. **Siddique, A**., Shahid, N., & Liess, M. (2021). Multiple Stress Reduces the Advantage of Pesticide Adaptation. Environmental Science & Technology, 55(22), 15100-15109. doi: https://doi.org/10.1021/acs.est.1c02669

3. **Siddique, A**., Shahid, N., & Liess, M. (2023). Persistent pesticides effects favour single population dominance and reduces biodiversity of vulnerable species. (Submitted).

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Abstract

The decline of sensitive species in agricultural streams is mainly attributed to pesticide contamination, even below the regulatory acceptable concentrations. Very low toxic pressure may thus determine ecological and evolutionary processes responsible for the current loss in biodiversity, and leading to adaption at community and individual level. In order to improve the current risk assessment, this dissertation aims to analyze factors that may shape the response of organisms to chemicals in the field.

To analyze the effect of long-term exposure to low pesticide concentrations in natural populations, a field investigation was conducted **(Chapter 2)**. We observed that populations from contaminated streams were up to 2.5-fold more tolerant to clothianidin. However, populations showing increased insecticide tolerance were characterized by reduced survival, per capita growth and mating when cultured under pesticide free conditions.

Given that multi-stress conditions may occur more often under global change scenarios, the adaptation to one stressor might shape the response to another stressor **(Chapter 3)**. We observed that agricultural populations are on average 2-fold more tolerant to insecticide clothianidin as compared to reference populations. After experimental pre-exposure to very low concentration (LC_{50}/ 1000), only reference populations showed increased pesticide tolerance. Under multiple stress of pesticides and elevated temperature, both reference and agricultural populations showed a similar tolerance to the combined stress of pesticides and warming due to stronger synergistic effects in adapted populations. However, agricultural populations were more sensitive to elevated temperature alone due to the hypothesized fitness cost of genetic adaptation to pesticides and as a result, pesticide adaptation loses its advantage.

Although pesticide tolerance enables the survival of tolerant species in contaminated streams, long-term exposure to pesticides may alter their genetic structure **(Chapter 4)**. *G. pulex* collected from 38 small streams showed that pesticide exposure increased the pesticide tolerance, reduced

the genetic diversity, resulted in an adopted genetic composition and compromised individual fitness in locally adapted populations. Specifically, an increased frequency of "high contamination alleles" and a decrease of "low contamination alleles" was observed with increasing contamination. Furthermore, the individual per capita growth decreased with increasing trade-offs of genetic adaptation. Nevertheless, *G. pulex* contributed an average of 44% of macroinvertebrate abundance and benefited from reduced interspecific competition with vulnerable species in contaminated streams.

Condering global change scenario and persistent stress leading to adaptation, the question arises: How can the combined effects of these apparently contradictory processes be predicted **(Chapter 5)**? We show that pesticide adapted *G. pulex* from agricultural streams were more tolerant to pesticides (clothianidin, prochloraz) as compared to non-adapted populations. However, joint exposure to both pesticides and temperature stress resulted in acute synergistic interactions, and the combined effects were stronger in adapted populations. We hypothesize that the pesticide adaptation reduces general stress capacity of individuals and trade-off process increases sensitivity to the combined stress. The general stress exerted by each of the individual factors was quantified using the Stress Addition Model (SAM). These studies showed that pesticide pollution triggers adaptation from sub-organismal to community level. Unraveling these processes explains effects from genes to ecosystem level.

Zusammenfassung

Der Rückgang empfindlicher Arten in landwirtschaftlich genutzten Fließgewässern wird hauptsächlich auf die Verunreinigung durch Pestizide zurückgeführt, selbst wenn diese unterhalb der zulässigen Konzentrationen liegt. Eine sehr geringe toxische Belastung kann daher ökologische und evolutionäre Prozesse bestimmen, die für den derzeitigen Verlust der biologischen Vielfalt verantwortlich sind und zu Anpassungen auf Gemeinschafts- und individueller Ebene führen. Um die derzeitige Risikobewertung zu verbessern, zielt diese Dissertation darauf ab, Faktoren zu analysieren, die die Reaktion von Organismen auf Chemikalien im Feld beeinflussen können.

Um die Auswirkungen einer langfristigen Exposition gegenüber niedrigen Pestizidkonzentrationen in natürlichen Populationen zu analysieren, wurde eine Felduntersuchung durchgeführt **(Kapitel 2)**. Wir stellten fest, dass Populationen aus kontaminierten Bächen eine bis zu 2,5-fach höhere Toleranz gegenüber Clothianidin aufwiesen. Die Populationen, die eine erhöhte Insektizidtoleranz aufwiesen, waren jedoch durch eine geringere Überlebensrate, ein geringeres Pro-Kopf-Wachstum und eine geringere Paarungsbereitschaft gekennzeichnet, wenn sie unter pestizidfreien Bedingungen gezüchtet wurden.

Da unter den Szenarien des globalen Wandels Mehrfachstressbedingungen häufiger auftreten könnten, könnte die Anpassung an einen Stressor die Reaktion auf einen anderen Stressor beeinflussen **(Kapitel 3)**. Wir haben festgestellt, dass landwirtschaftliche Populationen im Vergleich zu Referenzpopulationen im Durchschnitt eine 2-fach höhere Toleranz gegenüber dem Insektizid Clothianidin aufweisen. Nach einer experimentellen Vorexposition bei sehr niedrigen Konzentrationen (LC50/1000) zeigten nur die Referenzpopulationen eine erhöhte Pestizidtoleranz. Bei Mehrfachbelastung mit Pestiziden und erhöhter Temperatur zeigten sowohl die Referenzpopulationen als auch die landwirtschaftlichen Populationen eine ähnliche Toleranz gegenüber der kombinierten Belastung durch Pestizide und Erwärmung, was auf stärkere

Synergieeffekte bei den angepassten Populationen zurückzuführen ist. Die landwirtschaftlichen Populationen reagierten jedoch empfindlicher auf die erhöhte Temperatur allein, was auf die angenommenen Fitnesskosten der genetischen Anpassung an Pestizide zurückzuführen ist, wodurch die Anpassung an Pestizide ihren Vorteil verliert.

Obwohl die Pestizidtoleranz das Überleben toleranter Arten in kontaminierten Fließgewässern ermöglicht, kann die langfristige Exposition gegenüber Pestiziden ihre genetische Struktur verändern **(Kapitel 4)**. Bei *G. pulex* aus 38 kleinen Bächen zeigte sich, dass die Pestizidbelastung die Pestizidtoleranz erhöht, die genetische Vielfalt verringert, zu einer angepassten genetischen Zusammensetzung führt und die individuelle Fitness in lokal angepassten Populationen beeinträchtigt. Insbesondere wurde mit zunehmender Pestizidbelastung eine Zunahme der Häufigkeit von Allelen mit hoher Kontamination" und eine Abnahme der Allele mit geringer Kontamination" beobachtet. Darüber hinaus nahm das individuelle Pro-Kopf-Wachstum mit zunehmenden Abstrichen bei der genetischen Anpassung ab. Dennoch trug *G. pulex* durchschnittlich 44 % zur Makroinvertebratenabundanz bei und profitierte von der geringeren interspezifischen Konkurrenz mit anfälligen Arten in kontaminierten Bächen.

In Anbetracht des Szenarios des globalen Wandels und des anhaltenden Stresses, der zu einer Anpassung führt, stellt sich die Frage: Wie lassen sich die kombinierten Auswirkungen dieser scheinbar widersprüchlichen Prozesse vorhersagen **(Kapitel 5)?** Wir zeigen, dass pestizidadaptierte G. pulex aus landwirtschaftlich genutzten Bächen toleranter gegenüber Pestiziden (Clothianidin, Prochloraz) sind als nicht adaptierte Populationen. Die gemeinsame Exposition gegenüber Pestiziden und Temperaturstress führte jedoch zu akuten synergistischen Wechselwirkungen, und die kombinierten Effekte waren bei angepassten Populationen stärker. Wir stellen die Hypothese auf, dass die Anpassung an die Pestizide die allgemeine Stresskapazität der Individuen verringert und der Ausgleichsprozess die Empfindlichkeit gegenüber dem kombinierten Stress erhöht. Der allgemeine Stress, der von den einzelnen Faktoren ausgeht, wurde mit dem Stress-Additions-Modell (SAM)

quantifiziert. Diese Studien haben gezeigt, dass die Verschmutzung durch Pestizide eine Anpassung von der suborganismischen bis zur gemeinschaftlichen Ebene auslöst. Die Entschlüsselung dieser Prozesse erklärt die Auswirkungen von den Genen bis zur Ökosystemebene.

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List of Abbreviations

AChE	Acetylcholinesterase
ADaM	Artificial Daphnia medium
AFLP	Amplified fragment length polymorphism
AMOVA	Analysis of molecular variance
ANOSIM	Analysis of similarity
ANOVA	Analysis of variance
AOP	Adverse outcome pathways
CA	Concentration Addition
C_{ew}	Equilibrium water concentrations
$C_{w,eq}$	Estimated equilibrium water concentrations
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleicacidtriphosphate
DO	Dissolved oxygen
EA	Effect Addition
EU	European Union
EC	Electrical conductivity
ECx	Effective concentration
EDSs	Event-driven water samplers
EFSA	The European food safety authority
Fst	Genetic differentiation
Fis	Inbreeding Coefficient
FA	Fragment analysis
GCA	Generalized concentration addition model
He	Expected heterzygosity
I4A	Inbreeding for AFLP
KOC	Soil organic carbon-water partition coefficient
KgM	Klein gewasser monitoring
LC	Lethal concentration
LC-HRMS	Liquid chromatography–high-resolution mass spectrometry
LME	Linear mixed-effects
LOEC	Lowest observed-effect concentration
LSER	Linear solvation energy relationship
MDR	Model deviation ratio
MOA	Mode of action
N_a	Number of alleles per locus
nAChRs	Nicotinic acetylcholine receptors
NOEC	No observed-effect concentration

nP	Private alleles
OECD	Organisation of economic cooperation and development
OMPs	Organic micro pollutants
PCR	Polymerase chain reaction
PICT	Pollution induced community tolerance
PLP	Polymorphic loci
POCIS	Polar organic chemical integrative samplers
PPDB	Pesticide properties database
PPPs	Plant protection products
RAC	Regulatory acceptable concentration
SAM	Stress addition model
SIMPER	Similarity percentage analysis
SPEAR	Species at risk
SPMD	Semipermeable membrane devices
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TU	Toxic Unit
TU_{max}	Toxic unit value representing maximum toxicity
TU_{sum}	Toxic unit value representing the sum toxicity of chemicals
uHe	Unbiased expected heterozygosity
UVR	Ultraviolet radiation
WWTP	Waste water treatment plant

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Introduction

1.1 Pesticide use

During the past few decades, pesticides are widely applied to increase the global crop yield (Zhang et al., 2011). Pesticides are frequently cast-off in agriculture in order to ovoid crop devastation from plant diseases. Historically, pesticides production can be separated into three stages: (1) in the first stage (before 1870s) natural pesticides such as sulfur were used to control pests, (2) in the second stage (1870s-1945), inorganic synthetic pesticides such as natural materials and inorganic mixtures were mostly used; (3) in the third stage (since 1945) organic synthetic pesticides became common. Since 1945, the man-made organic pesticides, for example DDT, 2,4-D, and later HCH, dieldrin, have taken over the era of inorganic and natural pesticides. In the former period of organic manufactured pesticides, there were primarily three classes of insecticides, carbamated-, organophosphorus-, and organochlorined- insecticides. Soon after that herbicides and fungicides attained a substantial expansion as well. The revolution in the field of pesticides occurred in 1940s when the chemical industry started manufacturing synthetic compounds to control insects (insecticides), animal pests (rodenticides), unwanted weeds (herbicides), and fungal diseases (fungicides) (Casida and Quistad, 1998).

In the modern agriculture, a varied range of plant protection products (PPPs) have been applied regularly. However, the distribution of pesticide application throughout the world is rather uneven (Pimentel, 1996). On the basis of total pesticide consumption, Asia uses the most (53.2%) followed by America (29.4%) and Europe (14%).

About 51.1% land in Germany is cultivated with crops or grass for meadows. In agriculture, farming approaches are central for the environment protection. Although the Federal Government is trying to rise the share of organic farming up to 20%, so far it covers only 10.8% of total cultivated land. The conventional farming is major form of agriculture and consumes a lot of active ingredients. For the last 20 years, the demand of pesticides has been dramatically increasing. Germany is among the top five countries in Europe that is using enormous quantities of pesticides. Germany, Italy, France, and Spain together share over 50% of the total European pesticide sales. In 2014, pesticide sales in Germany accounted for approximately 12% of all sales in the European Union (EU, 2016).

1.2 Fate and transport of pesticides

Pesticides are available in several forms (e.g., liquid, solid and gaseous), and can be applied through numerous methods (spraying, with water or incorporated in soil) (Miller, 2002). The application time and pesticides selection are directly associated with crop type, crop stage, application method, intended target, chemical formulation of the product and weather conditions (Leonard, 1988). After pesticide application, distribution and fate of pesticides also depends on factors such as application method, physicochemical characteristics of compound (e.g., persistence, water solubility, sorption affinity), soil properties and climatic conditions (reviewed by (Alletto et al., 2011)). Pesticides in the environment are subjected to various biotic (microbial degradation) and abiotic (chemical degradation in soil, sorption, photo-degradation, hydrolysis) dispersal mechanisms. Additionally, major pathways for the loss of pesticides to the environment include aerial drift, volatilization, runoff to surface water, and leaching into the groundwater. Application method is not only vital for the pesticide's efficiency (Van Timmeren et al., 2011) but it also affect the fate

of pesticides as potential water pollution (Davis et al., 1996). For example, in case of soil incorporated pesticides, loss occurs largely through microbial transformation rather than volatilization and surface runoff. In contrast, for pesticides sprayed directly on crops or bare soils, volatilization and runoff events primarily contribute to the pesticide loss (Davis et al., 1996; Kronvang et al., 2004). Similarly, chemical properties of pesticides also impact their reactivities (Chaplain et al., 2011). For example, highly soluble compounds may leach out through soil to groundwater, and/or to be lost via surface runoff during extreme rainfall events. In contrast, volatile pesticides can simply evaporate in the atmosphere. Sorption is another key process that governs the distribution, fate, and ecological effects of the toxicants in the environment. Some pesticides can be sorbed strongly to the soil organic matter (Bondarenko and Gan, 2004; Kookana et al., 2014), and do not leach out easily. In this respect, pesticides degradation may take few minutes to several years. In the natural environment, pesticide degradation includes biogeochemical cycles.

Plant protection products enter into surface waters via point and non-point sources. The point source pathways consist of farmyard runoff, wastewater treatment plant effluents, and spillage where non-point pesticide contribution comes from air-borne and water-driven transport processes. After entering into a water bodies, behavior and fate of a pesticide is determined by its chemical properties (e.g., solubility and persistence) and the characteristics of the streams (e.g., current velocity, concentration of suspended particles, and constituents). Though freshwater covers only 0.8% of the Earth's surface, it supports a rich biological diversity. According to an estimate, about 100,000 of 1.8 million species inhabit freshwater ecosystem which is approx. 6% of the total biodiversity (Dudgeon et al., 2006). Rivers and streams are common freshwater sources that offer habitats for aquatic organisms. Most of the aquatic species are extremely important and provide numerous invaluable ecosystem services such as leaf litter degradation, water purification, food provision and climate regulation (Covich et al., 1999; Gamfeldt et al., 2008; Graça, 2001; Wallace and Webster, 1996). However, the stream ecosystems are highly

impacted by numerous toxicants such as organometallic compounds, pesticides, pharmaceuticals, detergents, personal care products, biocides, industrial chemicals, halogenated organic compounds and other organic micropollutants (OMPs) (Schwarzenbach et al., 2006). In agricultural areas, pesticide contamination is a well-known stressor that induce detrimental effects on aquatic macroinvertebrates (Schäfer et al., 2007). For instance, (Ippolito et al., 2015) modeled the runoff potential of pesticides worldwide and concluded that the freshwater systems within 40% of the global land surface area are likely to be ecologically affected by the insecticide exposure.

Pesticide exposure substantially affects the community composition of freshwater macroinvertebrates (Beketov et al., 2013; Liess et al., 2021b; Shahid et al., 2018). For instance, (Schäfer et al., 2007) investigated 29 streams in Finland and France, and reported a significant decline in sensitive species. Similarly, (Orlinskiy et al., 2015) observed significant reduction in sensitive species even at very low pesticide concentrations ($TU_{max} \leq -3$). Another study of (Beketov et al., 2013) revealed that the pesticide pollution in agricultural streams poses significant effects on species- and family richness in Australia and Europe, with up to 42% loss of the macroinvertebrate taxa. Recently, a nationwide monitoring comprised of more than 100 streams in Germany revealed altered community structure of macroinvertebrates even at regulatory compliant concentrations of pesticides (Liess et al., 2021b). In agricultural landscapes, pesticides are mainly responsible for the decline in invertebrate populations (Liess et al., 2021b; Stoate et al., 2001). Furthermore, the structural alterations of macroinvertebrate communities can compromise related ecosystem functions (Münze et al., 2015; Peters et al., 2013; Schäfer et al., 2012). In aquatic ecosystems, leaf litter breakdown and primary production are considered as a major source of energy for the freshwater food web. Recently, (Peters et al., 2013) reviewed available literature regarding the effects of pesticide on freshwater ecosystem structure and functioning. The meta-analysis revealed potential adverse effects even below the regulatory acceptable concentrations. Numerous field and laboratory investigations have

reported pesticide induced effects on leaf litter degradation, primary production and community respiration (Abelho and Graça, 2000; Widenfalk et al., 2004; Wilson et al., 2004).

1.3 Monitoring of pesticides

Field monitoring of pesticide exposure is particularly important in scientific understanding of environmental fate of pesticides. In agricultural landscapes, pesticides may enter the aquatic ecosystems through runoff in the form of pulses, and cause short term contamination (Liess et al., 1999; Russo et al., 2018). Therefore, linking observed effects of pesticides on aquatic invertebrates is not possible without adequate monitoring of pesticide peak concentrations (Friberg et al., 2003). Contamination load in agricultural streams mainly depends on the pesticide quantity applied in the field, timing and rainfall intensity, octanol-water partition coefficient (K_{ow}) (Neumann et al., 2002; Schulz, 2004) and the soil hydrology (Doppler et al., 2012; Leu et al., 2004). Further, partitioning of pesticides between water, sediments and atmosphere depends on their volatility and hydrophobicity. In aquatic ecosystems, contaminants may dissolve or bound to particulate organic matter, undergo further redistribution processes and change the exposure of the aquatic organisms accordingly.

For water quality monitoring, numerous sampling techniques have been employed. The conventional methods of water monitoring generally rely on grab water sampling at fixed time intervals. However, this strategy seems inappropriate to capture spontaneous and periodic fluctuations i.e. following pesticide application or after major rainfall events (Guo et al., 2004; Liess et al., 1999; Schulz, 2004), and therefore are known to underestimate the pesticide concentrations (Stehle et al., 2013; Xing et al., 2013). In comparison, event-driven sampling is a reliable technique to capture peak concentrations after rainfall events, and has commonly been applied for collecting water samples from streams (Beketov et al., 2013; Liess et al., 2021b; Shahid et al., 2018).

Passive sampling enables continuous monitoring of water contamination and represents an effective alternative to grab sampling. These simplified

and miniaturized sampling techniques do not require any energy supply for the entire setup. Passive samplers are specifically designed for selected target compounds according to the type of monitoring (Greenwood et al., 2007). In the last decade, various passive samplers have successfully been employed for the monitoring of different contaminants in freshwater systems (Münze et al., 2015; Vrana et al., 2005).

1.4 Biological monitoring and effects of pesticides

Ecological knowledge of streams provides an integrated picture of ecosystem health. Evaluating the quality status of waterbodies using biological information of the local biota in surface water is known as biological monitoring (Barbour et al., 1999). Biological monitoring approaches are principally based on the biological responses of aquatic communities to undesired natural or anthropogenic changes in water quality (Bonada et al., 2006) (Karr and Chu, 1998). Biological monitoring is one of the most appropriate approaches for the characterization of stream health (Karr, 1999), and can be performed at numerous levels (Allan et al., 2006). Indeed, assemblages of different aquatic populations are quite useful in biological assessment; stream macroinvertebrates are commonly used because of their abundance, easy sampling and identification, and quick response to a broad range of contaminants. Moreover, benthic macroinvertebrates are less mobile, and have aquatic life-cycle stages long enough to provide clear and localized picture of biological responses (Barbour et al., 1999). They have been employed for the biological monitoring of a wide array of stressors including pesticides (Malaj et al., 2014; Shahid et al., 2018), hydro morphological degradation (Lorenz et al., 2004; Nõges et al., 2016), acidification (Dangles and Guérold, 1999), habitat destruction (Von der Ohe and Goedkoop, 2013), salinity (Schäfer et al., 2012; Szocs et al., 2012) and eutrophication (Hering et al., 2006).

For biomonitoring of pesticide contamination in running waters, (Liess and von der Ohe, 2005) developed a trait-based approach SPEARpesticides (SPEcies At Risk). SPEAR index is based on four ecological traits: (1) sensitivity to toxicants; (2) generation time; (3) potential of pesticide exposure; and (4) recolonization ability. Generally, the SPEAR index

categorizes the aquatic taxa into sensitive- and tolerant species. SPEAR quantifies the proportion of sensitive macroinvertebrates to a total number of macroinvertebrates (Eq. 1.1).

$$\mathrm{SPEAR}_{\mathrm{pesticides}} = \frac{\sum_{i=1}^{n} \log(x_i+1)y}{\sum_{i=1}^{n} \log(x_i+1)} \times 100 \qquad (1.1)$$

where n is the total number of taxa; xi is the richness of taxon i, and y is 1 if taxon i is at risk, otherwise 0.

Biodiversity decline due to anthropogenic stressors in the last five decades is a record in human history (Assessment, 2005). In spite of general concern and numerous international initiatives (AREAS, 2003; Outlook, 2010), the present rate of biodiversity decline seems to be accelerating instead of reducing (Butchart et al., 2010; Walpole et al., 2009). The upcoming costs of this crisis may be dramatic, as the latest studies revealed the chances of planetary scale ecosystem shift to an unknown and irreversible state (Barnosky et al., 2012). Pesticide contamination is considered one of the key drivers for the worldwide decline in the richness and diversity of insects, plants and birds (Beketov et al., 2013). There is mounting evidence that the pesticide-driven impairment of biogenesis also disturbs ecosystem services extending from pollination (Rundlöf et al., 2015) to leaf-litter degradation (Schäfer et al., 2011) and to the biological control of agricultural pests (Roubos et al., 2014; Talebi et al., 2008) and of pathogens in freshwater.

On the other hand, pesticide pollution may lead to a community shifted towards a more tolerant community when stressors exert a selection pressure as formulated in the PICT (pollution induced community tolerance) concept, (Backhaus et al., 2004). However, induced tolerance is often associated with costs, as species shift may lead to the decline of diversity and functions of the biological community, and consequently result in impaired ecosystem functioning and stability (Rolf D. Vinebrooke, 2004; Schmitt-Jansen et al., 2008). Similarly, at the organism level, induced tolerance is associated with costs, since energy allocation into defense mechanisms requires trade-offs in other processes. Provided that organisms differ in their sensitivity and vulnerability to stressors, and

that complex stressor interactions occur in networks, it is not surprising that exposure of organisms to multiple stressors frequently leads to unforeseen effects.

1.5 Mechanisms of Tolerance development

Anthropogenic stressors are likely to cause major detrimental effects on aquatic organisms (Campos et al., 2016; Rodrigues et al., 2015). However, a substantial number of natural populations may still persist in highly contaminated areas by acquiring tolerance to stressors. Increased tolerance is mainly acquired through physiological acclimation and/or genetic adaptation to contaminants (Janssens et al., 2009; Medina et al., 2007; Ribeiro and Lopes, 2013). Physiological acclimation is an individual level reversible adaptive response where organisms acquire tolerance upon exposure to contaminants (Adeyemi and Klerks, 2013; Farwell et al., 2012). On the other hand, genetic adaptation involves variations at the genetic level, and development of tolerance at the population level by the selection of tolerant genotypes and/or removal of sensitive ones (Auld et al., 2010; Lopes et al., 2004; Ward and Robinson, 2005; Xie and Klerks, 2003).

Phenotypic plasticity serves a single genotype to show variable phenotypes in various environments, which allows adaptive traits to be induced within a single generation (Schlichting and Pigliucci, 1998). In a rapidly changing environmental conditions, the contribution of plasticity to the organismal adaptation has vital consequences for the expression of traits within individuals and the evolution of populations. With increasing anthropogenic activities, an important challenge is to consider the role of plasticity in shaping organism responses to disturbances. Upon occasional exposure, inducible traits are maintained as it facilitates the expression of adaptive phenotypes in response to variable environmental conditions (Weston et al., 2013). Physiological induction of tolerance has been observed after low insecticide exposure in mosquitoes and wood frogs from less-contaminated sites (Hua et al., 2013; Poupardin et al., 2008). In contrast, when populations experience long-term exposure over multiple generations, maternal effects result in increased tolerance, but a plastic

response eventually becomes constitutively expressed, resulting in genetic adaptation. For instance, some populations of *Hyalella Azteca* have shown a 550-fold greater resistance to pyrethroids as compared to wild-type populations (Weston et al., 2013). Further investigation revealed that one of three different single amino acid substitutions in the voltage-gated sodium channel (VGSC) was responsible for resistance to pyrethroids, with different mutations in different populations. This shows that several independent selection events arose in the different populations (Major et al., 2018). Furthermore, two amino acid substitutions (L925I and L925V) were proved to be completely recessive in *H. azteca*, signifying that these mutations were rapidly fixed in the populations due to high pyrethroid exposure (Sever et al., 2020).

There are numerous other factors that may affect the tolerance development. For example, (Becker and Liess, 2017) reported that the species diversity hampers adaptation to pesticides in *Gammarus pulex*. Similarly, (Shahid et al., 2018) revealed the effect of local contamination level and distance from the recovery area on resistance development. Life stage at the time of exposure is another important factor that may affect the evolution of resistance (Becker and Liess, 2017; Becker et al., 2020).

1.6 Impact of contaminants on genetic diversity

With wide-ranging farmland and application of pesticides, subsequent runoff into aquatic ecosystem has triggered evolutionary changes such as reduced genetic diversity throughout non-target invertebrate communities (Stuhlbacher and Maltby, 1992). When population-level processes of genetic adaptation to contaminants are not compensated by new genetic input, exposed populations may experience decreases in their levels of genetic diversity, the so-called contaminant-derived genetic erosion (Fasola et al., 2015; Ribeiro and Lopes, 2013; van Straalen and Timmermans, 2002). In this context, small and isolated populations are expected to be mainly susceptible to genetic erosion. As the probability of genetic drift and the increasing expression of recessive deleterious alleles is higher, populations may further loses genetic diversity and reproductive fitness, hence, increasing the risk of population extinction (Frankham,

2005; Reed and Frankham, 2003). Since the ability of natural populations to adapt to future environmental changes is directly associated with the amount of standing genetic diversity (Frankham, 2005, 2015), genetic erosion may affect the long-term survival of populations.

During recent years, investigations on genetic diversity have gained interest in evolutionary toxicology studies and neutral markers such as amplified fragment length polymorphisms (AFLP) or microsatellites (besides others) have been used to understand and predict long-term ecological impacts of contaminants (van Straalen and Timmermans, 2002). Neutral markers provides insight into the current status, dynamics and past selection pressures exerted on populations by investigating genetic variation in the non-coding region of DNA within and among populations (Hoffmann and Willi, 2008). Even though the suitability of neutral markers to assess the long-term consequences of contaminants has been established under laboratory settings (Athrey et al., 2007; Nowak et al., 2009), only a few studies have provided clear evidence of contaminant-driven genetic erosion under natural conditions (Maes et al., 2005; Paris et al., 2015). Laboratory studies showed that exposure to tributyltin decreased genetic diversity of *Chironomus riparius* populations within only few generations as a consequence of numerous life-history modifications including increased mortality and reduced fertility (Nowak et al., 2009). Likewise, laboratory populations of the killifish *Heterandria formosa* exposed to cadmium for eight generations exhibited 10–20% reduced genetic diversity as compared to control populations (Athrey et al., 2007). However, these laboratory indications contrasted with no reduction in genetic diversity in natural populations of the *Daphnia longispina* historically exposed to acid mine drainage (Martins et al., 2009), the killifish *Fundulus heteroclitus* populating highly contaminated urban harbors (McMillan et al., 2006) or the *Sander vitreus* inhabiting river sections polluted with endocrine active compounds (Miller et al., 2012). Investigating genetic diversity during and after contaminating events is difficult as the genetic diversity of natural field populations is highly dynamic and is affected by a multitude of interrelated biotic and abiotic components (Frankham, 2005; Moe et al., 2013). Patterns of genetic

variation within and among natural populations are impacted by various historical and recent factors, like past and present selection processes, migration and chance events. Most field studies regarding the effects of contamination on genetic variation, however, do not consider those natural processes adequately (Bickham et al., 2000), and therefore, fail to distinguish between natural ‘‘background noise’’ and anthropogenic impacts.

Genetic responses at the population level comprises of increased mutation rates and changes in genome-wide genetic diversity and allelic or genotypic frequencies due to contaminant-mediated selective pressures and altered gene flow between populations (Bickham, 2011). Even though our knowledge of the genetic effects of pesticides on freshwater species populations is still in its infancy, empirical evidence indicates that these effects are capable of altering neutral genetic diversity and structure (Inostroza et al., 2016) and can drive adaptive genetic variation (Weston et al., 2013). This suggests that agrichemical contamination may be a major driver of evolutionary change in freshwater ecosystems.

1.7 Fitness costs of adaptation

The acquisition of pesticide resistance in non-target aquatic organisms is an evolving issue, with a growing number of studies recording tolerance in both wild and laboratory populations to both current use and old compounds (Jansen et al., 2011a; Weston et al., 2018). While the development of resistance to contaminant may superficially reveal the adaptive capacity of organisms to survive exposure to anthropogenic contaminants, it is crucial to consider potential fitness costs associated with the resistance development. For example, pyrethroid resistance in *H. Azteca* acquired via mutations on the vgsc that decrease sensitivity to pyrethroids could lead to reduction in general fitness, because of reduced efficacy of the *VGSC* and metabolic costs related to the alteration or due to “genetic hitchhiking” of less desirable traits linked to the mutations (van Straalen and Timmermans, 2002; Zhao et al., 2000) . Furthermore, fitness costs related to mutations causing resistance to xenobiotics are not a new concept. (Fisher, 1958) established a model of adaptation that discusses

how autonomous selection pressures outline current phenotypes through gene co-evolution. Because of the complex gene interdependence, mutations with large phenotypic effects are likely to induce deleterious effects. Another study demonstrated that pyrethroid-resistant *H. azteca* were more sensitive to thermal stress as compared to non-resistant animals suggesting that future climate scenarios may lead to increased sensitivity to temperature variations in resistant organisms and intensify the potential fitness costs associated with resistance (Heim et al., 2018). Pyrethroid-resistant *H. azteca* may also be more susceptible to acute changes in salinity as compared to non-resistant individuals, though this could not be confirmed statistically (Heim et al., 2018). Alterations to thermal tolerance are known in other crustaceans raised under different salinity regimes, and can impact a wide range of processes including growth, acclimation rate, and survival (Kumlu et al., 2010).

1.8 Toxicant mixtures

In agricultural streams, organisms are often exposed to chemical mixtures. For instance, high loads of pesticide mixtures can be found in agricultural streams, especially after runoff events (Knillmann et al., 2018; Liess and von der Ohe, 2005). Therefore, the quantification of toxic effects is difficult and a key challenge for ecologists (Laetz et al., 2009). In general, pesticides can be classified on the basis of respective targets such as insecticides, fungicides and herbicides, chemical structure and modes of action (Casida, 2009). Organophosphates insecticides such as chlorpyrifos, temephos, malathion, dimethoate and diazinon contain phosphorus, and inhibit acetylcholinesterase (AChE) activity, an enzyme that hydrolyze the neurotransmitting agent acetylcholine (Carlock et al., 1999; Fukuto, 1990). Inhibition of AChE leads to acetylcholine buildup, that overstimulates neurological activity (Beauvais et al., 2000; Sismeiro-Vivas et al., 2007). Carbamates insecticides such as carbofuran and carbaryl also inhibit AChE activity (Fukuto, 1990). Pyrethroids insecticides such as esfenvalerate, deltamethrin and cypermethrin causes neurological damages, but the target site is Voltage-dependent sodium channels (Casida, 2009; Fukuto, 1990; Leahey, 1985). In contrast, neonicotinoids are systemic compounds, extensively applied for crop protection against pest insects (Nauen and

Denholm, 2005), and are well known substitute of carbamate and organophosphate insecticides, registered in more than 120 countries (Morrissey et al., 2015). The neonicotinoid insecticides affect the nervous system; specifically act agonistically on nicotinic acetylcholine receptors (nAChRs) (Casida and Durkin, 2013; Jeschke et al., 2010), responsible for post-synaptic neurotransmission in both invertebrates and vertebrates (Millar and Denholm, 2007). Due to systemic nature and effectiveness, neonicotinoids are most commonly applied insecticides worldwide (Pisa et al., 2015; Simon-Delso et al., 2015a).

However, the local mass mortality of bees has been associated with the large-scale use of neonicotinoids. In 2013, the European Food and Safety Authority (EFSA) affirmed neonicotinoids to be an inadmissibly high risk to bees (EFSA, 2018). Furthermore, a decrease of a broad variety of insects in neonicotinoid usage areas could already be reported (Sanchez-Bayo and Wyckhuys, 2019). As a consequence, EU limited the use of the neonicotinoids Clothianidin, Imidacloprid, and Thiamethoxam in plant protection and seed treatment products. In 2018, these limitations were supported by extensive data collection, and outdoor uses of all three products were banned. Other neonicotinoids registered as active substances in the EU remain unrestricted and EFSA recently concluded that one of these, acetamiprid, poses a low risk to bees (Bass and Field, 2018).

Nevertheless, pesticide mixtures especially with different mode of actions may act synergistically. Regarding the relevance of synergistic toxicant mixtures in the field, the funnel hypothesis suggests that the deviation from additivity decreases with an increasing number of components in a mixture. While pesticide mixtures in the field may contain several toxicants including insecticides, fungicides and herbicides (Münze et al., 2017) and are rarely equitoxic. Thus, pronounced interactions might be even more likely to occur in real ecosystems when few toxicants dominate the overall toxicity (Cedergreen, 2014).

Traditional ecotoxicology determines the inherent toxicity of chemicals using single species tests and a limited set of apical end points (survival,

growth, reproduction). However, in reality, aquatic ecosystems are challenged with multiple stress factors. Current approaches to assess the risk of anthropogenic stressors to aquatic ecosystems are developed for single stressors and determine stressor effects primarily as a function of stressor properties. The cumulative impact of several stressors, however, may differ markedly from the impact of the single stressors and can result in non-linear effects and ecological surprises. To meet the challenge of diagnosing and predicting multiple stressor impacts, assessment strategies should focus on properties of the biological receptors rather than on stressor properties. This change of paradigm is required because (i) multiple stressors affect multiple biological targets at multiple organizational levels, (ii) biological receptors differ in their sensitivities, vulnerabilities, and response dynamics to the individual stressors, and (iii) biological receptors function as networks, so that actions of stressors at disparate sites within the network can lead via indirect or cascading effects, to unexpected outcomes.

Assessment of pesticide combination effects is usually based on models of concentration addition (CA, assuming a similar mode of action for the mixture components) or independent action (IA, assuming a dissimilar mode of action). The predictive power of these models to estimate mixture toxicity has been documented in several studies (Backhaus et al., 2004; Faust et al., 2001; Tang and Escher, 2014). In the last decade, additional toxicological approaches Stress Addition Model (SAM) (Liess et al., 2016a) have been developed. The SAM is an appropriate approach to quantitatively predict the synergistic effects of multiple stressor combinations like contaminants and environmental stressors. Up until now, SAM is the only approach that reflects sensitivity distribution within a population, and predicts a range of effects for chemicals under different levels of environmental stress.

1.9 Environmental stressors

Ecotoxicological risk assessment of environmental chemicals is predominantly based on the results of laboratory studies where test organisms are exposed to a range of concentrations of single compounds.

This approach is useful for the generation of dose–response relationships and the derivation of toxicity data such as the concentration causing 50% impairment of a life history trait (e.g. LC_{50} or EC_{50}). In such laboratory experiments the test organisms are almost always kept in optimal conditions (temperature, moisture, food etc.) to optimize performance in the control treatment and isolate the effects of the chemical in question. This is the advantage of traditional laboratory testing, and methods have been greatly improved and standardized during the last decades (see e.g. (Walker et al., 2005). However, in their natural settings organisms rarely experience optimal conditions. On the contrary for most of their lifespan, organisms are forced to cope with sub-optimal conditions with frequent exposure to severe environmental stress (Holmstrup et al., 2010). Generally, sub-optimal conditions can be attributed to the physical, chemical and biological stressors (Lydy et al., 2004).These added environmental stressors may or may not alter the effects of chemical contaminants in comparison to the optimal laboratory test performed in well-controlled conditions. (Dinh et al., 2016; Heugens et al., 2001; Pieters et al., 2005). For instance, environmental parameters such as pH, temperature, or ultraviolet radiation (UVR) and food stress may affect the bioavailability of toxicants, toxicokinetic and physiological state of organisms (Clements et al., 2008; Franklin et al., 2000; Kashian et al., 2004). (Pettis et al., 2012) showed that the increased mortality of wild honey bee colonies was due to the interactions between pesticides and pathogens. In another study, pathogens amplified the effect of agrochemicals on amphibians (Rohr et al., 2008). Similarly, food limitation and UV-B radiations amplified the sensitivity of marine crustaceans to copper (Liess et al., 2001). Numerous additional environmental stressors such as elevated temperature, pH, salinity, and eutrophication are also recognized to interact and amplify the toxic effects of contaminants (Beermann et al., 2018; Matthaei et al., 2010). Lately, (Delnat et al., 2019) studied the effect of daily temperature variation on combined toxicity of chlorpyrifos and a biopesticide *Bacillus thuringiensis* variant in mosquito *Culex pipiens*. A high daily temperature variation altered the interaction between both stressors from additive to synergistic. Furthermore, streams draining agricultural land are often characterized by

loss of hydro morphological and habitat heterogeneity due to dredging and channelization, increased concentrations of macronutrients, and increased transport of suspended material (Friberg et al., 2010; Pedersen, 2009). These effects may additionally have profound effects on ecosystem structure and function (Pedersen and Friberg, 2009; Piscart et al., 2009; Rasmussen et al., 2011). Even though the so-called mixture-toxicity has been debated for a long time, understanding the interactive effects of multiple stressors is still limited. These interactive effects are of great interest for the management of aquatic ecosystems.

1.10 Objectives and outline

In the recent years, a considerable decline in biodiversity have been observed worldwide. However, a number of natural populations still persist by developing increased tolerance. Although adaptation to stressors helps individuals to survive under stressed conditions, the response may change under real field conditions confronted with pesticides mixtures and environmental stressors. Therefore, the aim of this doctoral research was to investigate pesticide adaptation and potential costs in natural populations that are not well understood. Objectives of the study were addressed through a series of activities, including field research, and laboratory studies. Specifically, this study includes several investigations to answer the following research questions:

1. Do natural populations of *G. pulex* from agricultural streams have developed increased tolerance to pesticides and have compromised general fitness?

2. Do pesticide adapted populations have benefit under multiple stress of pesticides and elevated temperature?

3. Are pesticides adapted populations affected in terms of reduced genetic diversity, and how individual trade-offs are linked at community level?

4. How the response of pesticide adapted populations is further shaped under pesticide mixtures and elevated temperature?

In order to achieve these objectives, first of all, changes in community structure and tolerance development in non-target invertebrates to pesticides were assessed experimentally using a neonicotinoid insecticide clothianidin and dominant crustacean *Gammarus pulex*. To analyze the fitness costs of increased pesticide tolerance, field populations were cultured in lab under non-contaminated conditions (CHAPTER 2). Additionally, field populations of *Gammarus pulex* from agricultural and reference streams with differential pesticides tolerance were exposed to combined stress of clothianidin and elevated temperature. We also quantified different mechanisms of tolerance and revealed fitness costs of tolerance under warming (CHAPTER 3). Further investigations focused on the long-term consequences of pesticide exposure on genetic structure and diversity in natural populations of *Gammarus pulex* using AFLP assay. We also addressed other factors that affect tolerance development and genetic diversity of the populations (CHAPTER 4). In order to investigate the benefit of pesticide adaptation in field exposed populations, the clothianidin tolerance was tested in combination with an azole fungicide prochloraz and elevated temperature (CHAPTER 5). From this dissertation, CHAPTER 2 was published in the journal Science of the Total Environment, CHAPTER 3 was published in Environmental Science and Technology, CHAPTER 4 is under review in Global Change Biology and CHAPTER 5 is under review in Environment International. Finally, a brief synthesis of obtained results, conclusions and outlook for future research are given in CHAPTER 6.

2

Increased pesticide tolerance and impaired performance in *G. pulex*

2.1 Abstract

Pesticide exposure in agricultural streams requires non-target species to adapt. However, pesticides may reduce performance in between exposure events due to long-term effects and physiological fitness costs of adaptation. Here, we investigated the long-term consequences of pesticide exposure to low concentrations in the widespread crustacean *Gammarus pulex*. We collected populations from six German streams covering no to moderate agricultural pesticide exposure. Peak concentrations ranged up to 1/400 of their acute median lethal concentration (Toxic Unit = -2.6), resulting in significant changes in the macroinvertebrate community composition ($SPEAR_{pesticides}$ = of 0.2). Acute toxicity tests revealed up to a 3-fold increased tolerance towards the most frequently found insecticide clothianidin compared to individuals collected from non-contaminated streams. However, populations showing increased insecticide tolerance were characterized by reduced survival, per capita growth and mating adults when cultured under pesticide-free conditions in the laboratory for three months. We conclude that pesticide pollution triggers adaptation both

at the species and the community level even at concentrations considered to be safe according to the European pesticide legislation. In *G. pulex*, adaptation is associated with impaired performance which potentially affects ecosystem functions such as leaf litter degradation. These long-term impairments need to be considered in deriving safe concentrations.

Key words: *Gammarus pulex*, Fitness costs, Pesticide exposure, Adaptation, Neonicotinoids, Ecotoxicology

2.2 Introduction

Macroinvertebrates in agricultural streams are exposed to pesticides, especially during the surface runoff following rainfall events (Kreuger, 1998; Liess and Schulz, 1999). This pesticide exposure may extend from a few minutes to several hours, depending on the intensity of rainfall, pesticide properties and the characteristics of the water body. Pesticide exposure poses deleterious effects on the structure and functions of macroinvertebrates in agricultural streams (Hunt et al., 2017; Liess and Ohe, 2005; Münze et al., 2017; Shahid et al., 2018). Furthermore, Beketov et al. (2013) reported significant effects of pesticide contamination on the species and family richness of macroinvertebrates. Significant change in the macroinvertebrate communitiy composition has been reported even at 3 to 4 orders of magnitude below the acute median lethal concentration (LC_{50}) of laboratory standard test species (Liess and von der Ohe, 2005; Schäfer et al., 2012). Reasons for these low environmental effect concentrations include an increased sensitivity of individuals to pesticides under multiple stress conditions (Liess et al., 2016a; Liess et al., 2019) and the culmination of effects from sequential exposure (Liess et al., 2013b). Accordingly, the exposure to pesticides may exert a considerable pressure for adaptation, which results in increased pesticide tolerance in exposed species (Becker and Liess, 2015; Delnat et al., 2020; Sparks and Nauen, 2015; Vigneron et al., 2015; Weston et al., 2013). Recently, Becker and Liess (2017) and Shahid et al. (2018) reported 3- to 4-fold increased pesticide tolerance in *Gammarus pulex* collected from agricultural streams.

However, adaptation to pesticides is often associated with considerable fitness costs under non-toxic conditions (Becker and Liess, 2015; Delnat et al., 2020; Medina et al., 2007; Meyer and Di Giulio, 2003). Fitness costs of increased pesticide tolerance (resistance) may reduce ability for population recovery in between events of pesticide exposure. Bach and Dahllof (2012) revealed cost of resistance to high pesticide concentrations (causing acute mortality in non-adapted populations) in marine amphipod collected from contaminated fjord. Similarly, (Heim et al., 2018) reported lower reproductive capacity and lower upper thermal tolerance in pyrethroid-resistant *Hyalella azteca* individuals compared to non-resistant individuals. Obviously, coping with the toxicity of high insecticide concentrations is costly, and requires energy and resource allocation for adaptation and survival. However, fitness costs of resistance to lower pesticide concentrations that typically cause no acute mortality but sub-lethal effects (TU ≤ -3) have never been investigated. Such concentrations have been observed regularly in the field (Knillmann et al., 2018; Liess and von der Ohe, 2005) and are generally considered to be safe according to the European pesticide legislation (EFSA, 2013). Unraveling these effects in real field conditions may contribute to our understanding of why pesticides affect the macroinvertebrate community composition in the field at concentrations much lower than those predicted to be safe based on the sensitivity tests in the laboratory (Liess et al., 2013b).

This study aimed at investigating the effects of pesticide exposure on the tolerance and fitness of *Gammarus pulex* which is one of the most common freshwater macroinvertebrate species in central European streams with high ecological relevance for leaf litter degradation (Schäfer et al., 2012). We were particularly interested in potential effects of increased pesticide tolerance on key life-history traits. For this purpose, populations from agricultural and reference streams with different tolerances to pesticides were cultured under uncontaminated conditions and the variable: survival, per capita growth and reproduction were monitored for three months.

2.3 Materials and Methods

2.3.1 Study design

We collected *Gammarus pulex* from six sites that covered a range of non-contaminated to moderately contaminated streams in central Germany. The test organisms were sampled from April to June 2018 during the period of peak pesticide application. Additionally, macroinvertebrates were sampled to quantify effects of pesticide exposure on the community composition using the $SPEAR_{pesticides}$ bioindicator. Pesticide exposure during the study period was measured from water samples collected during run-off events after heavy rainfalls. The organisms were acclimatized to test conditions for seven days and subsequently exposed to six different concentrations of the neonicotinoid insecticide clothianidin. Additionally, we cultured each population in a climate chamber under standardized pesticide free conditions for three months. Long-term endpoints such as survival, per capita growth and reproduction of cultured organisms were recorded.

2.3.2 Study sites

Three sites were located in less contaminated forested area and selected as reference sites, whereas streams close to agricultural fields were not protected and showed considerably higher pesticide exposure (Table 2.1). None of these sites were influenced by other sources of contaminants such as wastewater treatment plants (WWTPs), industrial effluents or mining drainage. During the sampling, different physico-chemical parameters such as electrical conductivity (EC), temperature, pH, and dissolved oxygen (DO) were also measured showing that these environmental parameters were well in the range of favourable conditions for *G. pulex* (McCahon and Pascoe, 1988b)(Table 2.1).

Table 2.1. Pesticide exposure and physico-chemical properties of the study sites.

Site	Coordinates	EC (µS)	Temp	pH	DO	SPEAR	TU_{max}	Most toxic compound	Compound class
Ref-1	50.59251 10.64666	728	14	8.5	-	0.91	-5.34	2_4_Dichlorophenoxy-acetic acid	Phenoxy herbicides
Ref-2	52.1656 10.83203	756	8.9	7.56	8.9	1.00	-5.83	Fluroxypyr	Pyridine herbicides
Ref-3	51.33528 12.97136	534	15.8	7.63	-	0.69	-3.69	2_4_Dichlorophenoxy-acetic acid	Phenoxy herbicides
Agri-1	51.28995 12.15237	2,060	15.4	8.08	11.5	0.09	-2.65	Thiacloprid	Neonicotinoids
Agri-2	51.46098 11.47198	1,033	15.7	8.5	11.8	0.34	-3.63	Thiacloprid, clothianidin	Neonicotinoids
Agri-3	52.27735 10.75138	1,839	15.9	8.27	14.7	0.37	-2.79	Thiacloprid, clothianidin	Neonicotinoids

2.3.3 Sampling of macroinvertebrates and test organisms

Macroinvertebrates were collected from 20 subsamples along a 50 m stream section, following the German nationalguidelines for the biological monitoring of streams (Gellert et al., 2014). In short, we collected organisms using a kick-sampler (25 x 25 cm) with a mesh size of 500µm, sorted specimens in a white tray and preserved them in 70 % EtOH for subsequent taxonomic identification in the laboratory. Individuals of *Gammarus pulex* were collected using a spoon or pipette and transported to the laboratory under constant aeration in a cooling box. Every week, a highly polluted and a lowly polluted population were sampled and tested to avoid a potential bias that may result from temporal variation in pesticide tolerance.

2.3.4 Quantification of pesticide exposure

Water samples from all sites were collected under the KgM project using two different techniques. The number of samples from each site varied from 1 to 6, depending on rainfall events. Briefly, computer triggered event samplers (Liess et al., 1999) and water level triggered event samplers (Liess and von der Ohe, 2005) were installed at each site. Water samples were collected within 24h following rainfall events, kept in a cool box at 4 °C and transported to the laboratory. Afterwards, 1 mL aliquots were

transferred into 2 mL autosampler vials and stored at −20 °C for analysis. A total of 108 chemicals, including insecticides, fungicides and herbicides were analysed in water samples collected from selected streams. Targeted substances were analyzed using LC-HRMS (Ultimate 3000 LC system coupled to a QExactive Plus MS equipped with a heated electrospray ionisation (ESI) source, all from Thermo Scientific).

To quantify toxicity, mean concentrations of pesticides detected in streams were converted into toxic units (TU) by relating the measured concentration of a pesticide to its median lethal concentration for a sensitive referece species (Sprague, 1970). Existing field studies in small freshwater streams show that pesticide effects on macroinvertebrate communities are mainly related to the maximum toxic unit (TU_{max}) exerted by the single most toxic compound. The summed up toxic units of all compounds (TU_{sum}, based on the assumption of additivity) does typically not increase the explained variance of biological effects significantly (Liess and von der Ohe, 2005; Orlinskiy et al., 2015; Schäfer et al., 2012). Therefore, we calculated the maximum toxic unit (TU_{max}) following the equation given below (Liess and von der Ohe, 2005; Tomlin, 2009) and used for further calculations.

$$TU_{max} = \mathrm{Max}_{i=1}^{n}\left[\log\left(\frac{Ci}{LC_{50i}}\right)\right] \qquad (1)$$

where TUmax is the highest value of *n* pesticides at each sampling site, C_i is the concentration (µg L^{-1}) of pesticide *i*, and LC_{50i} is the median lethal concentration (48 h, µg L^{-1}) of that pesticide for the reference organism. Here, we used LC_{50} values for *Daphnia magna*, *Chironomus riparius*, *Chironomus tentans*, *Hyalella azteca* obtained from the Ecotoxicology Database System (USEPA) and Pesticide Properties Database (PPDB), and the most sensitive species was selected as reference.

2.3.5 Effects of pesticide exposure on macroinvertebrate community

The $SPEAR_{pesticides}$ (SPEcies At Risk) bioindicator quantifies the toxic pressure of pesticides based on the proportion of macroinvertebrates classified as highly vulnerable to pesticides ("SPEcies At Risk") to the

total number of macroinvertebrates (Liess and Ohe, 2005). We calcuated $SPEAR_{pesticides}$ values using the software INDICATE (V 2019.11) that contains latest version of SPEAR index recently upgraded according to (Knillmann et al., 2018). A low proportion of vulnerable taxa (low SPEAR value) indicates high effects of pesticides and thus high pesticide exposure in a stream.

2.3.6 Acute toxicity tests for pesticide tolerance

The acute sensitivity of *Gammarus pulex* to neonicotinoid insecticide clothianidin was tested based on the OECD guidelines for the testing of chemicals (OECD 2004) and the guidelines for rapid tests for community-level risk assessment (Kefford, 2013). We selected the neonicotinoid insecticide clothianidin as test substance because it represents one of the most commonly applied classes of insectides in agriculture and has been frequently detected in surface waters (Knillmann et al., 2018).Test organisms were acclimatized to the test conditions in the laboratory for seven days before exposure. A 40 mg/L stock solution of clothianidin was prepared in distilled water using DANTOP (500g/kg, Spiess-Urania Chemical GmbH, Germany) and stirred overnight on magnetic stirrer. Required test concentrations i.e., 0, 5, 19, 72.2, 274.4, and 1042.6 µg/L were prepared by diluting the stock solution in ADAM (Artificial Daphnia medium) (Klüttgen et al., 1994). Random samples of the test solutions were analyzed for control; the average and maximum measured concentration of each nominal concentration ranged within acceptable boundaries (± 10%). Briefly, 16 individuals from each population were exposed to each test concentration of clothianidin for a period of 96 h to determine the effect of the toxicant. After every 24 h, the immobility and the mortality of the individuals were recorded as end points. Individuals were considered to be immobile if they did not move their bodies within 30 s of undisturbed observation or after probing with a rod. The fanning of gills and antenna was not counted as body movement.

2.3.7 Culture of Gammarus pulex

The long-term effects of field exposure were analysed by culturing *Gammarus pulex* under standardized pesticide-free conditions in the laboratory. In general, maintenance and culturing of organisms followed the descriptions given by (McCahon and Pascoe, 1988a, b) with modifications as follows: Approximately 100 individuals from each population including medium-sized gammarids were cultured in 5L glass tanks with a 3L aerated artificial stream water (ADaM) (Klüttgen et al., 1994) was used as culture medium. Organisms were fed with Alder leaves pre-conditioned in stream water for at least two to three weeks before use. Pre-conditioned stones of different size were added to increase the water quality of the culture medium and to provide juveniles with the opportunity to hide from omnivorous adults. Continuous aeration was provided using potable air pumps to avoid stress during experiments and cultures. Aeration and food availability was checked regularly. To maintain the quality of the medium, 500 mL of old culture medium was replaced by fresh medium after 14 days. Additionally, 1,500 mL of old medium was replaced with fresh medium after 30 days and dead organisms were removed. The culture was maintained at 16°C, with 60% humidity and artificial light (12 h light:12h dark) in a climate chamber. To analyze the long-term effects of local pesticide contamination in streams on respective populations, the survival, per capita growth and the proportion of mating adults were monitored every four weeks for three months.

2.3.8 Data analysis

All statistical analyses were carried out using R Studio for Windows (V 1.2.1) and R for Windows (V 3.5.1). For each acute sensitivity test and each observation time, the concentration that affected 75% of the exposed population (EC_{75}) was calculated using 5-parameter non-linear regression available with the package drc (V 3.0-1) (Ritz et al., 2015). We used a binomial error distribution in the models and set the lower and upper boundary to 0 and 1, respectively. EC_{75} was used instead of EC_{50} to quantify pesticide tolerance because we observed that the increase in tolerance with pesticide pollution was more pronounced at higher effective

concentrations. This is in accordance with the finding of Becker et al. (in preparation). To make use of all data, we then performed linear regression of all log-transformed EC_{75} values from the same test vs. the log-transformed observation time and interpolated the EC_{75} after the mean test duration (60h). This 60h EC_{75} was used as a measure of pesticide tolerance for further analyses. The long-term end points for the cultured populations after three months were derived from the introduced adults and their (pre-adult) offspring which could be differentiated based on size. Survival was calculated by simply dividing the number of adult individuals by the initial number of individuals at the start of the culture. For per capita growth, the total number of offspring was divided by the initial number of individuals. The proportion of mating adults ("reproduction") was calculated as 2 x the number of couples , divided by the overall number of adults in the culture. Simple linear regression was applied to analyze the correlation of pesticide pollution in the field (TU_{max}) with pesticide tolerance (log-transformed EC_{75}) and with the $SPEAR_{pesticides}$ bioindicator. The effects of of pesticide pollution and of pesticide tolerance on survival and reproduction after three months culture in the laboratory was analyzed using a binomial generalized linear model with a logit link function. The effects of pesticide pollution and of pesticide tolerance on the (log-transformed) per capita growth were analyzed using simple linear regression. The assumptions of homoscedasticity and of normally distributed residuals were confirmed by visual inspection, plotting residuals vs. fitted values residuals vs. leverage, and Q-Q plots.

2.4 Results

2.4.1 Pesticide contamination and community structure

Among investigated sites, the maximum toxic unit (TU_{max}) ranged from −5.8 to −2.6. In agricultural streams, most toxic compounds were neonicotinoids (clothianidin; $n = 1$, $TU_{max} = -3.6$ and thiacloprid; $n = 2$, mean $TU_{max} = -2.7$). In contrast, the forested streams that served as controls were only slightly contaminated. The maximum toxic unit ranged from −5.8 to −3.7 which is considered to be of lower ecotoxicological

relevance. In all forested sites, a herbicide (2, 4−Dichlorophenoxyacetic acid) was responsible for maximum toxicity (n=3, mean TU_{max}= −4.96).

The toxic pressure exerted by pesticides on the macroinvertebrate community structure was quantified using the $SPEAR_{pesticides}$ indicator. The change in $SPEAR_{pesticides}$ was strongly correlated to the TU_{max} of respective streams (linear regression, $F = 23.9$, residual df = 4, adjusted $R^2 = 0.82$, *p*-value = 0.008; Figure 2.1). Agricultural streams characterized by higher TU_{max} showed significantly lower $SPEAR_{pesticides}$ values (0.08 − 0.37) in comparison to the forested reference streams with lower pesticide contamination (0.7 − 1.0) (Welch two sample t-test, *p-value* < 0.01). These effects were observed even at concentrations in the range of 1/1000 to 1/100 of the acute LC_{50} of the most sensitive standard test organism that is generally considered to be safe by governmental risk assessment frameworks (European Commission, 2011).

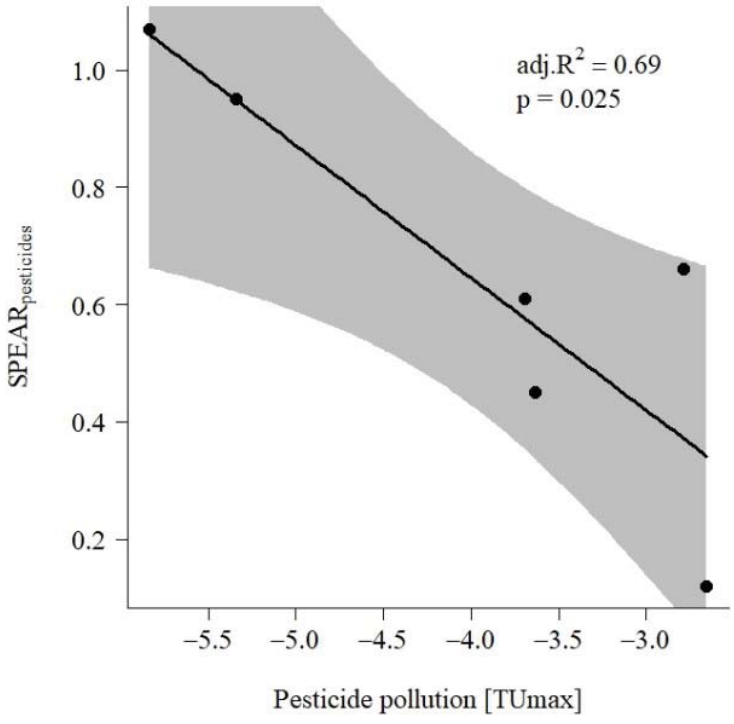

Figure 2.1. Correlation between the macro-invertebrate community structure indicated as $SPEAR_{pesticide}$ and the stream contamination expressed as maximum toxic unit (TU_{max}). The grey area corresponds to 95% confidence interval.

2.4.2 Increased tolerance to clothianidin in Gammarus pulex

The median effective concentration of the insecticide clothianidin after 96 h exposure (96h LC_{50}) was on average 33.28 µg/L across all populations. This was comparable to the 96h LC_{50} of clothianidin reported for the most sensitive standard test species used for the calculation of TU_{max} (*C. riparius*, 29 µg/L). Therefore, we consider pesticide effects in *G. pulex* as representative also for other sensitive macroinvertebrates. We observed pesticide adaptation already at low contamination in the range of TU_{max} = -3. *G. pulex* from agricultural streams was 2−fold more tolerant (mean effective concentration that immobilized 75 % of test individuals after 60h, EC_{75} = 158 µg/L) to clothianidin compared to populations from reference streams (mean EC_{75} = 71 µg/L; t = -3.03, residual df = 4, p-value = 0.038).

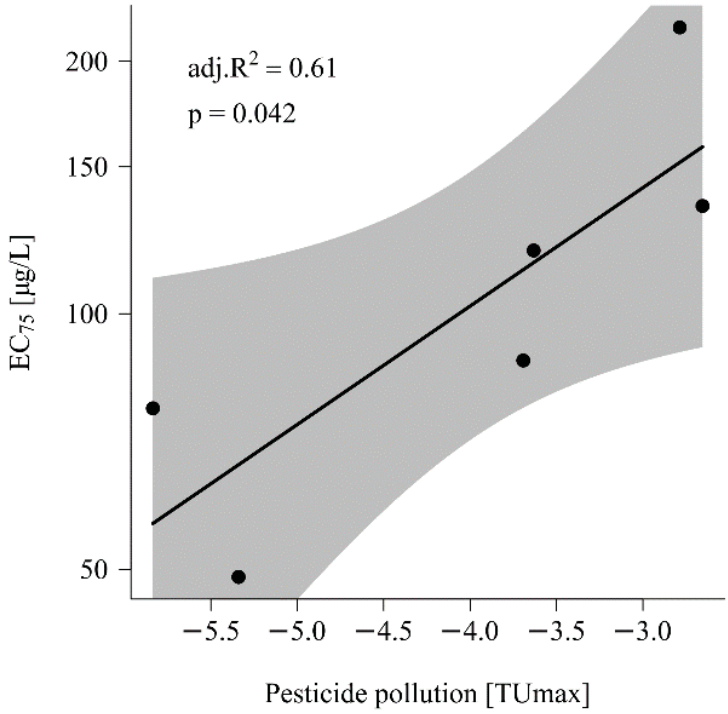

Figure 2.2. Correlation between pesticide tolerance in *G. pulex* (quantified as EC_{75} of the insecticide clothianidin after 60h constant exposure, log-transformed) and the level of pesticide pollution in the field (quantified as maximum toxic units, TU_{max}). Means ± 95 % confidence intervals are shown.

The tolerance to clothianidin was significantly correlated with the stream contamination expressed as TU_{max} (linear regression; $F = 8.6$, residual df = 4, adjusted $R^2 = 0.61$, p-value = 0.042, Figure 2.2). Further, clothianidin tolerance also showed a correlation with the macroinvertebrate community structure expressed as $SPEAR_{pesticide}$ (linear regression; adjusted $F = 5.67$, residual df = 4, $R^2 = 0.48$, p-value = 0.075) that also provides information about the local pesticide contamination.

2.4.3 Consequences of increased tolerance in G. pulex

We observed that the increased tolerance to clothianidin (60h EC_{75}) had consequences on the general fitness and long-term viability of *G. pulex*. Populations from agricultural streams with increased clothianidin tolerance showed reduced survival in culture after three months, as compared to populations from less contaminated reference streams (generalized linear regression; $Chi^2 = 7.87$, residual df = 4, Zavoina's R^2 = 0.23, p-value = 0.005, Figure 2.3a). Similarly, the per capita growth of gammarid populations significantly decreased with increasing clothianidin tolerance expressed as EC_{75} (linear regression; F = 13.72, residual df = 4, adjusted $R^2 = 0.72$, p-value = 0.021, Figure 2.3b). Furthermore, increased pesticide tolerance (EC_{75}) was significantly associated with a reduced proportion of mating adults in the populations (generalized linear regression; $Chi^2 = 6.12$, residual df = 4, Zavoina's Pseudo $R^2 = 0.32$, p-value = 0.013, Figure 2.3c).

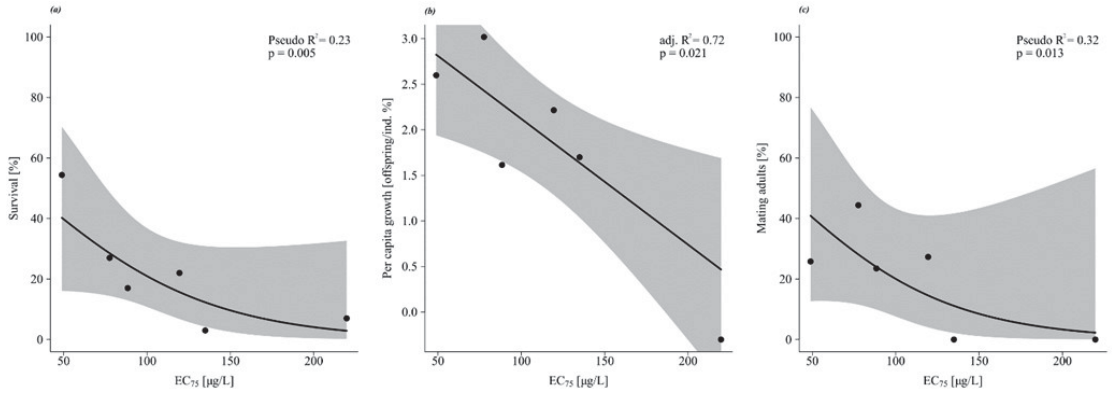

Figure 2.3. Effect of pesticide tolerance (60h EC_{75} of the insecticide clothianidin) on (a) survival, (b) per capita growth, and (c) reproduction (proportion of mating adults) of *G. pulex* populations. The grey area corresponds to 95% confidence interval. Performance in the cultured populations correlated also with the pesticide pollution observed in the field:

Increasing pesticide contamination (TU_{max}) was associated with reduced survial (generalized linear regression, $Chi^2 = 2.33$, residual df = 4, Zavoina's Pseudo $R^2 = 0.084$, p-value = 0.12), per capita growth (linear regression, $F = 5.11$, $R^2 = 0.45$, residual df = 4, p-value = 0.086) and a lower proportion of mating adults (generalized linear regression, $Chi^2 = 2.14$, residual df = 4, Zavoina's Pseudo $R^2 = 0.11$, p-value = 0.14, Table A.1).

2.5 Discussion

2.5.1 Quantification of pesticide effects on macroinvetebrates

In the present study, the pesticide contamination (TU_{max}) of agricultural streams ranged from −5.8 to −2.6. According to the most conservative first tier evaluation of European regulations for agricultural pesticides (EFSA, 2013), generally no effects should occur at concentrations 100 times below the LC_{50} of reference organisms (TU_{max} = -2). However, we found strong effects of pesticide pollution on the macroinvertebrate community composition at considerably lower TU_{max} levels. These ecological effects (quantified with the SPEAR index) are consistant with previous studies (Beketov et al., 2009; Bereswill et al., 2013; Hunt et al., 2017; Liess et al., 2008; Liess and von der Ohe, 2005; Münze et al., 2015; Orlinskiy et al., 2015). In the present study, neonicotinoid insecticides dominated the toxicity in agricultural streams. This is in accordance with several recent studies that reported effects of neonicotinoids on aquatic communities in agricultural streams (Becker and Liess, 2017; Münze et al., 2017; Shahid et al., 2018).

2.5.2 Adaptation to pesticides and consequences on the fitness

Our findings reveal that populations of *G. pulex* in agricultural streams have aquired increased tolerance to the neonicotinoid insecticide clothianidin. Even low insecticide concentrations ($TU \leq -3$) were sufficient to produce more tolerant populations in the field. Similar results were reported by Shahid et al. (2018).

Populations from agricultural streams with increased insecticide tolerance showed decreased survival, per capita growth and mating. The observed effects were not only based on initial decrease in performance but they

were persistent for at least four months (Table A.1). However, the observed effects became more pronounced and significant after three months of culture at pesticides free conditions. Persistent reduced performance observed in the present study may result in accumulative effects that get stronger until most individuals have died or stopped to reproduce. Persistent effects are an indicator for fitness costs from long-term adaptation rather than for delayed pesticide effects. Further, the persistent decrease in performance is of more concern than transient effects because it hinders population recovery and explains changes in the community composition over longer time scales at low concentrations. Our results indicate that in the field, even very low pesticide concentrations can considerably impair the performance of *G. pulex*, both directly through chronic effects and indirectly through fitness costs of genetic or physiological adaptation.

There are several investigations that report fitness costs of adaptation / increased tolerance to certain pesticides and metals in different organisms (Bach and Dahllof, 2012; Ffrench-Constant and Bass, 2017; Heim et al., 2018; Kliot and Ghanim, 2012; Xie and Klerks, 2004). It is suggested that molecular and physiological processes involved in tolerance development, and therefore also the associated fitness costs, depend on the intensity and duration of exposure (Amiard-Triquet et al., 2011). However, fitness costs of moderately increased tolerance caused by very low pesticide concentrations commonly observed in agricultural streams have never been investigated. Interference with energy allocation in an organism may impair life history traits such as survival, growth and reproduction (Novais et al., 2013). Therefore, the long-term negative effects investigated in *Gammarus pulex* might be due to their adaptation to pesticides and could potentially impact the performance of *G. pulex* in natural ecosystems.

Additionally, delayed (chronic) effects from field exposure may have contributed to the decreased performance of populations from agricultural streams. Several studies have investigated long term effects after pulsed exposure to pesticides on macroinvertebrates under laboratory conditions (Abel and Garner, 1986; Barros et al., 2017; Rasmussen et al., 2017). For example, Cold and Forbes (2004) observed effects on survival, mating

behavior, and reproductive output in *G. pulex* exposed to 0.1–0.6 µg/L of esfenvalerate for 1 h. These effects were even observed even after 2 weeks of pulse exposure, but much higher toxicity. Similarly, Liess and Schulz (1996) reported reduced survival and delays in emergence in the caddisfly, *Limnephilus lunatus*, during several weeks following 1h pulse exposure to fenvalerate. In another study, increase in the number of aborted broods and a decrease in viable offspring size in *G. pulex* females were observed in response to zinc exposure (Maltby and Naylor, 1990).

However, all these effects were observed after pulse exposure to high pesticide concentrations that are known to cause acute effects after constant exposure. The present study suggests that long-term effects can be observed also after pulse exposure to much lower concentrations during run-off events. These effects might be attributed to resource allocation for different metabolic processes in response to pesticide exposure (Baas et al., 2010; Jager et al., 2006). Resources used in detoxification processes significantly reduce the energy budget required for growth, fecundity, survival and reproduction.

2.6 Conclusions

Gammarus pulex shows moderate adaptation to pesticide pollution but also reduced long-term viability even at concentrations generally considered safe according to the European pesticide legislation. Long-term effects such as reduced survival, per capita growth, and reproduction may potentially impact the performance and ecosystem function of *G. pulex*, such as leaf litter degradation, in the field. Therefore, these long-term impairments need to be considered in deriving save concentrations.

2.7 Acknowledgments

We acknowledge the German Academic Exchange Service (Deutscher Akademischer Austauschdienst, DAAD) for financially supporting A.S through doctoral fellowship. Data on the macroinvertebrate community structure ($SPEAR_{pesticide}$) and TU_{max} was obtained from the KgM "Kleingewässer Monitoring" project including support by the Helmholtz

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2.8 Funding

See above

2.9 Conflict of interest statement

The authors that they have no conflict of interest.

3

Multi-stress reduces the advantage of pesticide adaptation

3.1 Abstract

Permanent exposure to a single stressor induces the development of genetic adaptation. However, adapted individuals are less tolerant to other stressors. Under global change scenarios, multi-stress conditions are expected to occur regularly. For predicting their ecological consequences, the question is how individuals that are genetically adapted to a single stressor respond to multiple stressors. Here we investigated the effect of multiple stress on the crustacean *G. pulex* from agricultural streams that are genetically adapted to pesticides compared with conspecifics from forested streams not genetically adapted to pesticides. We show that *G. pulex* from agricultural streams only have a survival advantage under pesticide stress in the absence of other environmental stressors. As expected under an optimum temperature, the genetically adapted agricultural populations were considerably more tolerant to pesticide as compared to the forest populations. Temperature stress alone only increased mortality in agricultural populations. In contrast, a combined stress of pesticides and warming revealed a similar tolerance of

agricultural and forested populations. Both stressors acted synergistically and these combined effects could be predicted with SAM (Stressor Addition Model). We conclude that under multi-stress conditions typical for global change scenarios, genetic adaption to pesticides loses its advantage.

3.2 Introduction

Pesticides affect macroinvertebrates in agricultural streams already below the regulatory acceptable concentrations, resulting in a considerable loss of vulnerable species,(Liess and von der Ohe, 2005; Shahid et al., 2018), Liess et al., 2021(Under review). However, surviving individuals may acquire pesticide tolerance through physiological acclimation (Sparks and Nauen, 2015) and genetic adaptation (Sun et al., 2014) . When populations experience occasional exposure, inducible traits are maintained as they facilitate the expression of adaptive phenotypes in response to variable environmental conditions (Weston et al., 2013). For example, physiological induction of tolerance after low insecticide exposure in mosquitoes and wood frogs from less contaminated sites (Hua et al., 2013; Poupardin et al., 2008). In contrast, when populations experience continuous exposure over multiple generations, a plastic response eventually becomes constitutively expressed, resulting in genetic adaptation (Fournier-Level et al., 2019; Lopes et al., 2008; Rivero et al., 2011). Examples include high tolerance to a pyrethroid insecticide (53-fold) in *H. azteca* (Weston et al., 2013) and moderate neonicotinoid insecticide tolerance (3 to 4-fold) in *G. pulex* from contaminated sites (Becker and Liess, 2017; Shahid et al., 2018; Siddique et al., 2020).

However, the development of genetic adaptation is often associated with reduced fitness (Siddique et al., 2020). Fitness costs in the absence of a primary stressor have already been observed (Bach and Dahllof, 2012; Fournier-Level et al., 2019). Under global change scenarios, organisms are exposed to multiple stressors such as exposure to agricultural pesticides and elevated temperatures (Meehl and Tebaldi, 2004; Russo et al., 2018). In the presence of one dominant stressor, organisms may adapt to it; however, the response of single-stressor adapted populations under

multiple stress conditions is rarely addressed. Only a few studies have investigated the combined effects of toxicants and temperature on populations adapted to thermal stress (Zhang et al., 2020; Zhang et al., 2018). Authors reported reduction in combined effect of temperature and toxicant in populations adapted to thermal stress. However, the multi-stress effects of pesticides and warming on populations adapted to pesticides have never been addressed. Unraveling these effects may provide insight on the consequences of pesticide adaptation under global change scenarios.

The present study was therefore designed to quantify the ecological consequences of different mechanisms of pesticide tolerance and the associated fitness costs. We performed our investigations with the crustacean *G. pulex*, one of the most common freshwater macroinvertebrate species in central European streams with a high ecological relevance for leaf litter degradation (Schäfer et al., 2012). Stressors investigated were the combined effect of the neonicotinoid insecticide clothianidin and elevated temperatures on agricultural and reference populations from forested areas. Further, we aimed at identifying the type of interaction between clothianidin and temperature, predicting results with concentration addition (CA, (Loewe and Muischnek, 1926b)), effect addition (EA, (Bliss, 1939b)), and stress addition (SA, (Liess et al., 2016a).

3.3 Methods

3.3.1 Description of study sites

In the present study, we investigated organisms from 17 sites in Germany (Supplementary Information, Figure B.1), each in spring and autumn 2019. Among them, 11 sites were contaminated with various levels of pesticides, whereas 6 were considered as forested reference streams. All sites covered a wide range of stream habitats including various types of sediments, plants, and stones and a variety of organic matter. As the study mainly focused on pesticide contamination, it was made sure that no other relevant sources of contamination such as wastewater treatment plants or industrial facilities were located upstream of the sampling sites. In contrast to

streams considered as polluted, reference sites were usually located within or at the edge of forests, at least with either non-agricultural landscape between the spring of the stream and the sampling site (description of sites, see SI and data repository at DOI: 10.1594/PANGAEA.924688).

3.3.2 Collection of macroinvertebrates and test organisms

The test organisms, G. pulex, were sampled in spring from March to April 2019 before the peak insecticide application period and in autumn from October to November 2019 after the main pesticide application period. Approximately 600 G. pulex individuals were collected from each site using a pipette and transported to the laboratory under constant aeration in a cooling box. Every week, two to three sites were visited, including a highly polluted and a lowly polluted population, and tested to avoid a potential bias that may result from temporal variation in the tolerance to pesticides. During sampling, the physicochemical parameters such as electrical conductivity (EC), temperature, pH, and dissolved oxygen (DO) were also measured showing that these environmental parameters were well in the range of normal conditions for gammarids (Table B.1). The test organisms were kept overnight at 16°C, 60% humidity, and constant aeration before pesticide exposure.

3.3.3 Quantification of pesticide contamination

The toxic pressure of pesticide contamination was quantified by applying water samples from the investigated sites collected within the Kleingewässermonitoring (KgM) project (https://www.ufz.de/kgm/) using computer triggered event samplers (Liess et al., 1999) and water level triggered event samplers (Liess and von der Ohe, 2005). During the sampling process, approx. 5 mL aliquots were pumped every 5 min from the stream at approx. 10-20 cm depth for 3 h 20 min, yielding unfiltered, cooled composite water samples of about 200 mL. The number of water samples from each site varied from one to six, depending on rainfall events. A total of 108 chemicals, including insecticides, fungicides, and herbicides were analyzed in the water samples. Targeted substances were analyzed using LC-HRMS (Ultimate 3000 LC system coupled to a Q Exactive Plus

MS equipped with a heated electrospray ionization (ESI) source, all from Thermo Scientific). The toxic units (TU) were calculated to determine the pesticide-induced water toxicity at each site. Existing field studies in small freshwater streams show that pesticide effects on macroinvertebrate communities are significantly related to the most toxic pesticide concentrations (maximum toxic unit − TU_{max}) (Liess and von der Ohe, 2005; Orlinskiy et al., 2015; Schäfer et al., 2012). Therefore, the maximum toxic unit (TU_{max}) was calculated as suggested by (Liess and von der Ohe, 2005) and used for further calculations. Invertebrate effects of pesticides were quantified by using the $SPEAR_{pesticides}$ (SPEcies At Risk) bioindicator, which quantifies the toxic pressure of pesticides based on the proportion of macroinvertebrates classified as highly vulnerable to pesticides ("SPEcies At Risk") to the total number of macroinvertebrates. A low proportion of vulnerable taxa (low SPEAR value) indicates high effects of pesticides and thus high pesticide exposure in a stream.

3.3.4 Selection of insecticide and temperature

In the present study, we used neonicotinoid insecticide clothianidin as a chemical stressor as it represents one of the most commonly applied classes of insecticides in agriculture and has been frequently detected in surface waters of the study area (Knillmann et al., 2018), Liess et al., 2021 (Under review). To identify the ecological relevance of an additional environmental stressor, we used 20°C and 24°C as temperature stress, in addition to the control temperature of 16°C. According to KgM data, the water temperature of approx. 34% of the sites reaches more than 18°C and among them, 10% range from 20°C to 23°C in June and July (data repository, DOI: 10.1594/PANGAEA.924688). However, none of the 17 sites investigated in the present study reached more than 18°C throughout the last two years, ruling out the possibility of co-tolerance in the field.

3.3.5 Acute sensitivity tests

The acute sensitivity of *G. pulex* to the neonicotinoid insecticide clothianidin was tested following the OECD guidelines for chemical testing with 100 individuals per 5 L beaker (OECD, 2004) and the rapid

tests for community-level risk assessment (Kefford, 2013). A 40mg/L stock solution was prepared in distilled water using DANTOP (500g/kg, Spiess-Urania Chemical GmbH, Germany) and stirred overnight on a magnetic stirrer. Organisms from each population were divided into two groups. In the first group, organisms were exposed to a sub-lethal clothianidin concentration (0.08µg/L) for 3 days (later described as pre-exposure). The pre-exposure concentration, i.e., 0.08 µg/L, was prepared by diluting the stock solution in ADaM (artificial stream water) (Klüttgen et al., 1994) for each population separately. 100 individuals of *G. pulex* for each treatment were added to clothianidin sub-lethal concentration and fed with ~6 g wet weight of pre-conditioned leaves (Siddique et al., 2020). The test vessels were kept in a climate chamber pre-set at 16°C, 60% humidity, a 12:12 light-dark cycle, and constant aeration. After pre-exposure, the organisms were removed from the contaminated medium and transferred to an uncontaminated fresh medium for recovery period and acclimation of 7 days. The organisms were kept again at 16°C in a climate chamber. After pre-exposure, 100 organisms from each population were also acclimatized at 20°C and 24°C to analyze the effect of temperature on physiological acclimation. The effect of temperature stress on pre-exposed populations was only investigated in autumn samples. In the second group, organisms were acclimated to test conditions for 10 days. To analyze the acute sensitivity to clothianidin, 100 organisms from each population were acclimatized at 16°C; to analyze the effect of temperature stress on clothianidin tolerance, 100 organisms from each population were acclimatized at 20°C and 24°C. After 10 days, both groups (pre-exposed and non-pre-exposed) were exposed to sub-lethal to lethal clothianidin concentrations. Five clothianidin concentrations (i.e., 0, 8, 40, 200 and 1000µg/L) were prepared by diluting the stock solution in ADaM. Briefly, 16 individuals from each population were exposed to each test concentration of clothianidin for a period of 96 h to determine the effect of the toxicant. The test vessels were placed in climate chambers at 16°C, 20°C and 24°C with 60% moisture, a 12:12 light-dark cycle, and constant aeration. After every 24 h, the immobility and mortality of the individuals were recorded as end points. Individuals were considered immobile if they did not move their bodies within 30 s of undisturbed observation or after

probing with a glass rod. All the experiments were performed with both spring and autumn populations unless otherwise mentioned.

3.3.6 Insecticide Testing

To determine the actual concentrations of insecticides used in this study, we collected 1000-mL samples of stock and working solutions. Samples were analyzed by Wessling GmbH, Landsberg OT, Oppin, Germany, using a Thermo Fisher Scientific TSQ 8000 Evo Triple Quadrupole GC−MS/MS. The detection limit of the instrument was 5.7 ng/L. The analytical column used was a TG-5HT guard column with a 0.53 mm ID and a 0.15 µm film thickness (Thermo Fisher Scientific, USA). The software TraceFinder 3.2 (Thermo Fisher Scientific) was applied for data processing. Actual concentrations recovered from the samples were in acceptable boundaries (±10%) to the nominal concentrations.

3.3.7 Data analysis

All statistical analyses were carried out using RStudio for Windows (V 1.2.1335) and R for Windows (V 3.5.1). For each acute sensitivity test and each observation time (24, 48h), the concentration that affected 50% of the exposed population (EC_{50}) was calculated using a generalized linear model with a quasi-binomial error distribution and a logit link function. (V 3.0-1) (Ritz et al., 2015). Pesticide tolerance was quantified using the 48 h EC_{50}. For the comparison of EC_{50} values among both seasons (winter vs. summer), t- test was applied. The TU_{max} values were derived from summer samples as the water samples for pesticide analysis were collected only in summer. However, as the TU_{max} data was available for 2018 and 2019 for most of the sites, average TU_{max} was used to derive the correlation between stream toxicity and the EC_{50} of *G. pulex* from spring and autumn populations. Similarly, for the quantification of ecological effects, the SPEAR was calculated only in summer. Linear regression was applied to analyze the correlation of pesticide pollution in the field (TU_{max}) with pesticide tolerance (log-transformed EC_{50}) and with the $SPEAR_{pesticides}$ bioindicator on spring populations. The dataset from spring and autumn populations for 48 h acute EC_{50} at 16°C, 20°C and 24°C, and pre-exposure

at 16°C were combined for further analysis. Whereas the pre-exposure at 20°C and 24°C was only performed with autumn populations, two sample t-tests (when the assumption of equal variances was fulfilled) and Welch's t-test (in case of non-equal variances) were applied to compare the different EC_{50} among reference and agricultural populations and to compare them among different temperature treatments. In case the data were not normally distributed, Wilcoxon's rank sum test was used. In case of three-group comparison, one-way ANOVA was applied. A p-value of < 0.05 was considered statistically significant.

We predicted the combined effects of insecticide clothianidin and temperature by applying different additive approaches (CA-concentration addition and EA-effect addition) and a synergistic approach (SA-stress addition model (SAM)). Both the EA (Bliss, 1939b) and CA (Loewe and Muischnek, 1926b) models are commonly applied to predict mixture effects and assume the additivity of concentrations or of effects. In contrast, the SA was developed to predict synergistic effects of independent stressors, such as a toxicant and an environmental stressor (Liess et al., 2016a). This approach allocates environmental stress and toxicant stress into general stress levels, which serve as the common currency to add various stressors (Liess et al., 2016a). We applied CA, EA, and the SA to predict LC_{50} using the software Indicate (Version 1.1.1; https://www.systemecology.de/indicate/). To quantify the predictive accuracy of the models, a model deviation ratio (MDR) was calculated for the CA, EA, and SA models by dividing the predicted LC_{50} values by the observed LC_{50} values. Belden (Belden et al., 2007) suggested the MDR as a simple measure of model accuracy and suggested the range of $0.5 < MDR < 2$ as a benchmark for the accuracy of CA or EA models. For an $MDR > 2$, interactions between stressors are interpreted as synergistic (Cedergreen, 2014). In the present study, we used CA as the null model and the combined effects were considered synergistic if the MDR values were >2. For the comparison of MDR values in agricultural and reference groups, bootstrapping was done to obtain 10 values for each group. After that, a t-test was applied to obtain statistical significance. For comparison of MDR values of SA and CA, a t-test was applied. Except for the

determination of observed and predicted LC_{50} values, we generated all figures and statistical analyses using the software RStudio.

3.4 Results

3.4.1 Pesticide contamination and the effects on macroinvertebrates

In the present study, the toxic pressure of pesticides in agricultural streams measured as a maximum toxic unit (TU_{max}) amounted up to −1.51. Neonicotinoids and carbamate insecticides were the most toxic compounds with TU_{max} ranging from −3.07 to −1.51. In contrast, forested streams were characterized by a much lower toxic pressure with TU_{max} ranging from −5.8 to −3.3. The ecological impact of the measured pesticide concentrations was identified by the effect on the invertebrate community structure. The macroinvertebrate-based indicator for pesticide pressure ($SPEAR_{pesticides}$) was significantly correlated to the pesticide contamination of respective streams (linear regression, F = 8.1, df = 1, residual df = 13, adjusted $R^2 = 0.34$, *p*-value = 0.013; Figure 3.1a). In addition, the pesticide tolerance of a dominant crustacean, *G. pulex*, increased with increasing pesticide pressure. The 48 h EC_{50} of *G. pulex* to insecticide clothianidin correlated with the TU_{max} (linear regression, adjusted $R^2 = 0.29$, F = 13.40, df = 1 and 30, and p-value < 0.001, Figure 3.1b). No seasonal variation (i.e., spring vs. autumn) in the clothianidin tolerance was observed among agricultural (Welch two-sample t-test, t = -0.098, df =17.59, p-value = 0.92) or reference populations (t-test, t = -0.21, df =7.16, p-value = 0.84).

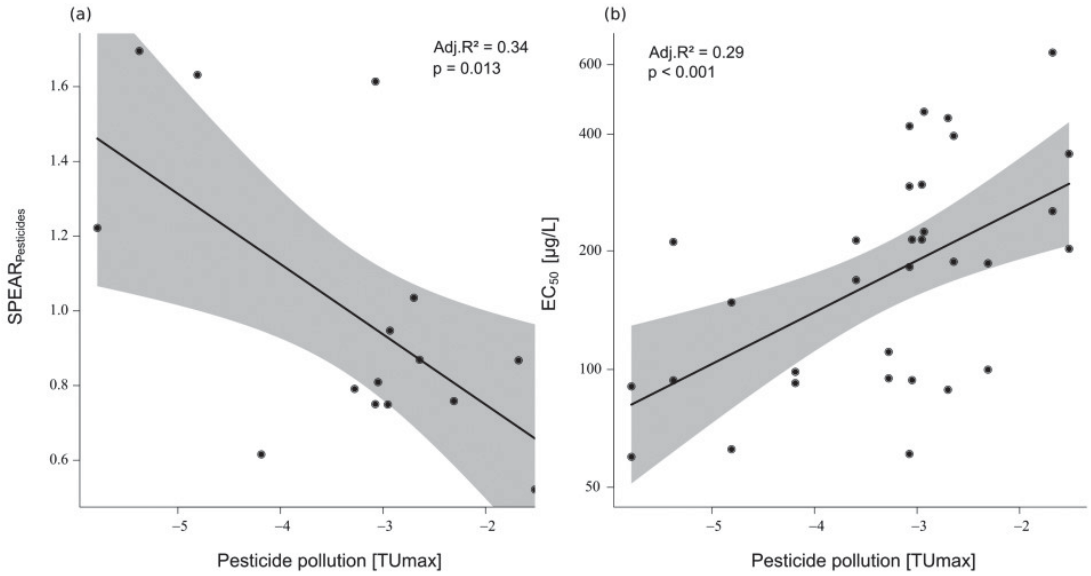

Figure 3.1. Correlation of toxic pressure (TU_{max}) with (a) macroinvertebrate community composition expressed as $SPEAR_{pesticides}$ and (b) pesticide tolerance in *G. pulex* (quantified as EC_{50} of the insecticide clothianidin after 48 h constant exposure, log-transformed) collected in spring and autumn. Means ±95% confidence intervals are shown.

3.4.2 Differential tolerance

Dose response curve analysis revealed that the gammarid populations from agricultural streams were significantly more tolerant to insecticide clothianidin as compared to reference populations at optimum temperature (Figure 3.2a, one-way ANOVA; $X^2 = 6.84$, df = 1, p-value = 0.0089). After pre-exposure to low clothianidin concentration (3 orders of magnitude below the acute EC_{50}), the reference populations showed increased tolerance (habituation) (Supplementary Information, Figure B.2a). In contrast, the tolerance of agricultural populations did not vary before and after pre-exposure (Supplementary Information, Figure B.2b). Therefore, after pre-exposure, the average tolerance of agricultural and reference populations was similar (Figure 3.2b).

3.4.3 Fitness costs of pesticide tolerance under non-contaminated conditions

We investigated the fitness costs of adaptation under elevated temperatures (20°C and 24°C) in the absence of pesticides.

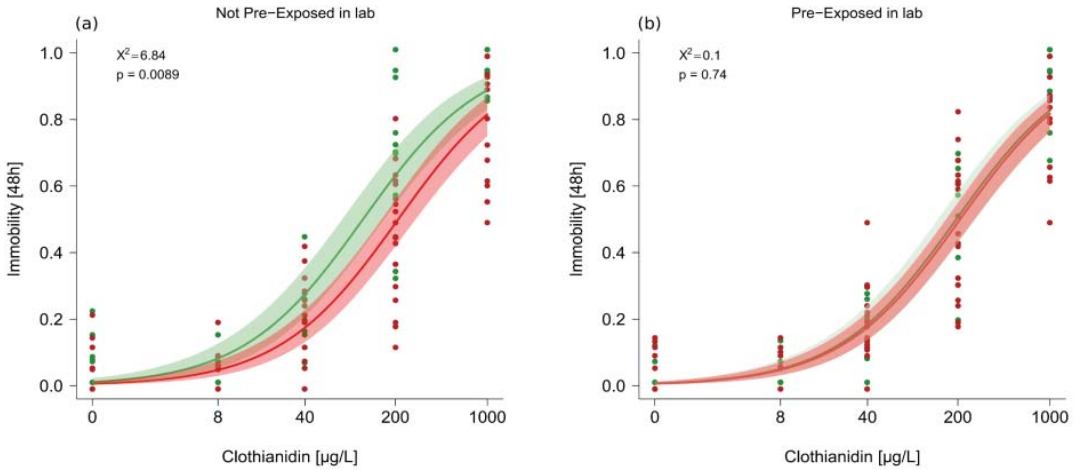

Figure 3.2. Dose response curves for *G. pulex* exposed to insecticide clothianidin for 48 h in laboratory. Figure (a) represents clothianidin tolerance without pre-exposure and (b) represents clothianidin tolerance after pre-exposure. In both figures, red curves denote the response of organisms from agricultural streams, whereas green curves show the response of organisms from reference streams. Shaded areas correspond to the 95% confidence intervals.

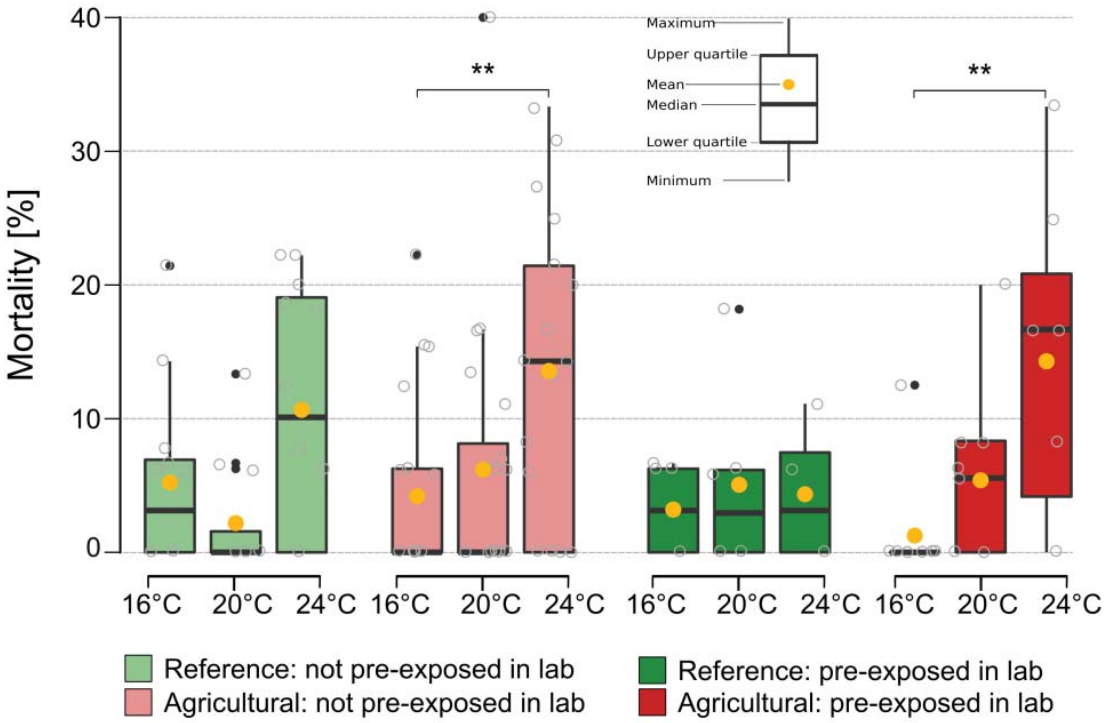

Figure 3.3. Comparison of the percent mortality of *G. pulex* collected from agricultural and reference streams after 48 h exposure to the neonicotinoid insecticide clothianidin in the laboratory at 16°C, 20°C and 24°C using one-way ANOVA. The grey dots represent data points.

We observed that the agricultural populations genetically adapted to pesticides (with and without pre-exposure) showed increased mortality

with increasing temperature (Figure 3.3, without pre-exposure, ANOVA; F = 8.66, df = 1, residual df = 55, p-value = 0.0047; with pre-exposure, ANOVA; F = 12.24, df = 1, residual df = 25, p-value = 0.0017). In contrast, reference populations, with and without pre-exposure did not show temperature dependent mortality (Figure 3.3).

3.4.4 Clothianidin tolerance under multi-stress.

Under an optimum temperature (16°C), the agricultural populations were on average 2.2-fold more tolerant to clothianidin as compared to reference populations (t = -3.85, df = 29.35, p-value < 0.001, Figure 3.4a). Under combined stress of clothianidin and temperature (20°C and 24°C), both agricultural and reference populations showed increased sensitivity with increasing temperature. As a result, the clothianidin tolerance of both populations was similar under temperature stress (Figure 3.4a). Clothianidin tolerance was reduced up to 5-fold in reference populations (t = 7.62, df = 19, p-value < 0.001), whereas 8.5-fold in agricultural populations (t = 10.13, df = 32.69, p-value < 0.001).

3.4.5 Clothianidin tolerance under multi-stress after pre-exposure.

We compared the multi-stress effect on habituated reference populations and genetically adapted agricultural populations (Supplementary Information, Figure B.2), observing that both populations showed sensitivity to temperature and similar clothianidin tolerance (Figure 3.4b). The clothianidin tolerance reduced up to 16.7-fold in habituated reference populations (t = 5.86, df = 4.34, p-value = 0.0032) and 15-fold in genetically adapted agricultural populations (t = 7.38, df = 8.4, p-value <0.001).

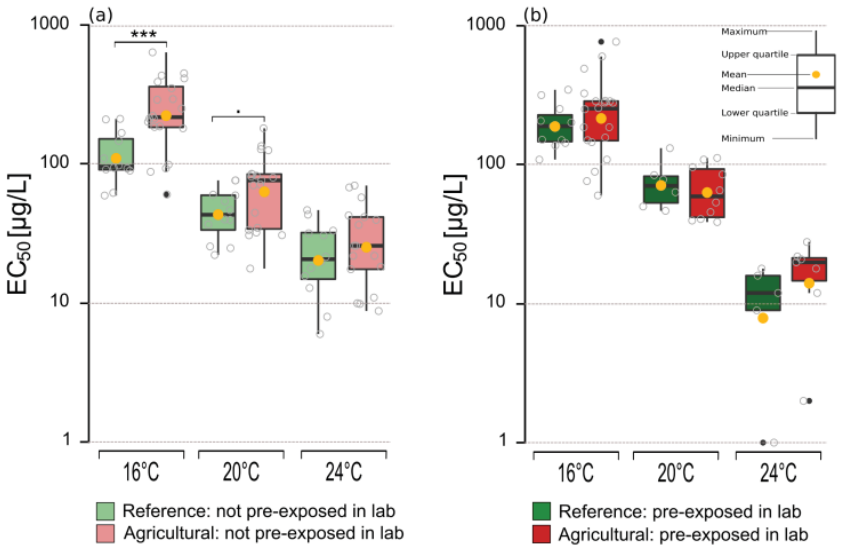

Figure 3.4. Comparison of the median effective concentrations (EC_{50}) of *G. pulex* collected from agricultural and reference streams after 48 h exposure to the neonicotinoid insecticide clothianidin in the laboratory at 16°C, 20°C and 24°C. The effect of temperature on the clothianidin tolerance of field-exposed populations is shown in (a), whereas the effect of temperature on increased clothianidin after pre-exposure is shown in (b). Grey dots represent data points.

3.4.6 Prediction of combined effect of clothianidin and temperature.

We used concentration addition (CA), effect addition (EA) and stress addition (SA) to predict the interactive effect between insecticide clothianidin and temperature. For CA and EA, the MDR values all above 2 indicated that the combined effect of the two stressors were greater than predicted and even pointed to synergistic effects of clothianidin and temperature in all the cases (Figure 3.5a, b and Table 3.1). We revealed that the CA and EA substantially underestimated the combined effect up to one order of magnitude for both reference and agricultural populations (Table 1). On average, the underestimation by EA and CA of the observed effect was 5.15 and 5.68 times, respectively. In contrast we observed that the SA model more accurately predicted the combined effect of clothianidin and temperature (Supplementary Information, Figure B.3), indicated by an average MDR value of 1.32 (Figure 3.5c, d and Table 3.1). Accordingly, predictions by SAM differed significantly from CA except for agricultural populations at 20°C (Table 3.1).

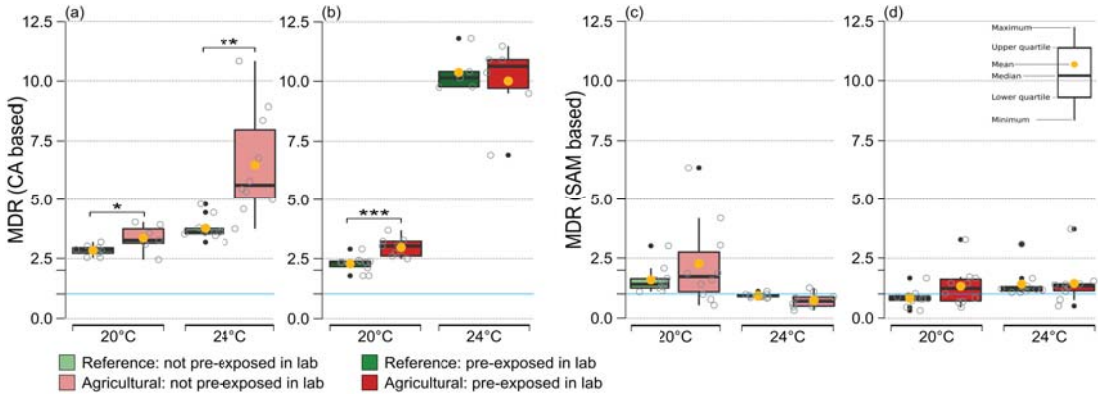

Figure 3.5. Comparison of the prediction and synergistic effects of clothianidin and temperature are presented in (a) CA-based MDR (model deviation ratio) values without pre-exposure and (b) after pre-exposure, and (c) SA-based MDR values without pre-exposure and (d) after pre-exposure. The grey dots represent data points. The grey background represents MDR > 2 and hence synergistic interaction between clothianidin and temperature in relation to CA as a null model. The blue line shows MDR of 1 – representing an optimal fit between observation and prediction.

Table 3.1. Experimental observations and predictions of *G. pulex* from agricultural and reference streams exposed to clothianidin at 20°C and 24°C with and without pre-exposure[a].

Sites	Pre-exposure	Temp. (°C)	Observed LC_{50}[b] (µg/L)	Predicted LC_{50}[c] (µg/L)	MDR			Statistical difference between SAM and CA[d]
					SA	CA	EA	p-value
Reference	No	20	43.53	124.14	1.58	2.83	2.86	***
Agricultural			71.37	237.51	2.26	3.35	3.68	0.093
Reference		24	33.54	124.89	0.92	3.76	3.74	***
Agricultural			47.30	287.35	0.73	6.48	5.72	***
Reference	Yes	20	74.07	167.03	0.86	2.29	2.25	***
Agricultural			87.90	264.68	1.33	2.98	2.95	***
Reference		24	16.25	183.19	1.42	11.31	10.1	***
Agricultural			26.64	290.09	1.45	11.17	9.86	***

[a] Values are based on the data from 48 h pesticide exposure. [b] The observed LC_{50} was calculated using the mean survival of all reference and agricultural populations separately. [c] The predicted LC_{50} was calculated by a CA model using the mean survival of all populations with respective conditions. Synergism was considered significant when the MDR value for CA was more than 2. [d] The statistical difference between predictions of SA and CA.

3.5 Discussion

3.5.1 Effects of pesticides on macroinvertebrates.

The pesticides toxicity (TU_{max}) of the investigated streams ranged from −5.8 to −1.5. According to the European regulations for pesticides (EFSA, 2013), generally no effects should occur at concentrations 100 times below the LC_{50} of reference organisms (TU_{max} = −2). Contrary to this, we observed altered a macroinvertebrate community structure at considerably lower TU_{max} levels (TU_{max} = −3). These ecological effects (quantified with the SPEAR index) are consistent with previous studies (Hunt et al., 2017; Liess et al., 2008; Münze et al., 2015) and also with the studies from same area (Becker and Liess, 2017; Shahid et al., 2018; Siddique et al., 2020).

3.5.2 Adaptation to pesticides and multi-stress.

Our findings revealed that the *G. pulex* crustaceans from agricultural streams were up to 5-fold more tolerant to insecticide clothianidin as compared to reference populations. However, only the reference populations could induce up to a 3-fold increased tolerance when pre-exposed to low pesticide concentration, suggesting that exposure to such low concentrations of pesticide (3 orders of magnitude below EC_{50}) can induce tolerance in non-adapted forest populations (Hua et al., 2013; Poupardin et al., 2008). These results are supported by plasticity theory (Debat and David, 2001) and previous findings that suggested the development of pesticide adaptation in regularly exposed populations (Becker and Liess, 2017; Shahid et al., 2018; Siddique et al., 2020) and a physiological response upon occasional exposure (Li et al., 2018; Schlichting and Pigliucci, 1998). Further, we observed that the pesticide tolerance acquired through genetic adaptation in agricultural populations is only slightly higher as compared to the tolerance acquired through habituation in reference populations under an optimum temperature. However, the downside of genetic adaptation is the decreased plasticity and fitness costs due to pleotropic effects of resistance conferring genes (Heim et al., 2018; Jansen et al., 2011a; Lagator et al., 2014; Siddique et al., 2020). In contrast, habituated populations maintain their plasticity to

respond under fluctuating environmental conditions. This was supported by the observed fitness costs in agricultural populations genetically adapted to pesticides as these populations that were more sensitive to increasing temperature under non-contaminated conditions and mortality of organisms increased with increasing temperature, whereas the reference populations (with and without pre-exposure) did not show any fitness costs. In a rapidly changing climate, organisms are often exposed to multiple stressors with different modes of action that may interact synergistically. Although a few studies have reported synergism between multiple stressors (Chen et al., 2015; Russo et al., 2018; Shahid et al., 2019), there are some cases where only few synergistic stressor combinations were reported (Birk et al., 2020). In contrast to the EA/CA approach, the SA model could predict the synergistic multiple stress effect of temperature and clothianidin, including the increasing strength of synergism at high temperature. Recently, Zhang et al. (2018) revealed that the synergistic interaction between a toxicant and temperature is reduced in populations adapted to thermal stress. Contrary to this, we found that the combined effect was stronger on agricultural populations genetically adapted to pesticides as compared to reference populations that are not genetically adapted and, hence, multi-stress resulted in similar pesticide tolerance in both populations. This indicates that the genetic adaptation is beneficial only under pesticide stress and that this advantage is offset under multi-stress conditions. The reduction in pesticide tolerance under thermal stress may be attributed to an energy trade-off as a result of multiple stress, thus revealing the costs of physiological adaptation (Rivero et al., 2011). The reduction in tolerance in genetically adapted populations could be due to extreme stress, which leads to the exhaustion of organisms since the additional stress may trigger strong effects even in adapted organisms (Verheyen and Stoks, 2019).

3.6 Conclusions

In rapidly changing climates, organisms are increasingly exposed to a multitude of stressors. The adaptation to stressors is beneficial under a single-stressor environment, such as adaptation to pesticides in agricultural streams. In contrast, under multiple stress of pesticides and elevated

temperatures, the adaptation (either genetic or physiological) loses its advantage. The general fitness of genetically adapted populations is compromised and the populations that exhibit a plastic response may perform better under fluctuating environmental conditions.

3.7 Acknowledgments

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3.8 Author contributions

A.S., N.S., and M.L. conceptualized the study, A.S. conducted the field collection of test organisms and experimental investigations, A.S. provided the data, A.S., N.S., and M.L. conducted formal analysis, A.S. wrote the first draft of the manuscript, N.S. and M.L. reviewed the manuscript and provided the necessary amendments, and M.L. provided supervision. All authors contributed to the final version of the manuscript.

3.9 Competing interests

The authors declare no competing interests

4

Pesticide selection reduces genetic diversity

4.1 Abstract

Low levels of pesticide contamination, already below the acceptable concentrations, affect vulnerable species. Insensitive species, on the other hand, can adapt to pesticide pressure. Although numerous studies have identified fitness costs in adapted individuals, the potential propagation of effects from individual genetic composition to community composition at the landscape level is unknown. Here we show for 38 small streams that pesticide exposure at the sub-individual level resulted in a reduced genetic diversity and altered genetic composition in adapted individuals of the dominant crustacean *Gammarus pulex*. Specifically, with increasing contamination, an AFLP–assay identified for 451 loci in 1035 individuals an increased frequency of "high contamination alleles" and a decrease of "low contamination alleles". This altered genetic structure was not explained by geographical distance between the sites but by pesticide exposure. At the individual level, this genetic adaptation of *G. pulex* from contaminated sites was linked to a lower per capita growth rate. Nevertheless, at community level, *G. pulex* contributed nearly one half to the total macroinvertebrate abundance at sites with high contamination. At

the same time, however, we have also observed a decline in competing vulnerable species. We conclude that pesticide contamination at already very low toxic pressure select for tolerant genotypes with less performant individuals, and further hypothesize that dominant species may nevertheless persist due to reduced interspecific competition with declining vulnerable species.

Key words: Pesticides, local adaptation, fitness, population genetics, macroinvertebrates

4.2 Introduction

The decline of vulnerable species in agricultural streams is mainly due to pesticide contamination, even below regulatory acceptable concentrations (Beketov et al., 2013; Galic et al., 2017; M Liess et al., 2021b). Very low toxic pressure may thus determine evolutionary and -ecological processes responsible for the current loss in biodiversity (Jaureguiberry et al., 2022) and leading to an adapted community as quantified by the SPEARpesticides (Matthias Liess & Ohe, 2005) and PICT concept (Rotter et al., 2015). These community changes are determined by direct effects of low concentrations on vulnerable species(Matthias Liess & Beketov, 2011), in conjunction with a comparative tolerance of insensitive species (Matthias Liess & Ohe, 2005), and altered competition between sensitive and insensitive species (Matthias Liess & Beketov, 2011; Matthias Liess et al., 2013) as well as sub-populations with contrasting pesticide sensitivity (Becker & Liess, 2017).

Individual tolerance is determined by physiological acclimation, epigenetic modifications (Wolf & Wade, 2009) and/or genetic adaptation (Medina et al., 2007; Ribeiro & Lopes, 2013). Physiological acclimation often provides a short–term solution to cope with stress (Ghalambor et al., 2007; Pigliucci, 2005) and is limited through resource allocation(Auld et al., 2010). In contrast, a long–term exposure requires evolutionary adaptation (Major et al., 2018). Among other mechanisms (Gaines et al., 2020), genetic adaptation involves evolution of tolerance at the population level with selection of the most tolerant genotypes (Lopes et al., 2004; Orr et al., 2022) that may initially lead to reduced genetic variability (Coors et al., 2009; Jansen et al., 2011) and/or changes in allele frequency in case of

specific alleles based adaptation (Gouin et al., 2019). Consequently, populations may suffer from inbreeding due to increased levels of homozygosity of deleterious alleles, potentially resulting in local extinctions (Frankham, 2005; Keller & Waller, 2002). Therefore, understanding whether tolerance to contaminants is transient or, instead, has a genetic basis, is crucial as genetic adaptive responses may carry substantial fitness costs that lower the performance of populations under fluctuating conditions (Convey & Peck, 2019; Heim et al., 2018). These adaptive responses might also be influenced by distance to nearby refuge sections. For example, adaptation to pesticide was reduced when distance to nearby refuge sections was low, allowing migration of individuals (J. M. Becker et al., 2020; Shahid et al., 2018). Furthermore, pesticide tolerance development in G. pulex was also reduced by the presence of competing species (Becker & Liess, 2017).

Although there are numerous investigations on contamination induced genetic adaptations, understanding their ecological consequences in natural populations is still in its infancy. This is because the potential propagation of effects from the individual trade-offs up to the community composition at the landscape level are unknown. To identify these processes, we quantified the ecologically relevant short-term peak exposure of agricultural pesticides in 38 small streams with a wide range of contamination. As biological endpoints, we determined the whole invertebrate community in streams, and for *G. pulex*, a common benthic shredder in European streams(Jażdżewski, 1980) we analyzed the genetic structure applying AFLP (Amplified Fragment Length Polymorphism) technique developed by Vos et al.,(Vos et al., 1995) as the genome of *G. pulex* is not sequenced yet. Furthermore, we quantified the per capita growth rate of *G. pulex* populations in streams with different pesticide loads. We hypothesized that: (i) Increasing pesticide exposure leads to increased tolerance of *G. pulex*, (ii) populations with increased pesticide tolerance are characterized by lower genetic diversity, (iii) the distribution of alleles in populations differs according to the level of contamination, (iv) genetic adaptation negatively impact individual fitness, (v) tolerance development, genetic diversity and allele frequency of *G. pulex* populations from contaminated sites is influenced by exchange with non-adapted population from nearby refuge areas , and (vi) the dominant crustacean *G. pulex* may compensate the negative consequences of

adaptation through a reduced interspecific competition with vulnerable species.

4.3 Materials and Methods

4.3.1 Study sites and sample collection

In the present study, we collected *G. pulex* from 38 streams in Germany during 2018–2019 (Sites details Figure C.1 and Table C.1, (Matthias Liess et al., 2021)). Among these sites, 25 were contaminated with environmentally relevant levels of pesticides (log $TU_{max} \geq -3.0$) (M Liess et al., 2021b), while 13 were contaminated with considerably low concentrations and thus considered as reference streams. The sites covered a variety of stream habitats such as numerous types of sediments, plants, organic matter and morphology (Matthias Liess et al., 2021). Since the present study focused on effects of pesticide contamination, therefore it was made sure there is no input from wastewater treatment plants or industrial facilities. Contrary to contaminated streams, the reference sites were located within or at the edge of forest (Matthias Liess et al., 2021). The test organism, *G. pulex*, were sampled using kick–nets (0.5 mm) in, (i) Summer 2018 (April–June): during the peak pesticide application period conforming high pesticide exposure, (ii) Spring 2019 (March–April): before the beginning of main pesticide application, and (iii) Autumn 2019 (October–November): 3 – 4 months after main pesticide application (Matthias Liess et al., 2021) both conforming to "low pesticide exposure" in the field. To avoid the possibility of pseudo-replicates, we correlated the pesticide toxicity of the year 2018 and 2019, where the contamination between the years differed by 41%. For acute sensitivity tests, approximately 100 individuals were collected from each site only in 2019 and transported to the laboratory under constant aeration and cool boxes. For genetic analysis, 30 individuals were collected from 38 sites and stored in absolute ethanol. Additionally, the physicochemical parameters such as temperature, pH, and dissolved oxygen (DO) were also measured to make sure that these environmental parameters were normal for gammarids (Table C.1). The organisms for acute tests were kept overnight at 16°C under continuous aeration before exposure to pesticide. For genetic analysis, samples were frozen at –20°C in absolute ethanol until further analysis. A detailed description of pesticide sampling and

analytics is provided in Liess et al. (2021) (M Liess et al., 2021b). A complete list of pesticide contamination and site description is also provided at the public data repository (Matthias Liess et al., 2021). In addition, forested stream sections were identified that may serve as potential refuge areas. The distance to the closest refuge sections was measured using Google Maps. We considered refuge sections both upstream and downstream as *G. pulex* can migrate in both directions.

4.3.2 Quantification of pesticide contamination

The pesticide contamination in the streams was measured by collecting rain–event–triggered water samples (Liess et al., 1999) under the Kleingewassermonitoring (KgM) project (https:// www.ufz.de/kgm/) (Liess et al., 2021b). During sampling, 5 mL aliquots were pumped every 5 min from the stream at 10–20 cm depth for 3 h 20 min, yielding unfiltered, cooled (4 °C) composite water samples of about 200 ml. The number of samples for each site varied from one to six, depending on the rainfall events. A total of 108 chemicals comprised of insecticides, fungicides, and herbicides, were analyzed in the water samples. Targeted substances were analyzed using liquid chromatography–high–resolution mass spectrometry [LC–HRMS, Ultimate 3000 LC system coupled to a Q Exactive Plus MS equipped with a heated electrospray ionization (ESI) source, all from Thermo Scientific]. The maximum toxic unit (TU_{max}) was calculated as suggested by Liess and Ohe (Liess and von der Ohe, 2005) since several field studies conducted in small streams show that pesticide effects on macroinvertebrate communities are best explained by TU_{max} (Liess et al., 2021b; Liess and von der Ohe, 2005; Schäfer et al., 2012). Effects of pesticides on macroinvertebrates were quantified using the $SPEAR_{Pesticides}$ (SPEcies At Risk) bioindicator, which quantifies the toxic pressure of pesticides based on the proportion of macroinvertebrates classified as highly vulnerable to pesticides (“SPEcies At Risk”) to the total number of macroinvertebrates (Liess and von der Ohe, 2005). A low SPEAR value indicates high effects of pesticides and hence high pesticide exposure. A detailed description of the approach is provided in Liess et al. (Liess et al., 2021b).

4.3.3 Selection of Insecticide and Acute Toxicity Tests

In order to analyze pesticide sensitivity of populations, we used clothianidin, a neonicotinoid insecticide commonly applied in agriculture and has often been detected in small streams of the study area (Knillmann et al., 2018; Liess et al., 2021b). The acute sensitivity of *G. pulex* to the neonicotinoid was tested following the OECD guidelines for chemical testing (OECD, 2004) and the rapid tests for community level risk assessment (Kefford, 2013). A 40 mg/L stock solution was prepared in distilled water using DANTOP (500 g/kg, Spiess–Urania Chemical GmbH, Germany) and stirred overnight on a magnetic stirrer. Individuals of *G. pulex* were acclimatized for 10 days in a climate chamber preset at 16 °C, a 16:8 light–dark cycle under constant aeration and fed with ~6 g of wet weight of preconditioned leaves (Siddique et al., 2020). After acclimation to lab conditions, organisms were exposed to a range of sublethal to lethal concentrations of clothianidin. Five clothianidin concentrations (i.e., 0, 8, 40, 200, and 1000 µg/L) were prepared by diluting the stock solution in ADaM. Briefly, 16 individuals from each population were exposed to each test concentration of clothianidin for 96 h and kept in climate chambers at 16°C with a 16:8 light–dark cycle and continuous aeration. Immobility was considered as endpoint and recorded after every 24 h. Individuals were considered as immobile when they did not show body movement within 30 s after probing with a glass rod. Acute toxicity experiments were only performed with populations collected in 2019.

To determine the actual concentrations of insecticides used for acute test, we collected 1000 mL samples of stock and working solutions. Samples were analyzed by Wessling GmbH, Landsberg OT, Oppin, Germany, using a Thermo Fisher Scientific TSQ 8000 Evo Triple Quadrupole gas chromatography–mass spectrometry (GC–MS/MS). The detection limit of the instrument was 5.7 ng/L. The analytical column used was a TG–5HT guard column with a 0.53 mm ID and a 0.15 µm film thickness (Thermo Fisher Scientific, USA). The software TraceFinder 3.2 (Thermo Fisher Scientific) was applied for data processing. Actual concentrations

recovered from the samples were in acceptable boundaries (±10%) to the nominal concentrations.

4.3.4 Population genetic diversity assessment

Genetic structure and diversity parameters of *G. pulex* populations were analyzed using amplified fragment length polymorphisms (AFLP) in a total of 1035 individuals from 38 sites (25–30 individuals per sites) during 2018 and 2019. AFLP is highly reproducible and larger numbers of amplified products can be generated in a single reaction(Villalobos-Barrantes et al., 2015), and have successfully been applied for the detection of genetic loci influenced by contamination showing potential for understanding the evolution deriving forces(Bach and Dahllof, 2012; Costa et al., 2012; Markert et al., 2010). Initially, we tested two primer combinations AAC/CTT and ACC/CTG (EcoRI/MseI) followed by Bach et al., (Bach and Dahllof, 2012) and selected one primer combination AAC/CTT for final analysis, Table C.2. A total of 451 polymorphic bands were obtained and used for further analysis.

4.3.5 DNA extraction and AFLP assay

Genomic DNA was extracted from each individual *G. pulex* using Dneasy Blood and tissue kit (Qiagen, Germany) following manufacturer's guidelines with minor modifications to increase the concentration of DNA required for AFLP. DNA integrity was checked on 1.5 % agarose gel, and DNA concentration was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies Inc.).

AFLP assay used in the present study is a modified version of (Vos et al., 1995) and (Paun and Schönswetter, 2012). We performed DNA restriction and adaptor ligation in a single step. Briefly, for each individual 400 – 500 ng of DNA was double–digested with 5 U EcoRI (Invitrogen) and 1 U MseI (New England Biolabs) and ligated in 10 µl total volume including 0.6 U T4 DNA ligase (Invitrogen), 10×T4 Ligase buffer (Invitrogen), 0.5 M NaCl, 1mg/ml BSA, 5 µM MseI adaptor pair (Invitrogen), 5 µM EcoRI adaptor pair (Invitrogen) for 3 h at 37 ◦C followed by 3 h at 17 °C. MseI

and EcoRI adapter pairs were heated at 95 °C for 5 minutes separately and cooled down at room temperature before adding to Restriction–Ligation reaction. The efficiency of the restriction reaction was checked on 1.5 % agarose gel in 1 × TBE buffer. The Restriction–Ligation products were diluted 10–fold with 0.1 × TE buffer and stored at –20°C until further processing. Pre–selective amplifications were then performed on 5 µl of diluted RL products in 10 µl volumes containing 10 mM dNTP–mix, 1 µM each of EcoRI and MseI preselective primers (premixed) (Invitrogen), 1 U Hotstart Taq polymerase (Invitrogen) all in 10 × PCR buffer (Invitrogen) and ddH_2O. The PCR conditions for the preselective amplification were: one hold of 72°C for 2 min, 20 cycles of (94 °C for 1 sec; 56 °C for 30 s, 72 °C for 2 minutes), and finish with a hold of 60 °C for 30 minutes. Preselective products were diluted 10–fold with 1 × 0.1M TE buffer. Selective amplifications were performed on 5 µl of diluted preselective products in 10 µl volumes containing 10 mM dNTP–mix, 1 µM of EcoRI and 5 µM MseI selective primers (Invitrogen), 1 U Hotstart Taq polymerase (Invitrogen) all in 10 × PCR buffer (Invitrogen) and ddH_2O. The cycling conditions for selective PCR were as follows: one hold of 94 °C for 2 minutes, 9 cycles of (94 °C for 1 sec, 65 °C; decreasing 1 °C every cycle for 30 sec and 72 °C for 2 minutes), followed by 23 cycles of (94 °C for 1 sec, 56 °C for 30 sec and 72 °C for 2 min) and finish with a hold of 60 °C for 30 minutes. Table C.4 provides information on choice of adaptor and primer constructions. Negative controls (TE buffer used as template) were processed in parallel. The final products were sequenced on the ABI Prism 3130XL Genetic Analyzer (Applied Biosystems) with Red 500 DNA size standard and analyzed in GeneMapper 5.0.

4.3.6 Genetic analysis

GeneMapper 5.0 (Applied Biosystems) was used to score AFLP alleles. DNA fragments less than 50 base pairs in length were excluded from the analysis to minimize the probability of including homoplastic AFLP bands. Among 451 loci, a maximum of 200 AFLP alleles were found in individuals. Peak data were converted to create a binary matrix by scoring for presence (1) or absence (0) of a DNA fragment. A data qualification selection was performed by which AFLP markers with less than 5%

abundance in total dataset (Bonin et al., 2005), and individuals with less than 140 AFLP markers were excluded. GenAlex v. 6.51 was used to calculate basic genetic statistics including private alleles (np), average number of alleles per locus (Na), percentage of polymorphic loci (PLP), unbiased expected heterozygosity (uHe), and expected heterozygosity (He). The Nei's genetic distances and genetic differentiation (PhiPT) were also calculated in GenAlEx v. 6.501(R Peakall & Smouse, 2020; ROD Peakall & Smouse, 2006). Population differentiation was further quantified by analysis of molecular variance (AMOVA). The program I4A, inbreeding for AFLPs, (Chybicki et al., 2011) was used to estimate the inbreeding coefficient (Fis) for each population. Estimates were calculated at three different values (0.1, 1, and 5) for each parameter, alpha and beta (Oleksa et al., 2013), using 10,000 rejected steps and 20,000 sampling steps. By computing different values of alpha and beta, we assess how much the inbreeding coefficients change when the shape parameters change. The accuracy of this estimate is determined by comparing the similarities between the loglikelihood values among the different B–distribution parameters. High similarity implies high accuracy.

The program Past 3.25 (Hammer et al., 2001) was used to access genetic diversity by applying Bray–Curtis similarity index on individuals within and between populations at each sampling site, thus creating a similarity matrix between all individuals. A Bray–Curtis similarity matrix was used as input for ANOSIM. To test whether the geographic distance accounted for eventual differences observed in genetic diversity between populations, the geographic distances between each pair of sites were measured based on the waterway GPS positions and correlated to the pairwise genetic dissimilarity based on ANOSIM computed by SIMPER.

4.3.7 Populations' per capita growth assessment

The long–term effects of pesticide exposure in field and adaptation were analyzed by culturing *G. pulex* under pesticide–free conditions in the laboratory(Siddique et al., 2020). In general, maintenance of culture followed the descriptions given by (McCahon and Pascoe, 1988a, b) with certain modifications. Briefly, 100 medium–sized individuals from each population were cultured in a 5 L covered glass tank filled with 3 L aerated artificial stream water (ADaM) (Klüttgen et al., 1994). Organisms were

fed ad libitum with pre–conditioned– alder leaves and stones (Siddique et al., 2020). Continuous aeration was provided in order to avoid oxygen shortage during experiments and cultures. To maintain the quality of the culture medium, 500 mL of medium was replaced by fresh medium every 14 days and 1500 mL of old medium was replaced with fresh medium every 30 days, and dead organisms were removed. The culture was maintained at 16 °C, with 60% humidity and artificial light (12 h light:12 h dark) in a climate chamber. Adults and offspring were counted every month until four months. For population growth, expressed as the per capita growth rate, the total number of individuals was divided by the initial number of individuals.

4.3.8 Macroinvertebrate abundance and population size

Under KgM project, macroinvertebrate from each site were collected from 20 sub-samples from each stream considering different types of habitat. The samples were stored in ethanol and were determined under microscope. Each individual of species in a sample was counted to determine abundance and population size.

4.3.9 Data analysis

All the statistical analyses were carried out using RStudio (V 1.2.1335) and R (V 3.5.1) for Windows, unless otherwise mentioned. For the acute sensitivity, the concentration that affected 50% of the exposed population (EC50) was calculated using a generalized linear model with a quasi–binomial error distribution and a logit link function (V 3.0–1)(Ritz et al., 2015). For the comparison of EC_{50} values (spring vs autumn), t–test was applied. The local contamination was based on TU_{max} values derived from water samples collected in summer 2018 and 2019. Similarly, for the quantification of ecological effects, the SPEAR was calculated based on invertebrate data collected in June for both years. Two sample t–tests (when the assumption of equal variances was fulfilled) and Welch's t–test (in the case of nonequal variances) were applied to compare genetic parameters among reference and agricultural populations. In case the data were not normally distributed, Wilcoxon's rank sum test was used.

Populations were divided into three groups based on TUmax range from –6 to –4.1, –4 to –2.1 and –2 to 0 and named as "Low Contamination", "Medium contamination" and "High contamination" respectively and compared using t–tests. The "low contamination alleles" were quantified based on the allele frequency significantly higher in the low contamination populations as compared to contaminated populations, and average frequency of these alleles for each population was used for further analysis. Similarly, the "high contamination alleles" were quantified based on the allele frequency significantly higher in the high contamination populations as compared to reference populations, and average frequency of these alleles for each population was used for further analysis.

For the population growth, expressed as the per capita growth rate, the total number of individuals was divided by the initial number of individuals. To analyze the association between different variables, linear regression was applied. The assumptions of homoscedasticity and of normally distributed residuals were confirmed by visual inspection, plotting residuals vs. fitted values, residuals vs. leverage, and Q–Q plots.

4.4 Results

4.4.1 Pesticide Exposure, invertebrate community structure and adaptation of G. pulex

The sites studied were characterized by pesticide concentrations resulting in a toxic pressure ranging from severe effects on macroinvertebrate communities to no effects (log TU_{max} –0.13 to –6.0). Neonicotinoids and carbamate insecticides exerted the highest toxic pressure. Their ecological effects were identified with the bio indicator $SPEAR_{pesticides}$ (proportion of vulnerable species), which negatively correlated to the in-stream pesticide contamination (adj. $R^2 = 0.24$, $p < 0.001$; Figure 4.1a). Also, pesticide tolerance of *G. pulex* increased with increasing toxic pressure. The 48 h

EC_{50} of *G. pulex* to insecticide clothianidin positively correlated with the TU_{max} (adj. R^2 = 0.51, $p < 0.005$, Figure 4.1b). Other field parameters potentially affecting the pesticides tolerance are shown in correlation plot Figure C.2.

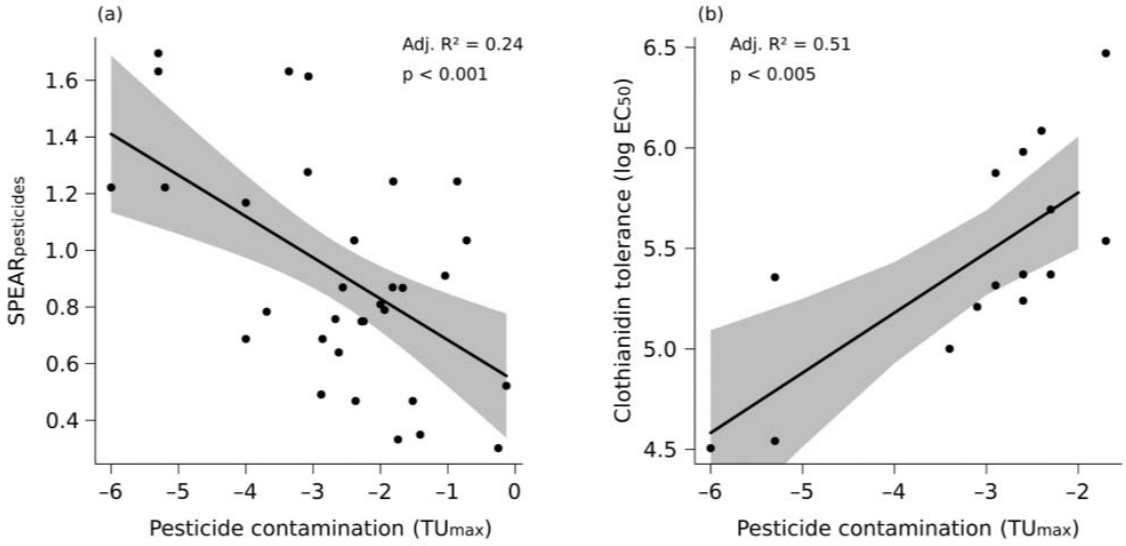

Figure 4.1. Effect of pesticide exposure. Relationship between site specific pesticide contamination (TU_{max}) and (a) macroinvertebrate community structure quantified as $SPEAR_{pesticides}$ and (b) pesticide tolerance of *G. pulex* (quantified as EC_{50} of the insecticide clothianidin after 48h constant exposure, log–transformed). Means ± 95% confidence intervals are shown.

4.4.2 Genetic diversity and genetic structure of G. pulex

The primer combinations selected in the present study yielded 451 polymorphic loci across 1035 individuals from 38 populations. Non-metric Multi-Dimensional Scaling (NMDS) showed organisms from agricultural and reference populations as two slightly overlapping clusters (Figure C.3). The organisms are designated to green and red group based on pesticide contamination with a cut-of value TU_{max} -3.2. Two agricultural sites were located within 20m to the refuge sections, therefore have high genetic diversity and similarity to reference sites. The overall within population genetic diversity based on Bray-Curtis index was significantly lower in contaminated populations as compared to reference populations (5.2% vs 17.4%, Table C.3). Also, the average between populations' genetic diversity was significantly lower in contaminated populations (34% vs. 50%, $p < 0.001$). Percentage of polymorphic loci (PLP), average

number of alleles per locus (Na), expected heterozygosity (He), and unbiased heterozygosity (uHe) was higher in reference populations (details; Table C.1). A significant, albeit weak relationship was observed between the pairwise genetic dissimilarity and geographic distance between populations (adj. $R^2 = 0.039$, $p < 0.001$, Figure C.4). Detailed pairwise genetic dissimilarity and geographic distance is provided in Table C.4. Although in general, populations were significantly differentiated from each other (overall PhiPT = 0.45, $p < 0.001$), the contaminated populations were more differentiated from each other (average PhiPT = 0.51) as compared to reference populations (average PhiPT = 0.33, pairwise PhiPT Table C.5). The AMOVA analysis revealed that in reference streams, on overage 67% of the variation was observed within population and 33% among populations. Whereas, in contaminated streams, 49% variation was observed within populations and 51% among populations.

4.4.3 Genetic diversity and adaptation of G. pulex

We observed a significant negative association between pesticide contamination in streams (TU_{max}) and population genetic diversity of *G. pulex* (adj. $R^2 = 0.63$, $p < 0.001$, Figure 4.2a). Based on the toxic pressure exerted by pesticides (TU_{max}), we divided the populations into three groups, i.e., low (TU_{max} –6 to –4.1), moderate (TU_{max} –4 to –2.1), and high contamination (TU_{max}; –2.0 to 0). We revealed a higher genetic diversity in populations from low contamination sites (mean 17.57%) followed by moderate (mean 9.45%, $p < 0.005$) and high contamination sites (mean 5.18%, $p = 0.015$, Figure 4.2a). Furthermore, adapted populations were characterized by the reduced genetic variability (adj. $R^2 = 0.504$, $p < 0.005$, Figure 4.2b), which also showed association with SPEARpesticides, shannon index, total number of insect taxa, and % EPT species (Figure C.5). Furthermore, correlation plot for additional

environmental parameters potentially affecting genetic diversity are shown in Figure C.6.

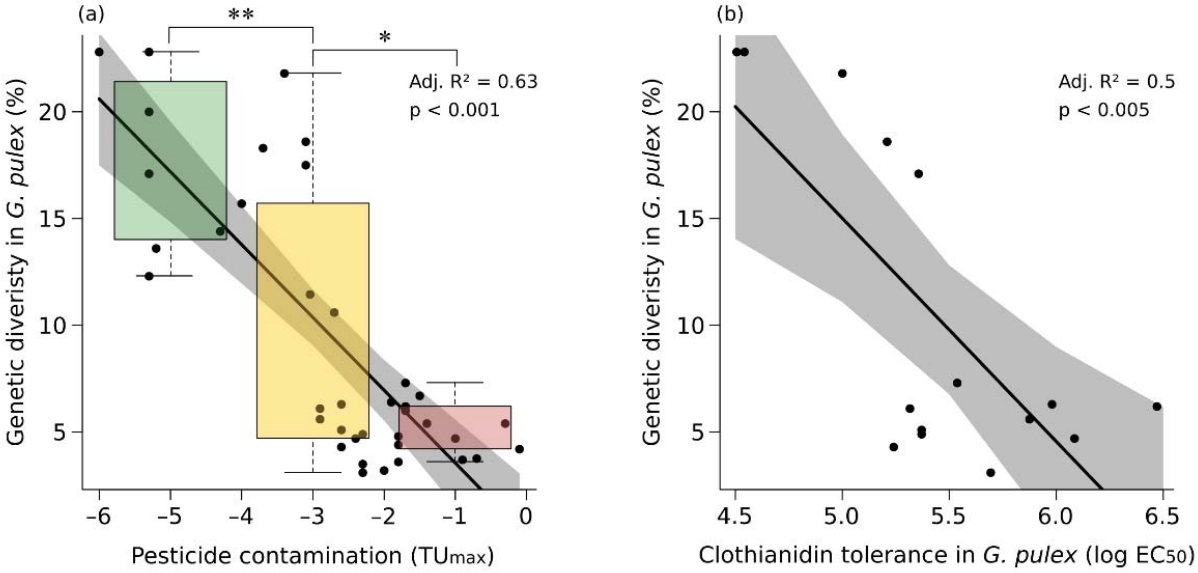

Figure 4.2. Pesticide exposure, genetic diversity and adaptation of *G. pulex*. Relationship between site specific pesticide contamination (TU_{max}) and within population genetic diversity in *G. pulex* (a). Total pesticide contamination: The green box represents populations with field exposure from TU_{max} –6 to –4.1, the yellow box represents populations from TU_{max} –4 to –2.1, and the green box represent populations from TU_{max} –2 to 0. Asterisks show significant differences between the groups. (b) Clothianidin tolerance (log EC_{50}) and within population genetic diversity in *G. pulex* populations. Grey area represents ± 95% confidence interval and dots represent data points.

4.4.4 Contamination specific alleles in G. pulex

The reference and contaminated populations showed different distribution of alleles over 451 loci. We observed that the average frequency of 52 alleles termed "low contamination alleles" was significantly higher in populations at sites with low pesticide contamination (0.54 vs. 0.34, $p < 0.05$), and also associated with site-specific pesticide contamination (adj. $R^2 = 0.43$, $p < 0.001$,). Accordingly, the average frequency of these alleles was significantly different among populations from low, moderate and high contamination sites revealing a toxic pressure dependent decline in allele frequency (Figure 4.3a).

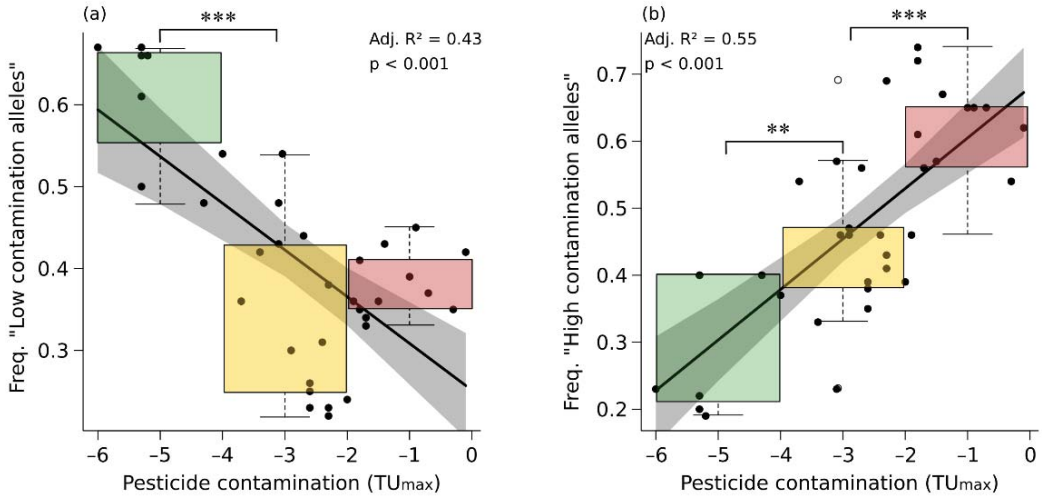

Figure 4.3. Pesticide exposure and contamination-specific alleles of *G. pulex*. Relationship between site specific pesticide contamination (TU_{max}) and (a) average allele frequency of "low contamination alleles" in *G. pulex* populations based on alleles significantly higher in streams with low toxic pressure (TU_{max} range from –6 to –4), (b) average allele frequency of "high contamination alleles" in *G. pulex* populations based on alleles significantly higher in streams with high toxic pressure (TU_{max} range from –2 to –0). The green box represents populations with field exposure from TU_{max} –6 to –4.1, the yellow box represents populations with field exposure from TU_{max} –4 to –2.1, and the red box represents populations with field exposure from TU_{max} –2 to 0. Grey area represents ± 95% confidence interval and dots represent data points.

For populations occurring in highly pesticide contaminated sites we observed that the average frequency of 14 alleles termed "high contamination alleles" was significantly higher (0.54 vs. 0.34, $p < 0.05$), and positively correlated with site-specific pesticide contamination (adj.

$R^2 = 0.55$, $p < 0.001$, Figure 4.3b). Accordingly, the average frequency of these alleles was significantly different among populations from low, moderate and high contamination sites revealing toxic pressure dependent increase in allele frequency. Additionally, the ratio of high to low contamination alleles increased with increasing toxic pressure at sites (adjusted $R^2 = 0.55$, $p < 0.001$, Figure C.7).

4.4.5 Fitness trade–offs in genetically adapted populations

Pesticide adapted populations of *G. pulex* were characterized by a reduced per capita growth when cultured under non–contaminated conditions (adj. $R^2 = 0.92$, $p < 0.005$, Figure 4.4a). Furthermore, average per capita growth was associated with within population genetic diversity (adj. $R^2 = 0.64$, $p < 0.05$, Figure 4.4b).

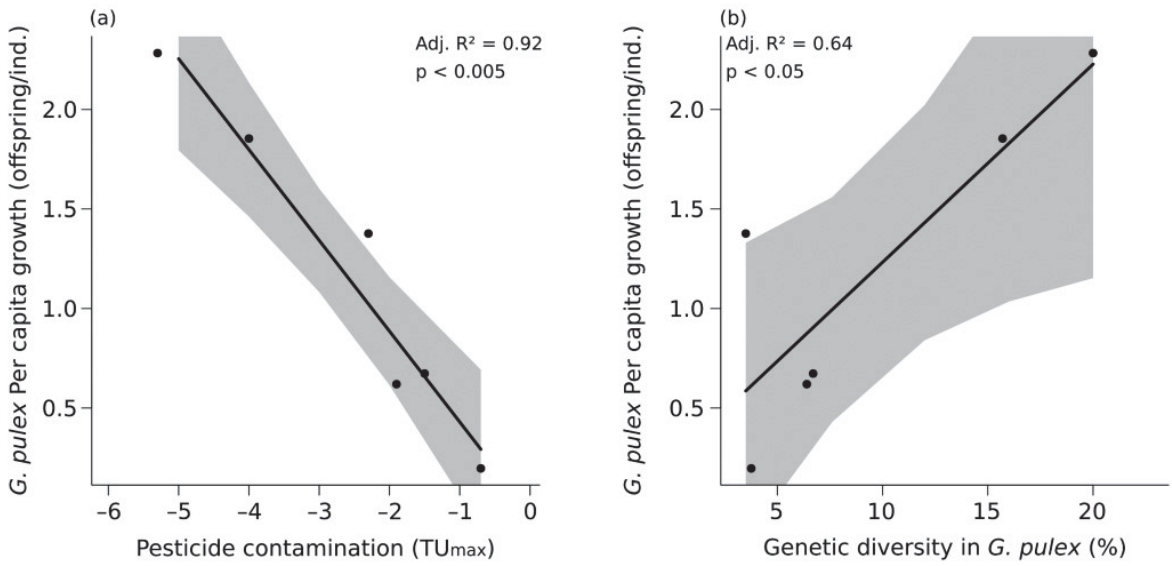

Figure 4.4. Fitness costs of exposure to pesticides in *G. pulex*. (a) Relationship between site specific pesticide contamination (TU_{max}) and per capita growth of gammarid populations cultured under non–contaminated conditions (average per capita growth in cultures from month 2 to 4), (b) Linear regression between genetic diversity and average per capita growth of gammarid populations cultured under non–contaminated conditions. Grey area represents ± 95 % confidence interval and dots represent data points.

4.4.6 Distance to refuge and genetic composition of G. pulex

G. pulex acquired a further increased tolerance when distance to nearby refuge area was high presumably decreasing the exchange rate between adapted and non-adapted populations (adj. R^2 = 0.22, p = 0.06, Figure 4.5a). Additionally, increasing distance to refuge sections reduced the genetic diversity (adj. R^2 = 0.36, $p < 0.001$, Figure 4.5b) and frequency of "low contamination alleles" (adj. R^2 = 0.42, $p < 0.001$, Figure 4.5c).

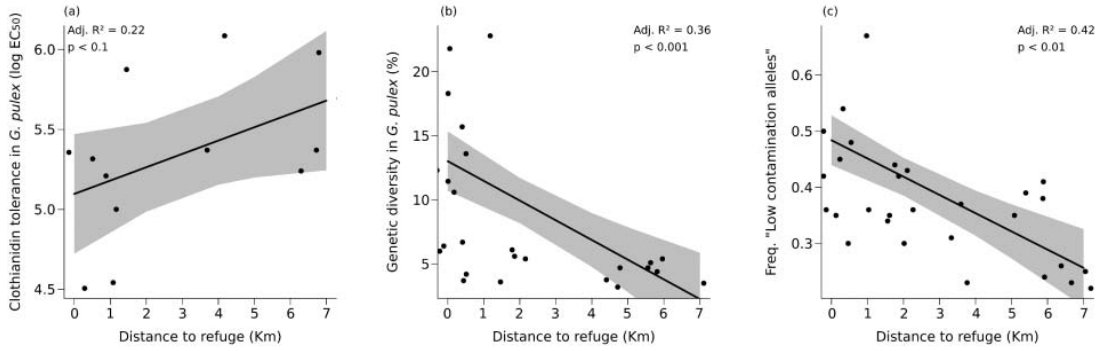

Figure 4.5. The effect of nearby refuge areas. Relationship between distance to refuge and (a) Clothianidin tolerance (48h EC_{50}) in *G. pulex*, (b) Within population genetic diversity and (c) Frequency of low contamination alleles in the studied populations of *G. pulex*. Grey area represents ± 95% confidence interval and dots represent data points.

4.4.7 Inbreeding in G. pulex and relation to vulnerable competitors

With increasing abundance of *G. pulex*, inbreeding coefficient of respective populations slightly decreased (R^2 = 0.12, $p < 0.05$, Figure C.8). In contrast, the inbreeding coefficient was not associated with pesticide contamination or genetic diversity ($p > 0.1$, Figure C.9). Also, abundance of populations was not affected by pesticide exposure (Figure C.10). However, *G. pulex* population density decreased with the increasing occurrence of vulnerable competitors. Such a relationship was established for EPT species (Ephemeroptera, Plecoptera, Trichoptera; adj. R^2 = 0.21, $p < 0.01$) and also for the proportion of vulnerable species (SPEARpesticides; adj. R^2 = 0.13, $p < 0.01$) Figure 4.6a and b. Correlation plot of abundance of macroinvertebrates groups is given in Figure C.11.

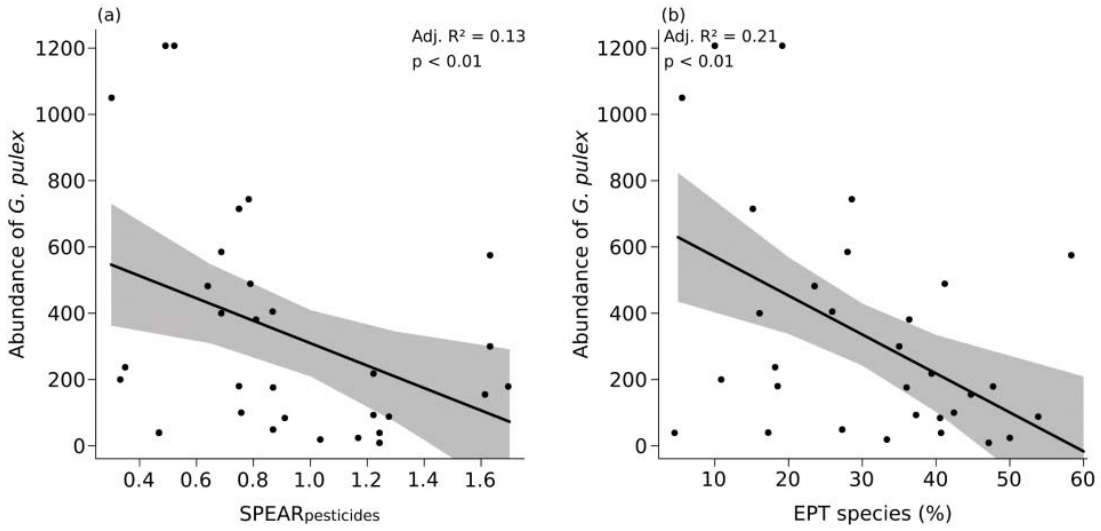

Figure 4.6. Abundance of *G. pulex*. Relationship between Abundance of *G. pulex* and (a) $SPEAR_{Pesticides}$, and (b) Percentage of vulnerable species. Grey area represents ± 95% confidence interval and dots represent data points.

4.5 Discussion

Pesticide exposure triggers evolutionary processes in natural populations (Medina et al., 2007), and also alters the macroinvertebrate community composition (M Liess et al., 2021b; Rumschlag et al., 2020; Stehle & Schulz, 2015). There are numerous investigations on contamination induced genetic adaptations, however, understanding their ecological consequences in natural populations is still in its infancy. This is because the potential propagation of effects from the individual genetic composition up to the community composition at the landscape level are unknown. In detail, relevant biological effects include: (i) increased tolerance and a reduction in genetic diversity as an outcome of contamination (Bach & Dahllof, 2012), (ii) toxic pressure dependent distribution of alleles at ecological relevant ultra-low exposure concentrations, (iii) fitness trade-offs associated with genetic adaptation (Heim et al., 2018), (iv) impact of geographical distance to the non-contaminated refuge populations (J. M. Becker et al., 2020; Shahid et al., 2018) and (v) the interspecific competition with vulnerable species. A few of these processes have been investigated to some extent. However, to the best of our knowledge, this is the first study that integrates the processes linked to pesticide exposure on all these levels of biological organization.

At community level, we observed a decline in vulnerable species already below the regulatory compliant concentrations ($TU_{max} \geq -3$), mostly contributed by neonicotinoids and carbamate insecticides. These results are comparable with other investigations that identified strong impacts of nonpoint-source pesticide pollution on streams in Australia (Beketov et al., 2013), Europe (Beketov et al., 2013; M Liess et al., 2021b; Matthias Liess & Ohe, 2005), North America (Chiu et al., 2016) and South America (Hunt et al., 2017). A situation that is in contrast to the aims of the regulatory authorization of pesticides that is supposed to prevent unacceptable effects in the environment (Australian Environment Agency, 2009; EFSA (2013); US Government, 2004).

At individual level, we observed up to 4.5–fold (average 2.2–fold) increased pesticide tolerance in comparatively less sensitive specie *G. pulex* collected from contaminated streams. Such a development of pesticide tolerance in different aquatic species such as *G. pulex*(Siddique et al., 2021) and *Daphnia magna*(Almeida et al., 2021) have been observed. Similarly, local adaptation to ore mine effluents have also been observed in *Dugesia gonocephala* (Weigand et al., 2018). The acquisition of increased tolerance in a natural population may occur through environmentally induced physiological adjustments (acclimation)(Maxwell et al., 2014; Siddique et al., 2021), epigenetic modifications (Wolf & Wade, 2009), loss of sensitive individuals, and selection of more tolerant genotypes which may reduce the genetic diversity (Forbes & Calow, 1996; Van Straalen et al., 2011). The latter could be the reason for reduced genetic diversity in the current study. Furthermore, reduced genetic diversity explained by toxic pressure reveal that even such low concentrations may significantly shape genetic metrics of populations and may describe the loss of sensitive species under long–term exposure that have so far not been studied.

In general, genetic erosion is widely accepted as a potential evolutionary outcome of long–term exposure to contaminants(Nowak et al., 2009; Ribeiro & Lopes, 2013; Ungherese et al., 2010). For example, low genetic diversity in >700 aquatic species from extensive cropland(Crossley et al., 2022), and reduction in allelic richness was observed in *G. pulex* from

wastewater contaminated rivers(Švara et al., 2022). However, in these cases, investigations only focused on high exposures and did not investigate the effect of low exposure and possibility of local adaptation. Furthermore, tolerance acquired through genetic adaptation based on specific alleles may change allele frequency as a result of increased number of tolerant individuals in a population (Gouin et al., 2019). Likewise, in the present study, we observed a decrease in average alleles frequency of "low contamination alleles" with increasing toxic pressure. This change in frequency was significantly different among populations from low, moderate and high pesticide exposure indicating loss of alleles already at concentrations 3 to 4 orders of magnitude below the EC_{50}. Reduction in frequency of some alleles could be attributed to long-term consequence of contamination that selects for tolerant genotypes as mentioned earlier(Ribeiro & Lopes, 2013). On the other hand, an increasing average frequency of "high contamination alleles" with pesticide contamination observed in the present study indicates effects on allelic distribution on such a low range of pesticide contamination. Further, this suggests that the "high contamination alleles" might be functionally associated with tolerance conferring mechanisms required to cope with pesticide stress. For instance, the allele frequency of two outlier SNP loci involved in insect cuticle resistance in natural populations of mayfly *Andesiops torrens* were significantly correlated with high pesticide exposure (Gouin et al., 2019).

Although pesticide exposure was mainly responsible for the observed effects in the investigated streams (M Liess et al., 2021b), several other environmental factors may affect the genetic diversity of populations. For example, geographic distance between sites partially explained the pairwise genetic dissimilarity. Other studies also reported genetic structure of populations partially or completely explained by geographic distance(Svara et al., 2022; Whitehead et al., 2003). Furthermore, historical colonization events in *Gammarus fossarum* (Weiss & Leese, 2016) and barrier effects in *Ancylus fluviatilis* and *D. gonocephala* (Weiss et al., 2022) have been associated with populations genetic structure and differentiation. In addition, abundance of sensitive species may also impact the evolutionary process as tolerance development can be affected by

species diversity (Becker & Liess, 2017). Likewise, we observed a higher genetic diversity in *G. pulex* populations with species richness in the respective streams indicating that the conditions that promotes species diversity may also preserve genetic diversity within species. Such a positive association between genetic– and species diversity has also been reported by Manel et al. (Manel et al., 2020).

Fitness costs are often associated with increased tolerance due to energy trade–offs (Fournier-Level et al., 2019; Siddique et al., 2021). However, it has rarely been reported in natural populations (Jansen et al., 2011; Siddique et al., 2020). We observed that pesticide exposure and genetic adaptation to pesticides were associated with reduced per capita growth in natural populations of *G. pulex* revealed under non-contaminated conditions. This finding suggests that survival in contaminated streams infers energetic costs reducing the energy available for vital functions and reduces plasticity for survival under slightly different conditions(Marchand et al., 2004). Also other studies revealed that pyrethroid resistant *Hyalella azteca* exhibited reduced thermal tolerance(Heim et al., 2018), and lower survival and lipid levels(Fulton et al., 2021). However, these trade-offs were revealed in the presence of stressors, unlike our study where the fitness costs were observed under non-contaminated conditions.

Migration of organisms from nearby refuge area often support recovery for pesticide affected individuals (Jeremias Martin Becker et al., 2020; Shahid et al., 2018) and populations (Matthias Liess & von der Ohe, 2005). Likewise, we observed that the increase in clothianidin tolerance was reduced with increasing distance to the nearby refuge area. However, genetic diversity and frequency of "low contamination alleles" increased with decreasing distance to refuge sections, suggesting that migration events from nearby refuge sections supports populations to retain genetic diversity.

Inbreeding coefficient of *G. pulex* populations increased with decreasing abundance. However, nor local pesticide contamination neither reduced genetic diversity did induce inbreeding depression. Similarly, Svara et al. (Svara et al., 2022) reported higher inbreeding coefficient in *G. pulex* from

wastewater contaminated sites as compared to pristine sites, which could also be due to a smaller and thus genetically impoverished population.

On the ecosystem level, we observed that the abundance of *G. pulex* was independent of pesticide contamination (Fig C.10) despite their apparent fitness trade-offs. We hypothesize that this non-existent association of *G. pulex* abundance and pesticide exposure is due to the exposure-induced decline in competing pesticide-vulnerable SPEAR and EPT species. In contrast, species with low or even declining dominance may not be able to compensate individual trade-offs from pesticide effects at the community level (Matthias Liess & Beketov, 2011).

Thus, pesticide exposure at already low toxic pressure causes decline in vulnerable species, while on the other hand, selects tolerant genotypes and individuals with reduced per capita growth in dominant species. We hypothesize that dominant species, however, benefits from reduced interspecific competition with declining vulnerable species. This, we suggest, is a relevant process of species selection to site-specific environmental conditions. Unravelling these underlying mechanisms enables to link effects from genes towards the ecosystem-level.

4.6 Acknowledgements

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4.7 Authors contributions

A.S., N.S., and M.L. conceptualized the study; A.S. conducted the field collection of test organisms, experimental investigations and genetic analyses; A.S. performed the initial analysis; M.L. guided the analytical cognition process; A.S. wrote the first draft of the manuscript, extended by M.L; All authors contributed to the final version of the manuscript.

4.8 Competing interests

The authors declare no competing interests

5

Predicting the combined effects of multiple stressors

5.1 Abstract

Global change confronts aquatic organisms with multiple stressors causing synergistic effects. Persistent stress, however, leads to adaptation and related trade-offs. The question arises: How can the combined effects of these apparently contradictory processes be predicted? Here we show that pesticide adapted *Gammarus pulex* from agricultural streams were more tolerant to pesticides (clothianidin, prochloraz) as compared to non-adapted populations. However, joint exposure to both pesticides and temperature stress resulted in acute synergistic interactions, and the combined effects were stronger in adapted populations. We hypothesize that the pesticide adaptation reduces general stress capacity of individuals and trade-off process increases sensitivity to the combined stress. The general stress exerted by each of the individual factors was quantified using the Stress Addition Model (SAM). Both pesticides, warming and the reduced stress capacity of adapted populations acted synergistically. We

conclude that acute effects of multiple stressors including trade-offs are synergistic and can be predicted by SAM.

5.2 Introduction

Planetary Boundaries for climate change, chemical pollution, land use change, and nutrients are transgressing safe limits for maintaining biodiversity (Persson et al., 2022; Rockström et al., 2009). However, these boundaries do not explicitly consider the interactions between different stressors, instead, it focuses on individual stressors in isolation. This is mainly because the interactions between different stressors are complex, the interaction cannot be predicted as no general framework is existing to calculate these interactions. Accordingly, the Planetary Boundaries framework will increase its usefulness for understanding the global risks when stress interactions are predictable.

The combined effects of multiple stressors are generally shaped by their magnitude, modes of action, exposure sequence, and overlap duration, and can be additive (equal to the sum of individual stressors calculated with the respective null model), antagonistic (less than additive), or synergistic (more than additive). Concentration addition is generally observed when two or more chemicals affect the same mode of action or target the same biological pathway (Folt et al., 1999). If organisms are adapted at population and community level (Liess et al., 2013a; Liess et al., 2021c), or show co-variability to stressors, antagonistic interactions can be observed (D. Vinebrooke et al., 2004; Folt et al., 1999). Several investigations have already showed antagonistic effects of multiple stressors at population and community level (Chara-Serna et al., 2019; Cornejo et al., 2019; Mehler et al., 2011). In contrast, synergistic interactions are more likely to be prevalent when the stressors are independent, act on different biological pathways or physiological systems (Darling and Côté, 2008). Acute synergistic interactions have been reported by several investigations (Raby et al., 2019; Shahid et al., 2019; Walker et al., 2022).

Several common pesticides with different modes of action are often applied together as tank mixtures or sequential applications, and hence, co-

occur in the environment. A plethora of chemicals can be found in freshwaters, especially after rainfall events (Liess et al., 2021c; Riise et al., 2004; Werner et al., 2004). In the last decade, neonicotinoids were the most commonly applied class of insecticides in the study area and worldwide (Jeschke et al., 2011; Sánchez-Bayo et al., 2016; Simon-Delso et al., 2015b). Azole fungicide prochloraz has also been detected frequently in European surface waters (Kreuger et al., 2010; Liess et al., 2021c; Munze et al., 2017) and are known to interact synergistically with different insecticides such as neonicotinoids (Iwasa et al., 2004), pyrethroids (Kretschmann et al., 2015; Wieczorek et al., 2018) and organophosphates (Sejerøe, 2011). These pesticide mixtures have frequently been detected in small streams located in agricultural landscapes (Liess et al., 2021c; Munze et al., 2017; Shahid et al., 2018). Further, organisms in the field experience sub- or supra-optimal conditions and are forced to cope with complex environmental stress (Holmstrup et al., 2010). Under climate change scenario, extreme temperature is one of the most relevant stressors that can further enhance the effects of pesticides (Piggott et al., 2015; Russo et al., 2018). Increased temperature may pose physiological stress to aquatic organisms by increasing metabolic rate associated with the mechanisms of thermal tolerance (Cherkasov et al., 2006; Feder and Hofmann, 1999). Even though several studies have shown that environmental stress may act synergistically on the biological effects of single toxicants (Liess et al., 2013a; Macaulay et al., 2021a; Meng et al., 2022), it is not clear how adaptation to pesticides influences the interaction between pesticide mixtures and environmental stressors. Adaptation depends on trade-offs between the benefits of immediate stress responses and their long-term fitness costs. Therefore, predicting the combined effects of stressors is not possible without understanding the relevant factors such as mixture toxicity, environmental stress, adaptation, and associated fitness costs. To enable an effective ecosystem management, we need to determine the ecological relevance of each of these factors.

Accordingly, we aim to reveal complex interactions of a frequently detected insecticide clothianidin, and an azole fungicide prochloraz in combination with warming – a most relevant environmental stressor under climate change. Further, we aim to investigate how pesticide adaptation

shapes multiple stress–response relationships. For this purpose, we investigated populations of the widespread crustacean *G. pulex* from contaminated and reference streams and exposed them to a mixture of pesticides and temperature stress. Furthermore, we predicted combined impacts using traditional models for toxicant mixtures (i.e., concentration addition (CA) (Bliss, 1939a) and effect addition (EA) (Loewe and Muischnek, 1926a)) and stress addition model (SAM (Liess et al., 2016b)) designed to predict synergistic interactions between independent stressors. To our best knowledge, this is the initial study to reveal complex interactions of pesticide mixtures, environmental stress, and the fitness cost of pesticide adaptation which may act as a stressor under multi-stress conditions. Due to its high topical relevance, we expect that the approach presented here will be the starting point for a fundamental expansion of our understanding of the effect of multiple stressors.

5.3 Materials and Methods

5.3.1 Sampling of test organisms and characterization of pesticide pollution

Individuals of *G. pulex* were collected from 12 sites, 8 from pesticide contaminated agricultural streams, and 4 from un-contaminated forested stream sections (considered as the reference group; Supporting Information, Figure D.1). During sampling of test organisms, physicochemical parameters such as dissolved oxygen (DO), pH, electrical conductivity (EC), water temperature (°C), and water level (cm) were measured.

This investigation was conducted as part of the nationwide small stream monitoring (KgM) project(Liess et al., 2021c), and data pertaining to community structure and pesticide pollution were obtained from this dataset. The toxic pressure of pesticide pollution was quantified by analyzing the event-driven runoff samples and grab samples in 2018, 2019, and 2021 during the peak application of pesticides (April – July). Rain-event-triggered water samples were collected using automated (MAXX TP5, Rangendingen, Germany) and bottle samplers (EDS) (Liess and Von

der Ohe, 2005b) to capture run-off-induced peak exposures after rainfall (Liess et al., 1999). Run-off events raise the water level of streams that trigger the samplers to capture peak concentrations. Automated samplers take 5 mL water every 5 min from the stream for 3.3 h, yielding 200 mL of water samples. Collected samples were kept at 4°C in samplers until they were transported to the laboratory within 48 h. Grab samples were collected regularly after every three weeks which is similar to the monitoring practices suggested by the Water Framework Directive (WFD).

A wide range of pesticides (108) and urban toxicants (257) was analyzed. All pesticides were quantified using liquid chromatography MS/MS, whereas, urban toxicants using liquid chromatography − high-resolution mass spectrometry − LC−HRMS as mentioned earlier (Liess et al., 2021c) (for details, see (Reemtsma et al., 2013)).

5.3.2 Calculation of toxicant exposure

To estimate pesticide-induced water toxicity, measured concentrations were transformed into toxic units (TUs) by dividing them with their respective acute LC_{50} for the standard reference organisms (Sprague, 1970). To further quantify the highest toxic pressure per site, we calculated the maximum toxic unit (TU_{max}) (Eq. 5.1, Liess and Ohe (2005)).

$$TU_{max} = \mathrm{Max}_{i=1}^{n}\left[\log_{10}\left(\frac{Ci}{LC_{50i}}\right)\right] \qquad (5.1)$$

where TU_{max} is the highest value of the toxic unit, *Ci* is the detected concentration of the pesticide (µg L^{-1}), and the LC_{50i} is the respective acute median lethal concentration (µg L^{-1}) for the most sensitive reference organism. For the calculation of TU_{max}, LC_{50} values of the most sensitive species were used and obtained from the Pesticide Properties Database (PPDB) (University of Hertfordshire, 2014) and Ecotoxicology Database System (USEPA, 2014).

5.3.3 Characterizing the ecological effects of pesticides

We used a bio-indicator $SPEAR_{pesticides}$ to quantify the long-term impact of pesticides on macroinvertebrate community structure (Liess and Ohe,

2005). The SPEAR index quantifies the toxic pressure of pesticides by classifying macroinvertebrates as vulnerable and nonvulnerable taxa based on different ecological traits. We calculated SPEAR values using the Indicate software (https://systemecology.de/indicate/).

Benthic macroinvertebrates were collected using multi-habitat sampling technique. Briefly, 20 subsamples were collected from representative microhabitats along a 50 m stretch of the stream, using a square-frame kick-net with a surface area of 625 cm^2 (mesh size: 500 µm). Collected organisms were filtered through a set of sieves, and preserved in 90% ethanol. Taxa were determined to the family level in the laboratory under stereo-microscope (Zeiss Discovery V20; Oberkochen, Germany). For the calculation of SPEAR values, we used population densities (per m^2) - the abundance of each family divided by the sampling area.

5.3.4 Exposure to pesticide mixture and temperature stress

The acute toxicity experiments were conducted following the rapid testing and OECD guidelines for testing of chemicals (Kefford, 2013; OECD, 2004). We selected clothianidin and azole fungicide prochloraz for the pesticide mixtures. To prepare clothianidin stock solution, we used DANTOP® (Spiess-Urania Chemical GmbH, Germany) in distilled water, and mixed it overnight on a magnetic stirrer. Whereas, prochloraz stock solution was prepared using DMSO as a solvent. The maximal solvent concentration in treatments was 0.001% [vol/vol] which is far below the LOEC (Bowman et al., 1981) and the solvent limit recommended by OECD test guidelines (OECD, 2000). Stock solutions were further diluted in Artificial Daphnia medium (ADaM; (Klüttgen et al., 1994) to prepare the required test concentrations. For mixture toxicity and multiple stress, we set up a full factorial design with nine clothianidin concentrations (0, 0.01, 0.1, 1, 10, 100, 215, 465 and 1000 µg/L) × three prochloraz treatments (0, 1, 10 µg/L) × three temperatures (16, 19 and 22°C). Before pesticide exposure, 350 individuals from each population were acclimatized to three different temperatures (16, 19, and 22 °C) for 10 days. Organisms were exposed to the stressors for 48 h, and the immobility

was recorded an end point. If organisms did not move their bodies within 20 seconds, they were considered immobile.

To find out the exposure concentrations of clothianidin and prochloraz, we collected 250 mL of the stock and test concentrations using GC−MS/MS by SGS GmbH. Actual concentrations recovered from the samples were within acceptable boundaries (±10%) to the nominal concentrations.

5.3.5 Data analyses and prediction of combined effects

For the data analyses and figures, we used RStudio version 2022.2.3.492 for Windows (RStudio, 2022) and the basic R version 4.2.1 for Windows (R Core Team, 2022). To compare clothianidin tolerance of gammarid populations under different stress conditions, we calculated EC_{50} (median effective concentration) from the toxicity experiments using five-parameter log-logistic model (Ritz and Streibig, 2005). Further, we compared clothianidin tolerance (EC_{50}), toxic pressure (TU), and ecological status (SPEAR index) of agricultural and reference streams using two-sample t-test (data with equal variances) and Welch's t-test (data with non-equal variances). For the association between different factors such as toxic pressure (TU_{max}) and the change in macroinvertebrate community composition or the clothianidin tolerance, we applied linear regressions. Before analyses, we confirmed normal distribution and homoscedasticity of residuals, and ln(x) transformed EC_{50} values to obtain normal distribution.

For the mixture toxicity and multiple stress, survival per treatment was averaged for agricultural and reference groups. First, we investigated interactions between clothianidin and prochloraz under different temperature regimes. For this, we compared the EC_{50} of clothianidin for two prochloraz concentrations (i.e., 1 and 10 µg/L) at 16, 19, and 22°C with the respective controls (i.e., agriculture and reference control at 0 µg/L prochloraz). Second, we investigated the joint toxicity of both toxicants and sub-optimal temperatures. For this, we compared all treatments of prochloraz under higher temperature regimes (i.e., 19 and 22 °C) with respective control of agricultural and reference populations at 16 °C and without prochloraz (best case). The individual stress of prochloraz

and elevated temperature was determined based on the mortality of agricultural and reference populations observed in respective treatments. To determine the stress due to the fitness cost of pesticide adaptation, we used the difference in mortality caused by additional stressors (i.e., 22°C with 10 µg/L of prochloraz) in adapted and non-adapted populations.

To predict cumulative response to mixture toxicity and multiple stressors, two additive approaches for mixture toxicity such as concentration addition (CA; Loewe and Muischnek (1926a)) and effect addition (EA; Bliss (1939a)), and the stress addition model (SAM; Liess et al. (2016b)) was employed. In comparison to CA and EA, the SAM model is designed to predict the cumulative impacts of toxicants and environmental stressors (Liess et al., 2016b). These models were further compared for their predictive accuracy.

According to the EA model, the combined effect was predicted by the following mathematical equation (Eq. 1).

$$E(c_{mix}) = 1 - \prod_{i=1}^{n}(1 - E(c_i)) \qquad (1)$$

where E(cmix) is the joint effect of E(ci) stressors.

For the concentration addition model (CA), the sum of the toxic units corresponding to mixture components was calculated by the following equation (Eq. 2).

$$ECx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i}\right)^{-1} \qquad (2)$$

where ECx_{mix} is the sum of concentrations of toxicants present in the mixture, *pi* represents the relative fraction of toxicant *i*, and *ECxi* is the concentration of the toxicant *i* posing × % effect.

For the prediction of combined effects (i.e., EC_{50}), we applied EA, CA, and SAM models using a web-based application (Indicate, version 2.2.1; http://www.systemecology.eu/indicate/). For the predictive accuracy of these models, we divided predicted EC_{50} values by the observed EC_{50} values and calculated the model deviation ratios (MDR). We used EA as a

null model for the combined effects. MDR values < 0.5 indicated antagonistic response from exposure to a toxicant mixture and values > 2 indicated synergism (Cedergreen, 2014).

5.4 Results

5.4.1 Pesticide exposure and ecological effects

In total, 365 targeted substances were analyzed in the water samples collected from selected streams 5. In terms of toxic units (log TU_{max}, see methods), pesticide contamination ranged from −3.0 to −1.4 TU in agricultural streams, with a mean of −2.5 TU which is known to cause ecological effects. In contrast, reference streams were contaminated only to a minor extent (log TU_{max}: −5 to −3.6, mean: −4.2) which is considered safe for the ecosystem. Pesticides causing the highest toxic pressure (TU_{max}) include neonicotinoids (7 sites), fungicides dimoxystrobin, fludioxonil and azoxystrobin (one site each), and fipronil (one site).

We quantified the ecological impacts of pesticide contamination by the change in macroinvertebrate community composition using the SPEARpesticides bioindicator, and observed lower SPEAR values (i.e., 0.2 to 0.8; median 0.5) in agricultural streams, indicating a reduced proportion of species vulnerable to pesticides. In contrast, reference streams showed higher SPEAR values indicating an increased proportion of species vulnerable to pesticides (i.e., 0.8 to 1.7; and median 1.21). Accordingly, the macroinvertebrate community structure significantly depended on local pesticide contamination (TU_{max}; adjusted $R^2 = 0.84$, $p < 0.001$, F-statistic: 59.05 on 1 and 10 DF; Figure 5.1). Non-contaminated reference streams (log $TU_{max} < -3.5$) were characterized by significantly higher SPEAR values in comparison to pesticide contaminated agricultural streams (Wilcoxon's rank sum test, $W = 30$, $p < 0.01$).

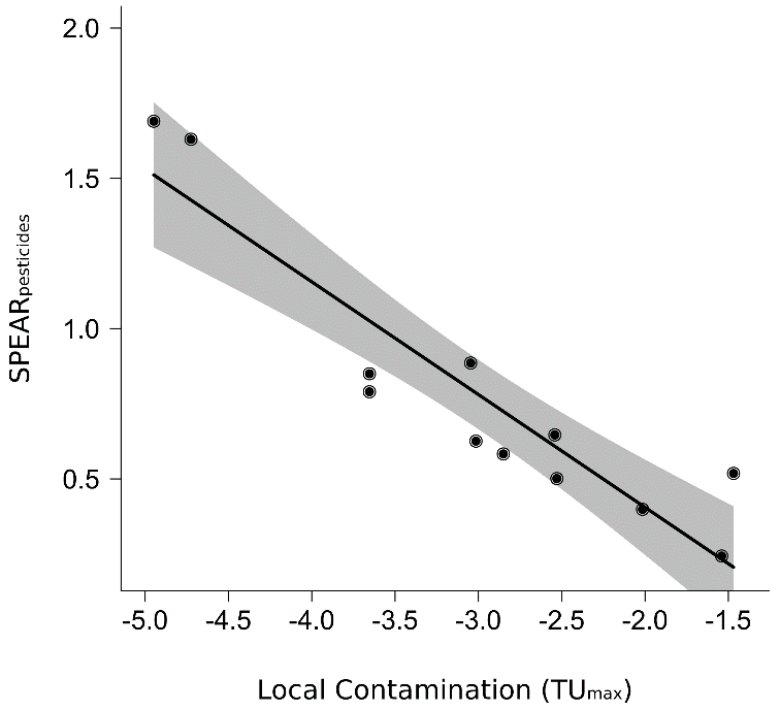

Figure 5.1. Effects of pesticide contamination on macroinvertebrate community structure: Local pesticide contamination changes the macroinvertebrate community structure (linear regression, adjusted R^2 = 0.83, F = 54.06, residual d.f. = 11, $p < 0.001$). Shaded areas represent 95% confidence intervals.

G. pulex from agricultural streams showed higher tolerance to pesticides as compared to those from non-contaminated reference streams. Laboratory investigations revealed that under optimal temperature (16°C), agricultural populations were 2.2-fold more tolerant to clothianidin as compared to reference populations (reference = 67 µg/L, agricultural = 150 µg/L, t = −4.7547, df = 8.8442, and p < 0.001, Figure 5.2). The tolerance of both, the adapted- and the non-adapted populations, significantly decreased with increasing temperature. Agricultural populations exposed at 19°C and 22°C were respectively 1.9- (EC_{50}: 79 µg/L) and 3.0-fold (EC_{50}: 50 µg/L) less tolerant to clothianidin as compared to the optimal temperature of 16°C (150 µg/L). However, the reference populations showed less decrease in tolerance with increasing temperature. The average EC_{50} decreased by 1.5-fold (EC_{50}: 45 µg/L) at 19°C and 1.7-fold (EC_{50}: 39 µg/L) at 22°C. Thus, the difference in tolerance between the two groups also decreased with increasing temperature. - from 2.2-fold at 16°C

($p < 0.001$) to 1.7-fold at 19°C; $p < 0.05$) and finally 1.2-fold at 22°C which was not significantly different anymore ($p > 0.5$) (Figure 5.2).

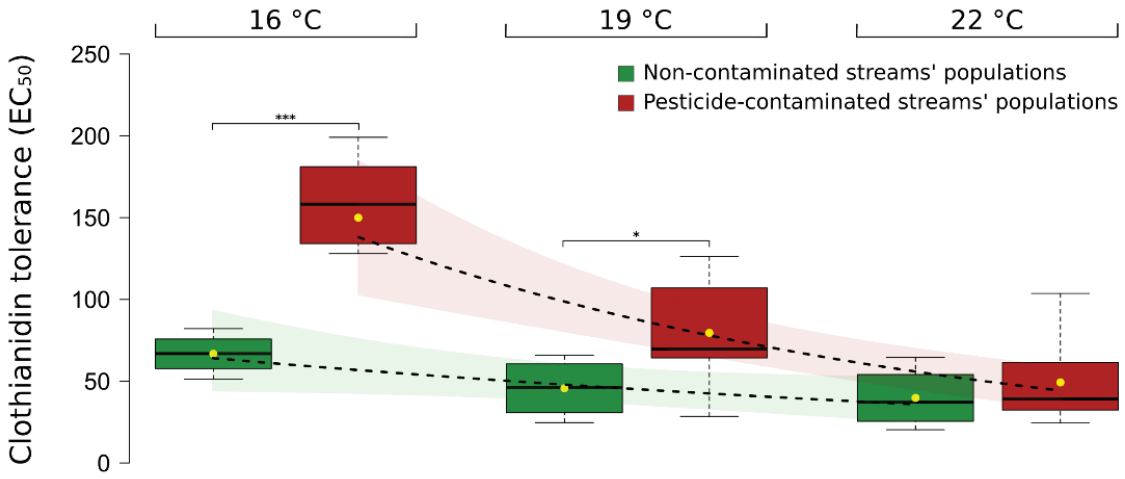

Figure 5.2. Pesticide tolerance of populations from contaminated and non-contaminated streams quantified with their Clothianidin tolerance (EC50) under different warming conditions: EC50 of G. pulex collected from control (green) and agricultural streams (red) after exposure (48-h) to clothianidin under different temperature regimes (16, 19, and 22°C). The lower and upper boundaries of the box represent 25th and 75th percentile, the horizontal line denotes the median, and the whiskers correspond to the lowest and highest values. Dashed lines represent fitted regressions with confidence intervals displayed by shaded areas. The significance level is displayed as: "*" for $p < 0.05$, "**" for $p < 0.01$, and "***" for $p < 0.001$.

Increasing local pesticide contamination showed a significant adaptation related to the sensitivity of gammarid populations to clothianidin. Among all environmental factors measured, the increased clothianidin tolerance was best explained by the toxic pressure calculated as toxic unit (TU_{max}, Figure D.2). However, the association between toxic pressure and clothianidin tolerance was reduced by an additional toxicant (prochloraz) and temperature stress. Under optimal temperature (16°C), the pesticide tolerance of *G. pulex* individuals exposed to clothianidin alone was better explained by the toxic pressure (adjusted $R^2=0.66$, $p < 0.001$) as compared to those exposed at 19 and 22°C (19°C: $R^2=0.26$, $p < 0.05$; 22°C: $R^2=0.21$, $p = 0.07$), especially in the presence of multiple stress (high prochloraz concentration and temperature), this association was further decreased.

Both indicators of pesticide effects, ecological impact of pesticide at ecosystem level (SPEAR), and clothianidin tolerance of *G. pulex* in the

laboratory were significantly correlated. However, this correlation decreased with increasing multiple stress (Supporting information, Figure D.3).

5.4.2 Interaction between clothianidin and prochloraz

Overall, prochloraz slightly increased the sensitivity of *G. pulex* to clothianidin (Figure D.4, R^2=0.1, $p < 0.001$). To identify the joint toxicity of the pesticide mixture, we compared the toxicity of clothianidin at 0, 1, and 10 µg/L of prochloraz under three temperature regimes (16, 19, and 22°C). All treatments were compared with their respective controls. In both populations, prochloraz showed weak synergistic interaction (i.e., MDR < 2) with clothianidin even at the highest concentration (10 µg/L; Figure 5.2, Table 5.1). However, the combined effects of this pesticide mixture were significantly stronger in reference populations (paired sample t-test; $p < 0.05$).

5.4.3 Interaction between multiple stress

For the combined effect of multiple stressors, we performed similar analyses as in the previous chapter "Interaction between clothianidin and prochloraz". However, here we compared all treatments of prochloraz under higher temperature regimes (i.e., 19 and 22 °C) with respective controls of agricultural and reference populations at 16 °C and without prochloraz (best case).

In reference populations, temperature stress caused weak synergistic interaction (MDR < 2) with clothianidin. However, in agricultural populations, elevated temperature increased the sensitivity of individuals to clothianidin strongly synergistic which is shown by MDR values of 2.0 and 3.0 at 19 and 22°C respectively. Further, the combination of prochloraz and temperature stress notably increased clothianidin sensitivity of individuals from both agricultural and reference populations (Table 5.1). Pesticide adaptation acted as an additional stress factor for adapted organisms. The interaction of multiple stress was significantly stronger in agricultural populations (paired sample t-test; $p < 0.05$) and

Table 5.1. Prediction of joint effects of neonicotinoid clothianidin alone and in combination with a fungicide prochloraz under different temperature regimes.

	Temp °C	Prochloraz (µg/L)	Observed EC_{50}[a]	Predicted EC_{50}[b]	MDR CA	MDR EA	MDR SAM
			Reference populations				
Mix-tox[c]	16	0	66.8				
	16	1	58.2	66.8	1.1	1.1	1.1
	16	10	46.3	66.8	1.4	1.4	1.4
	19	0	45.7				
	19	1	34.4	45.7	1.3	1.3	1.2
	19	10	24.1	45.7	1.9	1.9	1.8
	22	0	39.2				
	22	1	31.7	39.2	1.2	1.2	1.2
	22	10	25.9	39.2	1.5	1.5	1.5
Multi-stress[d]	*Control of 16 °C*		*66.8*				
	19	0	45.7	65.8	1.4	1.5	0.3
	19	1	34.4	66.4	1.9	1.9	0.8
	19	10	24.1	66.3	**2.8**	**2.8**	1.0
	22	0	39.2	66.2	1.7	1.7	0.5
	22	1	31.7	66.2	**2.1**	**2.1**	0.7
	22	10	25.9	66.4	**2.6**	**2.6**	1.1
			Agricultural populations				
Mix-tox	16	0	146.4				
	16	1	175.1	149.3	0.9	0.8	0.3
	16	10	117.4	149.3	1.3	1.2	0.4
	19	0	75.9				
	19	1	67.2	75.9	1.1	1.1	1.1
	19	10	60.7	78.6	1.3	1.2	0.3
	22	0	50.6				
	22	1	36.7	53.9	1.5	1.4	0.2
	22	10	36.9	50.6	1.4	1.4	1.4
Multi-stress	*Control of 16 °C*		*146.4*				
	19	0	75.9	149.3	**2.0**	1.9	0.6
	19	1	67.2	147.5	**2.2**	**2.2**	1.0
	19	10	60.7	152.9	**2.5**	**2.4**	0.4
	22	0	50.6	150.1	**3.0**	**2.9**	0.8
	22	1	36.7	154.2	**4.2**	**4.0**	0.6
	22	10	36.9	150.1	**4.1**	**4.0**	1.0
Average prediction of the combined effects of Mix-tox (two toxicants)					1.3	1.3	1.0
Average prediction of the combined effects of Multi-stress					2.5	2.5	0.73

[a]The observed LC_{50s} for clothianidin are based on the average survival of the respective populations (i.e., agricultural and reference) and calculated using five-parameter log-logistic model, whereas, the [b]predicted LC_{50} values are calculated by the CA model. [c]Under mixture toxicity, we compared the EC_{50} of clothianidin for prochloraz concentrations (i.e., 1 and µg/L) at 16, 19, and 22°C in relation to their respective controls without prochloraz. For [d]Multi-stress, we compared all treatments of prochloraz under higher temperature regimes (i.e., 19 and 22 °C) with respective control of agricultural and reference populations at 16 °C and without prochloraz (best case).

caused up to 2-fold higher synergism of multiple stressors (using CA as a null model) in agricultural populations (Table 5.1). To identify the association between synergism and general stress, we used general distribution of stress in the SAM framework to calculate the individual stress posed by different stressors including clothianidin, prochloraz, elevated temperature, and fitness cost of pesticide adaptation, and added them according to SAM to quantify the total general stress. Overall, synergism compared to the both null models (i.e., CA and EA), increased with increasing total stress of all the stressors (Null model CA: Figure 5.3, $R^2 = 0.64$, $p < 0.001$; Null model EA: $R^2 = 0.52$, $p < 0.001$).

5.4.4 Prediction of Multi-stress Effects

We used concentration addition (CA), effect addition (EA), and stress addition model (SAM) to predict the combined effects of pesticide mixture and multiple stress including elevated temperature. Among these models, SAM predicted best in terms of an average MDR values of 1.0 and 0.73 (Table 5.1), and the modeled curves (Supporting Information, Figure D.5, D.6). Whereas, CA and EA considerably underestimated the combined effects of all multiple stressors by up to 4-fold (Table 5.1, Figure D.5, D.6).

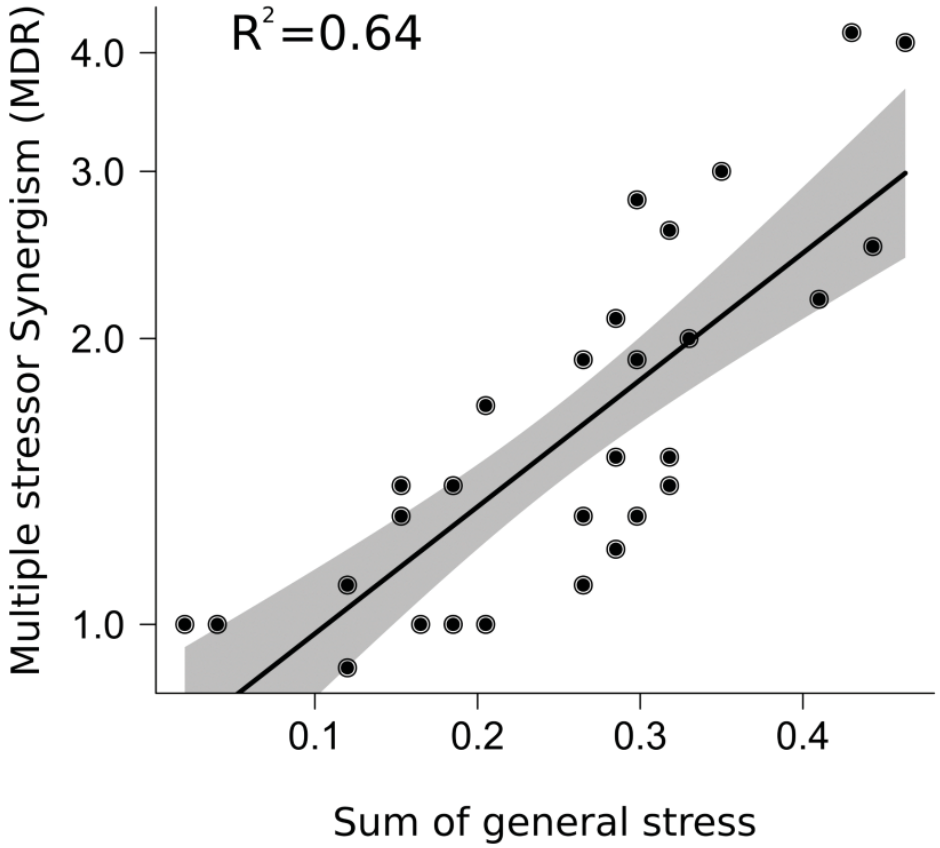

Figure 5.3. Relationship between total general stress and strength of synergistic effects: Combined stress of multiple stressors including prochloraz, sub-optimal temperature and fitness cost of pesticide adaptation significantly increased the clothianidin sensitivity expressed by acute synergism (linear regression, adjusted $R^2 = 0.64$, $F = 56.76$, residual d.f. = 30, $p < 0.001$). Shaded areas represent 95% confidence intervals.

5.5 Discussion

Here we found that pesticide adaptation costs act as a stressor under multiple stress conditions. Moreover, all the stressors with different modes of action, including clothianidin, prochloraz, sub-optimal temperature and fitness cost of pesticide adaptation, can be added to calculate the overall general stress and predict their synergistic effects. This is substantially extending on most studies on multiple stressors that focus on binary stressors (as reviewed by Jackson et al. (2016)). It is also contrasting the

approach that the stronger stressor overrides the effect of weaker stressors (Morris et al., 2022; van Dijk et al., 1994).

Our results show that the agricultural populations were significantly more tolerant to the pesticide mixture as compared to the reference populations. This higher tolerance develops due to pesticide adaptation resulting from prior exposure in the field (Shahid et al., 2018; Siddique et al., 2021). However, both the agricultural and reference populations showed acute synergistic responses to the joint stress of pesticides and temperature (Figure 5.2). Synergism was up to 2-fold stronger in agricultural populations adapted to pesticide pollution (Table 5.1). In general, environmental stressors are expected to interact synergistically with chemical stressors (Heugens et al., 2006; Liess et al., 2016b; Meng et al., 2022). For example, Delnat et al. (2019) observed synergistic interaction of high variation in daily temperature with a mixture of chlorpyrifos and *Bacillus thuringiensis* towards *Culex pipiens*. Similarly, Macaulay et al. (2021b) reported synergistic combined effects of the heatwave and a neonicotinoid insecticide imidacloprid on mayfly nymphs. However, these investigations did not focus on stress adaptation. Recently, Siddique et al. (Siddique et al., 2021) and Heim et al. (Heim et al., 2018) reported increased sensitivity of pesticide resistant populations to the temperature stress. It is suggested that the mechanisms of tolerance development cause energetic constraints which may result in trade-offs between different fitness related functions. Therefore, the stronger synergistic response of pesticide adapted populations might be attributed to the lack of a plastic response, suggesting higher costs to maintain pesticide tolerance (Callahan et al., 2008).

A critical challenge in predicting the combined effects of multiple stresses is to establish a "common currency" to quantify and integrate different stressors (Segner et al., 2014). The Stress Addition Model (SAM) assumes that each organism has a "general stress capacity" towards all types of specific stressors (Liess et al., 2016b). This concept enables us to transform different stressors into general stress levels. Accordingly, here we calculated the individual stress posed by different stressors including clothianidin, prochloraz, elevated temperature, and fitness cost of pesticide

adaptation, and added them to quantify total general stress. Each stressor reduced the common stress capacity of individuals. Thus, the acute synergism of multiple stressors was getting stronger with increasing total general stress (Figure 5.3). So far, SAM has been employed to assess the interaction between toxicants and environmental stressors (Liess et al., 2016b; Shahid et al., 2019), and toxicant mixtures (Shahid et al., 2019). This is the first study where the fitness-cost of pesticide adaptation is included as an additional stressor and predicted the combined effects of four stressors.

Overall, traditional models (CA and EA) underestimated the combined impacts of clothianidin and prochloraz under higher temperature regimes (multiple-stress conditions; Table 5.1, Figure D.5, D.6). These results are not surprising for the interacting multiple stressors because CA and EA assume additive effects (Bliss, 1939a; Loewe and Muischnek, 1926a). Whereas, SAM predicted the cumulative impacts better than CA and EA even in pesticide adapted populations (Table 5.1, Figure D.5, D.6). SAM presumes that the combined impact of stressors with different modes of action can be calculated by adding up individual effects transformed to general stress and then compared with the general stress capacity of the individuals within a population (Liess et al., 2016b). Obviously, this approach is comparably successful to predict combined effects of different stressors.

5.6 Conclusions

The current study is an important step toward ecological realism in risk assessment by revealing complex interactions of pesticide mixtures, environmental stress, and the fitness costs of pesticide adaptation. Our results show that multiple stressors such as clothianidin, prochloraz, elevated temperature and pesticide adaptation can interact synergistically, and therefore, pesticide adapted populations become more vulnerable to global warming. Although predicting the combined impacts of multiple stressors was a great challenge so far, we successfully used the stress addition model (SAM) to calculate total general stress, and showed that

the synergism increases with increasing total stress of the interacting stressors.

5.7 Author Contributions

Conceptualization: All; Study design: All; Investigation: NS and AS; Statistical analysis: NS; Interpretation of results: All; Writing Original Draft: NS; Review & Editing: All.

5.8 Acknowledgements

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6

Discussion and outlook

In freshwater ecosystems, organisms are frequently exposed to a range of stressors that result in decline of vulnerable species even at very low pesticide concentrations (Liess and Ohe, 2005; Schäfer et al., 2012). At the same time, some less vulnerable species may develop increased pesticide tolerance. Effects at such low concentrations confirms that the current environmental risk assessment of pesticides are not protective enough in determining the risk. This could be attributed to the fact that the possible adaptation to stressors and associated trade-offs are not considered in risk assessment. Therefore, in this dissertation, important factors that may shape the response of organisms to pesticide such as adaptation to pesticides, fitness costs in terms of compromised performance, exposure to mixtures and multiple stress, and population genetic diversity were studied.

6.1 Pesticide adaptation and compromised fitness

The effects of local pesticide contamination on dominant crustacean were quantified in terms of acute EC_{50} of Clothianidin. *G. pulex* from

agricultural streams were up to 2.5-fold more tolerant as compared to conspecifics from reference streams. The magnitude of tolerance increased with increasing toxic pressure of the pesticides. Furthermore, fitness costs were associated with increased pesticide tolerance and pesticide exposure. The populations from agricultural streams had compromised general fitness in terms of reduced survival, per capita growth and mating when cultured under pesticide free conditions in the laboratory for three months.

Generally, depending on the extent and intensity of exposure, individuals may acquire pesticide tolerance through physiological acclimation (Sparks and Nauen, 2015), maternal effects, and/or genetic adaptation (Sun et al., 2014). When populations experience occasional exposure, inducible traits are maintained as they facilitate the expression of adaptive phenotypes in response to variable environmental conditions (Weston et al., 2013). In contrast, when populations experience longterm exposure over multiple generations, maternal effects result in increased tolerance, but a plastic response eventually becomes constitutively expressed, resulting in genetic adaptation (Lopes et al., 2008). However, fitness costs are often associated with increased tolerance.

The fitness costs of adaptation observed as adverse population effects were not based on an initial decrease in performance that diminished over time, but were persistent and built up for at least four months under non-contaminated conditions. This persistence in the observed effects suggests that the decrease in performance results rather from fitness costs associated with the adaptation to pesticide exposure, than from delayed effects of the pesticides themselves. Delayed adverse effects of pulsed exposure to pyrethroid have been observed to last at least for 15 d in *G. pulex* (Cold and Forbes, 2004), and even for three months in insects (Liess and Schulz, 1996). In contrast, pulse exposure to a neonicotinoid insecticide decreased survival and per capita growth of the crustaceans *G. pulex*, *Daphnia magna* and *Asellus aquaticus* only for 14–21 d (Beketov and Liess, 2008). There

are several investigations that report fitness costs of adaptation to certain pesticides and metals in different organisms (Bach and Dahllof, 2012; Heim et al., 2018). For example, *Gammarus fossarum* developed increased tolerance to cadmium following exposure to low concentrations in the field, but showed decreased feeding in laboratory conditions (Vigneron et al., 2015). Similarly, (Piola and Johnston, 2006) detected differential tolerance to copper in laboratory-grown populations that resulted in reduced growth rates of a colonial bryozoan, and *D. magna* that evolved insecticide resistance in the laboratory showed higher susceptibility to parasites (Jansen et al., 2011b). However, fitness costs of moderately increased tolerance caused by very low pesticide concentrations commonly observed in agricultural streams have never been investigated. These results suggest that in the field, even very low pesticide concentrations can considerably impair the performance of *G. pulex* through fitness costs of genetic or physiological adaptation. These long-term impairments need to be considered in deriving safe concentrations under risk assessment.

6.2 Pesticide adaptation under multiple stress

As multi-stress conditions may occur regularly under global change scenarios, the ecological consequences of pesticide adaptation were quantified under multiple stress. Under optimum temperature, *G. pulex* from agricultural streams were significantly more tolerant to pesticides as compared to the reference populations. The increased pesticide tolerance in agricultural populations could be due to acclimation, epigenetic changes, and genetic evolution. Further, reference populations showed increased pesticide tolerance upon lab pre-exposure to very low concentration (LC_{50}/ 1000). The induction of tolerance upon such a low toxic pressure of a pesticide in non-adapted reference populations is also observed in other species by several studies (Hua et al., 2013; Poupardin et al., 2008).

The increased tolerance after pre-exposure to neonicotinoid insecticide clothianidin can be due to the upregulation of detoxification mechanisms that may neutralize the effect of irreversible binding and cumulative effect of neonicotinoid insecticide clothianidin (Hua et al., 2013). In contrast, no further increase in tolerance in the agricultural populations might be due to evolved pesticide resistance (Lopes et al., 2008). In general, inducible traits are maintained under variable environmental conditions as they facilitate the expression of adaptive phenotypes (Li et al., 2018; Schlichting and Pigliucci, 1998). However, consistent exposure over multiple generations results in evolution of genetic resistance (Debat and David, 2001; Lopes et al., 2008) and is often associated with fitness costs (Heim et al., 2018; Jansen et al., 2011a; Lagator et al., 2014; Siddique et al., 2020). Similarly, in the present study, we observed that agricultural populations showed increased sensitivity to increasing temperature under noncontaminated conditions due to the hypothesized fitness costs of adaptation to pesticides and other potential stressors. In contrast, the reference populations (with and without preexposure) did not show fitness costs in terms of decreased survival at elevated temperature under noncontaminated conditions.

Organisms in the field are often exposed to multiple stressors that may interact synergistically. Several studies have reported synergism between multiple stressors, (Shahid et al., 2019; Shahid et al., 2021) including a meta-analysis study(Liess et al., 2016a). However, under some “multistress” conditions, stressors did not interact as the populations were already adapted to one stressor at low fitness costs (Chen et al., 2015; Dinh Van et al., 2013; Zhang et al., 2018). Both reference and agricultural populations showed a similar tolerance to the combined stress of pesticides and warming due to stronger synergistic effects in adapted populations. The combined effect was further increased after pre-exposure in both agricultural and reference populations. The energetic costs of pesticide tolerance in genetically adapted populations can be the consequence of

hyperactivation of the nervous system rather than resource-based trade-off (Bourguet et al., 1997; Djogbénou et al., 2010; Rivero et al., 2011; Verheyen and Stoks, 2019). In contrast, the overproduction of detoxifying enzymes (up to 50 times more in some cases) in physiologically acclimated populations is widely assumed to deplete the energetic stores of insects, generating energetic trade-offs between insecticide resistance and key life history traits(Roush and McKenzie, 1987).Therefore, adaptation to pesticides increased the synergistic effect of pesticides and warming. This offset the advantage of adaptation to stressors and increases sensitivity under changing environmental conditions. As the trade-off processes are mathematically well-represented in the SAM approach, the interaction between chemical and environmental stressors was predicted well using the SAM as compared to CA and EA.

6.3 Pesticide adaptation and reduced genetic diversity

Pesticide exposure triggers evolutionary processes in natural populations (Medina et al., 2007), and also alters the macroinvertebrate community composition (M Liess et al., 2021b; Rumschlag et al., 2020; Stehle & Schulz, 2015). There are numerous investigations on contamination induced genetic adaptations, however, understanding their ecological consequences in natural populations is still in its infancy. This is because the potential propagation of effects from the individual genetic composition up to the community composition at the landscape level are unknown. In detail, relevant biological effects include: (i) increased tolerance and a reduction in genetic diversity as an outcome of contamination (Bach & Dahllof, 2012), (ii) toxic pressure dependent distribution of alleles at ecological relevant ultra-low exposure concentrations, (iii) fitness trade-offs associated with genetic adaptation (Heim et al., 2018), (iv) impact of geographical distance to the non-contaminated refuge populations (J. M. Becker et al., 2020; Shahid et al., 2018) and (v) the interspecific competition with vulnerable species. A few of these processes have been investigated to some extent. However, to the

best of our knowledge, this is the first study that integrates the processes linked to pesticide exposure on all these levels of biological organization.

At community level, we observed a decline in vulnerable species already below the regulatory compliant concentrations ($TU_{max} \geq -3$), mostly contributed by neonicotinoids and carbamate insecticides. These results are comparable with other investigations that identified strong impacts of nonpoint-source pesticide pollution on streams in Australia (Beketov et al., 2013), Europe (Beketov et al., 2013; M Liess et al., 2021b; Matthias Liess & Ohe, 2005), North America (Chiu et al., 2016) and South America (Hunt et al., 2017). A situation that is in contrast to the aims of the regulatory authorization of pesticides that is supposed to prevent unacceptable effects in the environment (Australian Environment Agency, 2009; EFSA (2013); US Government, 2004).

At individual level, we observed up to 4.5–fold (average 2.2–fold) increased pesticide tolerance in comparatively less sensitive specie *G. pulex* collected from contaminated streams. Such a development of pesticide tolerance in different aquatic species such as *G. pulex* (Siddique et al., 2021) and *Daphnia magna*(Almeida et al., 2021) have been observed. Similarly, local adaptation to ore mine effluents have also been observed in *Dugesia gonocephala* (Weigand et al., 2018). The acquisition of increased tolerance in a natural population may occur through environmentally induced physiological adjustments (acclimation)(Maxwell et al., 2014; Siddique et al., 2021), epigenetic modifications (Wolf & Wade, 2009), loss of sensitive individuals, and selection of more tolerant genotypes which may reduce the genetic diversity (Forbes & Calow, 1996; Van Straalen et al., 2011). The latter could be the reason for reduced genetic diversity in the current study. Furthermore, reduced genetic diversity explained by toxic pressure reveal that even such low concentrations may significantly shape genetic metrics of populations and may describe the loss of sensitive species under long–term exposure that have so far not been studied.

In general, genetic erosion is widely accepted as a potential evolutionary outcome of long–term exposure to contaminants(Nowak et al., 2009; Ribeiro & Lopes, 2013; Ungherese et al., 2010). For example, low genetic

diversity in >700 aquatic species from extensive cropland(Crossley et al., 2022), and reduction in allelic richness was observed in *G. pulex* from wastewater contaminated rivers(Švara et al., 2022). However, in these cases, investigations only focused on high exposures and did not investigate the effect of low exposure and possibility of local adaptation. Furthermore, tolerance acquired through genetic adaptation based on specific alleles may change allele frequency as a result of increased number of tolerant individuals in a population (Gouin et al., 2019). Likewise, in the present study, we observed a decrease in average alleles frequency of "low contamination alleles" with increasing toxic pressure. This change in frequency was significantly different among populations from low, moderate and high pesticide exposure indicating loss of alleles already at concentrations 3 to 4 orders of magnitude below the EC_{50}. Reduction in frequency of some alleles could be attributed to long-term consequence of contamination that selects for tolerant genotypes as mentioned earlier(Ribeiro & Lopes, 2013). On the other hand, an increasing average frequency of "high contamination alleles" with pesticide contamination observed in the present study indicates effects on allelic distribution on such a low range of pesticide contamination. Further, this suggests that the "high contamination alleles" might be functionally associated with tolerance conferring mechanisms required to cope with pesticide stress. For instance, the allele frequency of two outlier SNP loci involved in insect cuticle resistance in natural populations of mayfly *Andesiops torrens* were significantly correlated with high pesticide exposure (Gouin et al., 2019).

Although pesticide exposure was mainly responsible for the observed effects in the investigated streams (M Liess et al., 2021b), several other environmental factors may affect the genetic diversity of populations. For example, geographic distance between sites partially explained the pairwise genetic dissimilarity. Other studies also reported genetic structure of populations partially or completely explained by geographic distance(Svara et al., 2022; Whitehead et al., 2003). Furthermore, historical colonization events in *Gammarus fossarum* (Weiss & Leese, 2016) and barrier effects in *Ancylus fluviatilis* and *D. gonocephala* (Weiss et al., 2022) have been associated with populations genetic structure and

differentiation. In addition, abundance of sensitive species may also impact the evolutionary process as tolerance development can be affected by species diversity (Becker & Liess, 2017). Likewise, we observed a higher genetic diversity in *G. pulex* populations with species richness in the respective streams indicating that the conditions that promotes species diversity may also preserve genetic diversity within species. Such a positive association between genetic– and species diversity has also been reported by Manel et al. (Manel et al., 2020).

Fitness costs are often associated with increased tolerance due to energy trade–offs (Fournier-Level et al., 2019; Siddique et al., 2021). However, it has rarely been reported in natural populations (Jansen et al., 2011; Siddique et al., 2020). We observed that pesticide exposure and genetic adaptation to pesticides were associated with reduced per capita growth in natural populations of *G. pulex* revealed under non-contaminated conditions. This finding suggests that survival in contaminated streams infers energetic costs reducing the energy available for vital functions and reduces plasticity for survival under slightly different conditions(Marchand et al., 2004). Also other studies revealed that pyrethroid resistant *Hyalella azteca* exhibited reduced thermal tolerance(Heim et al., 2018), and lower survival and lipid levels(Fulton et al., 2021). However, these trade-offs were revealed in the presence of stressors, unlike our study where the fitness costs were observed under non-contaminated conditions.

Migration of organisms from nearby refuge area often support recovery for pesticide affected individuals (Jeremias Martin Becker et al., 2020; Shahid et al., 2018) and populations (Matthias Liess & von der Ohe, 2005). Likewise, we observed that the increase in clothianidin tolerance was reduced with increasing distance to the nearby refuge area. However, genetic diversity and frequency of “low contamination alleles” increased with decreasing distance to refuge sections, suggesting that migration events from nearby refuge sections supports populations to retain genetic diversity.

Inbreeding coefficient of *G. pulex* populations increased with decreasing abundance. However, nor local pesticide contamination neither reduced

genetic diversity did induce inbreeding depression. Similarly, Svara et al. (Svara et al., 2022) reported higher inbreeding coefficient in *G. pulex* from wastewater contaminated sites as compared to pristine sites, which could also be due to a smaller and thus genetically impoverished population.

On the ecosystem level, we observed that the abundance of *G. pulex* was independent of pesticide contamination despite their apparent fitness trade-offs. We hypothesize that this non-existent association of *G. pulex* abundance and pesticide exposure is due to the exposure-induced decline in competing pesticide-vulnerable SPEAR and EPT species. In contrast, species with low or even declining dominance may not be able to compensate individual trade-offs from pesticide effects at the community level (Matthias Liess & Beketov, 2011).

6.4 Pesticide adaptation under mixture toxicity and multiple stress

Here we found that pesticide adaptation costs act as a stressor under multiple stress conditions. Moreover, all the stressors with different modes of action, including clothianidin, prochloraz, sub-optimal temperature and fitness cost of pesticide adaptation, can be added to calculate the overall general stress and predict their synergistic effects. This is substantially extending on most studies on multiple stressors that focus on binary stressors (as reviewed by Jackson et al. (2016)). It is also contrasting the approach that the stronger stressor overrides the effect of weaker stressors (Morris et al., 2022; van Dijk et al., 1994).

Our results show that the agricultural populations were significantly more tolerant to the pesticide mixture as compared to the reference populations. This higher tolerance develops due to pesticide adaptation resulting from prior exposure in the field (Shahid et al., 2018; Siddique et al., 2021). However, both the agricultural and reference populations showed acute synergistic responses to the joint stress of pesticides and temperature (Figure 5.2). Synergism was up to 2-fold stronger in agricultural populations adapted to pesticide pollution (Table 5.1). In general, environmental stressors are expected to interact synergistically with

chemical stressors (Heugens et al., 2006; Liess et al., 2016b; Meng et al., 2022). For example, Delnat et al. (2019) observed synergistic interaction of high variation in daily temperature with a mixture of chlorpyrifos and *Bacillus thuringiensis* towards *Culex pipiens*. Similarly, Macaulay et al. (2021b) reported synergistic combined effects of the heatwave and a neonicotinoid insecticide imidacloprid on mayfly nymphs. However, these investigations did not focus on stress adaptation. Recently, Siddique et al. (Siddique et al., 2021) and Heim et al. (Heim et al., 2018) reported increased sensitivity of pesticide resistant populations to the temperature stress. It is suggested that the mechanisms of tolerance development cause energetic constraints which may result in trade-offs between different fitness related functions. Therefore, the stronger synergistic response of pesticide adapted populations might be attributed to the lack of a plastic response, suggesting higher costs to maintain pesticide tolerance (Callahan et al., 2008).

A critical challenge in predicting the combined effects of multiple stresses is to establish a "common currency" to quantify and integrate different stressors (Segner et al., 2014). The Stress Addition Model (SAM) assumes that each organism has a "general stress capacity" towards all types of specific stressors (Liess et al., 2016b). This concept enables us to transform different stressors into general stress levels. Accordingly, here we calculated the individual stress posed by different stressors including clothianidin, prochloraz, elevated temperature, and fitness cost of pesticide adaptation, and added them to quantify total general stress. Each stressor reduced the common stress capacity of individuals. Thus, the acute synergism of multiple stressors was getting stronger with increasing total general stress (Figure 5.3). So far, SAM has been employed to assess the interaction between toxicants and environmental stressors (Liess et al., 2016b; Shahid et al., 2019), and toxicant mixtures (Shahid et al., 2019). This is the first study where the fitness-cost of pesticide adaptation is included as an additional stressor and predicted the combined effects of four stressors.

Overall, traditional models (CA and EA) underestimated the combined impacts of clothianidin and prochloraz under higher temperature regimes

(multiple-stress conditions; Table 5.1, Figure D.5, D.6). These results are not surprising for the interacting multiple stressors because CA and EA assume additive effects (Bliss, 1939a; Loewe and Muischnek, 1926a). Whereas, SAM predicted the cumulative impacts better than CA and EA even in pesticide adapted populations (Table 5.1, Figure D.5, D.6). SAM presumes that the combined impact of stressors with different modes of action can be calculated by adding up individual effects transformed to general stress and then compared with the general stress capacity of the individuals within a population (Liess et al., 2016b). Obviously, this approach is comparably successful to predict combined effects of different stressors.

6.5 Implications and outlook

Under climate change, organisms are increasingly exposed to multitude of stressors, and adaptation to one stressor may shape the response to another stressor. Hence, risk assessment based on single toxicant might not be protective enough without considering the adaptation to stressors. In this dissertation, adaptation to pesticides was studied from individual genetic composition to macroinvertebrate community at the landscape level under different stress scenarios. Therefore, the obtained results may have several implications for the ecological risk assessment of pesticides.

6.5.1 Adaptation to pesticides and compromised fitness

Adaptation to pesticides in non-target species may have consequences for the local biodiversity. Especially, it is imperative for key species that are often used for biomonitoring and risk assessment. The current study shows the magnitude of adaptation in a dominant crustacean from pesticide contaminated agricultural streams, and associated fitness costs characterized by reduced survival, per capita growth and mating adults under non-contaminated conditions. The persistent decrease in performance is potentially of more concern it may hinder population recovery and explain changes in the community composition over longer time scales at low concentrations. Unraveling the fitness costs of moderate pesticide exposure and adaptation has several implications for ecological

risk assessment and biodiversity conservation. Depending on the mechanisms of adaptation, the consequence could be short term or long term. It is suggested that when populations experience occasional exposure, inducible traits are maintained as they facilitate the expression of adaptive phenotypes in response to variable environmental conditions. In contrast, when populations experience longterm exposure over multiple generations, maternal effects result in increased tolerance, but a plastic response eventually becomes constitutively expressed, resulting in genetic adaptation. Hence, it is imperative to understand how species with reduced performance to due associated trade-offs of adaptation compete with other species. Further, it would be interesting to monitor such adapted species over longer period to understand the consequence of adaptation in species fully evolved under pesticide stress.

6.5.2 Adaptation to pesticides under multiple stress

Under global change scenarios, multi-stress conditions are likely to occur more frequently, and it could have severe consequence for populations adapted to pesticides. Present study reveals different mechanisms of pesticide tolerance in agricultural and reference populations of non-target species, and further shows increased sensitivity to increasing temperature. Under climate change, organisms may be exposed to several stressors such as elevated pesticide pressure, sedimentation, and low dissolved oxygen. Additionally, under global warming, exposure to elevated temperatures may be a key stressor. The combined effect of toxicants and warming are often investigated by space for time substitution where temperature did not act as a stressor because the populations were already adapted to the respective thermal conditions. If, on the other hand, a directional change in temperature is assumed, temperature-induced stress is to be expected through adaptation processes at the level of individuals and communities. Hence, the fitness costs of pesticide adaptation in terms of increased sensitivity to elevated temperature reveals trade-offs of pesticide adaptation. Unraveling the costs of adaptation under single and multiple stress scenarios is imperative in understanding the fate of adapted species under global warming. Hence, the sensitivity of pesticide adapted population under multiple stress has several implications for ecological

risk assessment of chemicals as populations adapted toxicant could be more sensitive to another chemical or non-chemical stressors. An extension of this study could be analyzing the effect of pesticides with different modes of action in pesticide adapted populations.

6.5.3 Adaptation to pesticides and genetic diversity

Altered genetic composition in a species under contamination pressure may have substantial consequences for the species' ecological performance and survival. Reduced genetic diversity in the populations can impact the species' key life traits, hence its abundance, ecological function, interspecific competition, and the ability to recover from disturbance. In the current study, pesticide concentrations even below regulatory thresholds affected the tolerance, genetic diversity and allelic distribution in a dominant crustacean *G. pulex*. The populations showed increased frequency of high contamination alleles and a low frequency of low contamination alleles with increasing pesticide contamination. This genetic structure was not explained by geographic distance but by pesticide contamination. The tolerance and genetic diversity and low contamination alleles were further affected by distance to refuge sections as non-contaminated refuge sections often act as recovery. Furthermore, Individuals characterized by reduced genetic diversity showed reduced per capita growth under non-contaminated conditions. Nevertheless, *G. pulex* contributes nearly one half to the total macroinvertebrate abundance in highly contaminated streams. While, on the other hand, we observed a decline in vulnerable species. Hence, *G. pulex* even with negative consequences of adaptation in terms of reduced genetic diversity and performance seems to benefit from reduced competition from declining vulnerable species in its ecological niche. Furthermore, unravelling these underpinning processes reveal that the costs of pesticide adaptation at individual level are compensated at the community level due to reduced interspecific competition and enables to link the effects from genes to ecosystem level.

6.5.4 Pesticide adaptation under mixture toxicity and multiple stress

Understanding and predicting the combined impacts of multiple stressors in the field is a great challenge. The current study is an important step toward ecological realism in risk assessment by revealing complex interactions of pesticide mixtures, environmental stress, and the fitness costs of adaptation. Our results show that the agricultural populations were significantly more tolerant to the pesticide mixture. This higher tolerance might be due to pesticide adaptation resulting from prior exposure in the field. Although toxicants with different modes of action are expected to act synergistically, in the present study, we observed additive effects. Such additive interactions may occur especially when individual effect sizes of both toxicants are highly asymmetric (e.g. (Loewen et al., 2020)). Our results show that heat stress can synergistically interact with pesticide mixtures, and populations that are already adapted to pesticides are more vulnerable to global warming. The synergistic response of adapted populations implies that global warming will jeopardize freshwater biodiversity, especially in agricultural streams, and therefore, we cannot overlook additional impacts of agrochemicals under climate change.

A

Supporting Information for chapter 2

Ayesha Siddique, Matthias Liess, Naeem Shahid, Jeremias Becker 2020. Insecticides in agricultural streams exert pressure for adaptation but impair performance in *Gammarus pulex* at regulatory acceptable concentrations. Published in "Science of the Total Environment".

Table A.1: Survival and long-term traits of *G. pulex*

Population	Initial *n* individuals	TU_{max}	EC_{75} [µg/L]	Survival [%]				Mating [%]				Per-capita growth [offspring / ind.]			
Month of culture				1	2	3	4	1	2	3	4	1	2	3	4
Ref-1	57	-5.34	48.9	78.9	68.4	54.4	31.6	40.0	15.4	25.8	11.1	0.18	1.40	1.83	1.65
Ref-2	100	-5.83	77.6	100.0	41.0	27.0	7.0	34.0	24.4	44.4	28.6	1.00	2.00	2.77	1.67
Ref-3	100	-3.69	88.4	100.0	20.0	17.0	22.0	31.4	30.0	23.5	18.2	1.50	0.40	0.68	0.58
Agri-1	100	-2.65	134.9	40.0	5.0	3.0	13.0	20.0	0.0	0.0	0.0	2.50	0.40	0.74	0.83
Agri-2	100	-3.63	119.4	78.0	63.0	22.0	15.0	20.5	28.6	27.3	26.7	0.80	0.90	1.24	1.36
Agri-3	100	-2.78	219.7	50.0	10.0	7.0	3.0	24.0	20.0	0.0	0.0	1.80	0.20	0.19	0.10
Effect of EC_{75}															
Intercept				11.75	7.05	7.93	3.86	1.15	-0.05	4.58	-2.51	-1.19	0.94	6.84	1.52
Slope				-2.23	-1.68	-2.04	-1.23	-0.37	-0.16	-1.18	-0.77	0.01	-0.01	-1.49	0.015
X^2 / F				3.09	2.28	13.94	4.88	7.12	0.15	4.13	1.65	2.94	7.64	8.32	13.52
df				1 and 4	1 and 4	1 and 4	1 and 4	1 and 4	1 and 4	1 and 4	1 and 4	4	4	1 and 4	4
p				0.078	0.13	<0.001	0.027	0.0075	0.69	0.041	0.19	0.16	0.051	0.0039	0.021
Significance				(.)		***	*	(**)		*			(*)	**	*
R^2				0.25	0.16	0.22	0.092	0.030	0.0061	0.23	0.11	0.27	0.58	0.59	0.71
Effect of TU_{max}															
Intercept				-1.35	-1.72	-3.3	-2.24	-1.88	-1.29	-3.60	-2.92	1.46	-2.44	-2.21	-2.22
Slope				-0.65	-0.24	-0.46	-1.08	-0.22	-0.021	-0.53	-0.28	0.36	-0.51	-0.53	-0.48
X^2 / F				1.49	0.29	2.33	0.14	7.19	0.0062	2.14	0.51	1.45	5.70	5.11	2.21
df				4	4	4	4	4	4	4	4	4	4	4	4
p				0.22	0.58	0.12	0.70	0.007	0.93	0.14	0.47	0.29	0.075	0.086	0.21
Significance								**					(*)	(*)	
R^2				0.16	0.025	0.084	0.0051	0.022	0.0002	0.11	0.033	0.083	0.48	0.45	0.19

For survival and mating: Binomial GLM with logit link function
For per-capita growth: Normal LM with per-capita-growth log-transformed
Log-transformed EC_{75}

B

Supporting Information for chapter 3

Ayesha Siddique, Naeem Shahid, Matthias Liess 2022. Multiple stress reduces the advantage of pesticide adaptation. Published in "Environmental Science and Technology".

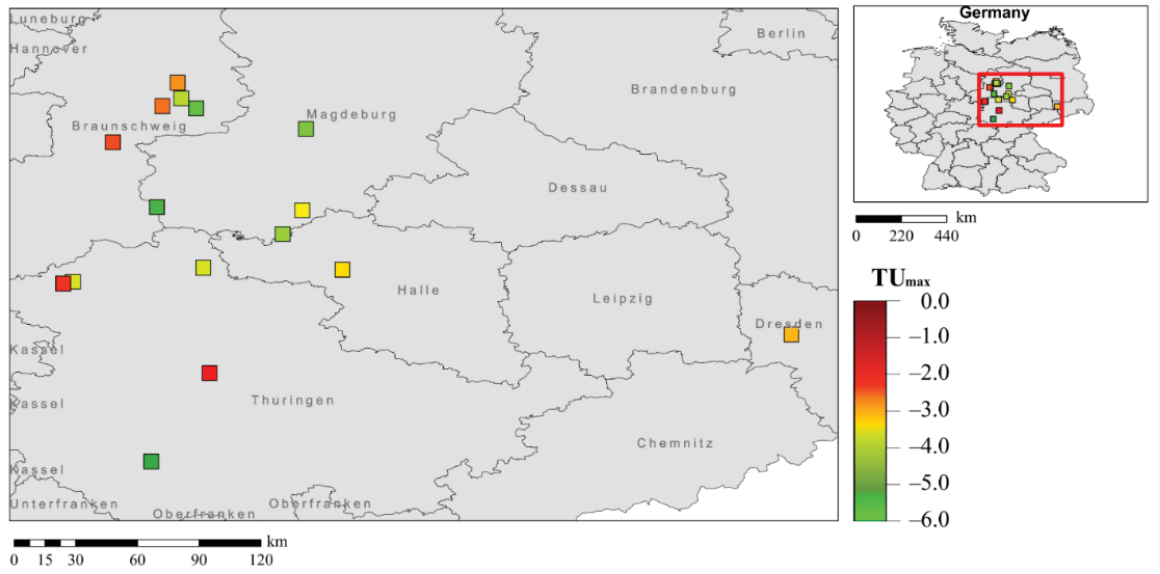

Figure B.1. Location of the sampling sites that cover a range of non-contaminated to moderately contaminated streams in central Germany. Square shapes represents the sampling sites and are coloured according to the TU_{max} values ranging from -6 (low pesticide pollution; green) to 0 (high pesticide pollution; red).

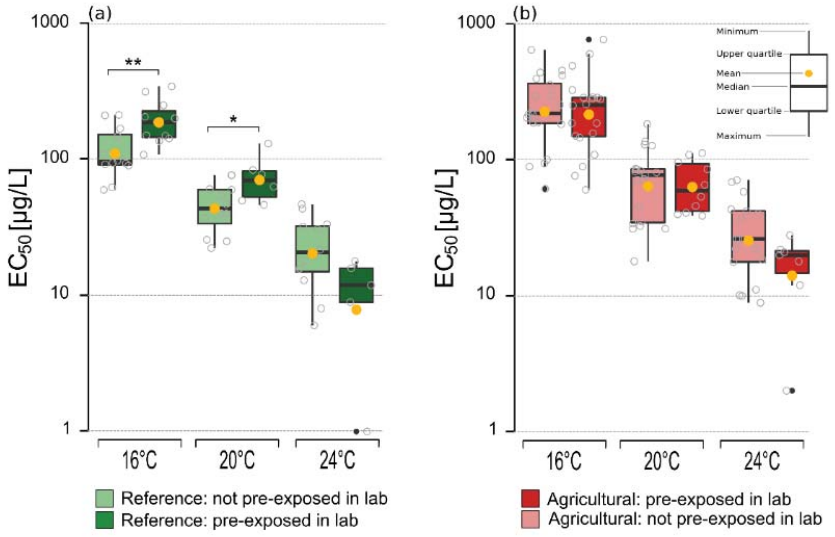

Figure B.2. Comparison of the median effective concentrations (EC_{50}) after 48 h exposure to the neonicotinoid insecticide clothianidin before and after pre-exposure of *G. pulex* collected from agricultural and reference streams in the laboratory at 16°C, 20°C and 24°C. The effect of temperature on the clothianidin tolerance of forest populations is shown in (a) whereas the effect of temperature on increased clothianidin of agricultural populations is shown in (b). Grey dots represent data points.

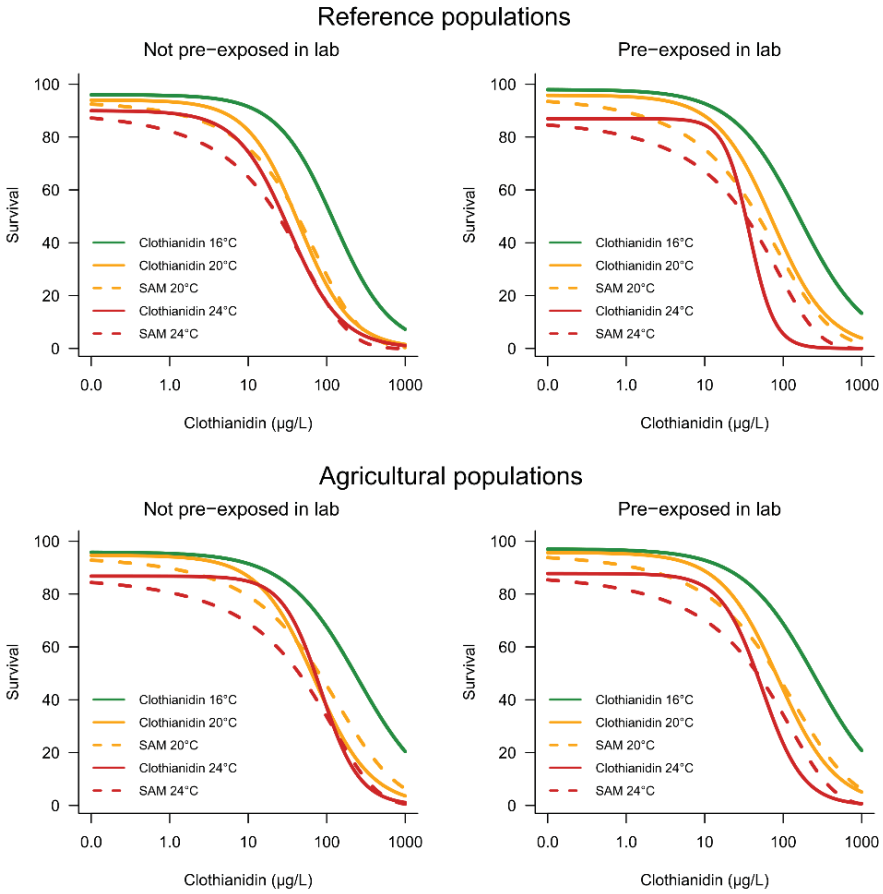

Figure B.3. Survival of *G. pulex* after 48h exposure to clothianidin at 20- and 24°C in reference and agricultural population with- and without pre-exposure in lab using 16°C respective reference as control. The solid lines show the fitted observed concentration−response relationships, and the dashed lines represent the modeled concentration−response relationship under additional stress using the SAM.

Table B.1. Pesticide exposure and EC_{50} of the study sites.

Site ID	SPEAR	TU_{max}	Temperature (°C)		48h EC_{50} Spring				48h EC_{50} Autumn					
			Spring	*Autumn*	16 °C	20 °C	24 °C	P.E 16 °C	16 °C	20 °C	24 °C	P.E 16 °C	P.E 20 °C	P.E 24 °C
S35	0.86	-2.64	9	13	395.6	85.9	40	289.9	188.67	22	22	77	41	28
S39	0.75	-2.31	9	13.5	187	129.3	58	158.5	100	26	26	110	54	21
S43	1.03	-2.69	9	12.6	439.4	183	36.8	254	88.85	10	10	259	40	18
S66	0.94	-2.93	9.8	10	456	85	41.9	778.9	225	NA	NA	292	66	NA
S73	NA	-3.59	16.2	9.3	169.4	60.7	47	209.3	214	8	8	351	133	NA
S76	0.74	-2.95	15	4	215	80.7	68.5	188.3	297	43	43	498	86	22
S78	0.61	-4.19	11	5.6	92.4	25	21.6	191.7	98.7	43.6	43.6	153	85	12
S79	0.80	-3.04	18	9.3	215	32.4	70.7	610	94	22	22	283	NA	NA
S83	0.86	-1.68	10.3	7.1	254	17.8	9.9	151.8	646	NA	NA	290	110	2
S85	0.52	-1.51	6.3	15.4	203.6	136.1	26	147	356	8.8	8.8	90	113	20
S102	0.75	-3.07	12	5.6	60.9	33.8	17.6	60.6	294	11	11	284	97	12
S105	1.63	-4.81	7.5	10	148.5	76.9	32	152	62.6	15.7	15.7	27	50.5	18
S109	0.79	-3.28	8.2	5.6	111	40.3	33.4	321	95	20	20	110	47	9
S115	1.69	-5.37	6.4	13.5	93.9	54.6	18	144.9	212	6	6	255	79	1
S116	1.22	-5.78	5.4	12	90.6	25.7	30.9	203.7	59.87	12.9	12.9	139	64	16
S118	1.61	-3.07	8.1	5.6	183	31	18.3	188.3	419	NA	NA	330	46	NA

Table B.2. Percentage mortality in pesticide-free controls.

Site ID	Spring				Autumn					
	16 °C	20 °C	24 °C	PE16 °C	16 °C	20 °C	24 °C	PE16 °C	PE20 °C	PE24 °C
S35	12	0	0	10	0	6	33	0	0	0
S39	15	7	5	6	0	0	8	12	20	0
S43	6	0	25	0	0	0	13	0	0	16
S66	0	13	30	0	0	6	NA	0	8	NA
S73	0	6	6.25	0	6	6	22	0	0	NA
S76	0	0	0	0	0	0	0	0	5	16
S78	21	13	18	0	7	0	0	6	18	0
S79	0	11	16	0	22	0	20	0	NA	NA
S83	0	40	14	14	5	0	NA	0	6	33
S85	0	0	27	0	0	0	0	0	0	25
S102	15	16	21	15	6	0	14	0	8	8
S105	0	0	0	0	0	0	0	6	0	0
S109	0	0	0	0	0	0	7	6	6	11
S115	6	0	18	11	0	0	22	0	5	41
S116	14	0	12	0	6	0	20	0	0	6
S118	0	16	0	0	0	6	NA	0	0	NA

C

Supporting Information for chapter 4

Ayesha Siddique, Naeem Shahid, Matthias Liess 2023. Persistent pesticides effects favour single population dominance and reduces biodiversity of vulnerable species. Submitted.

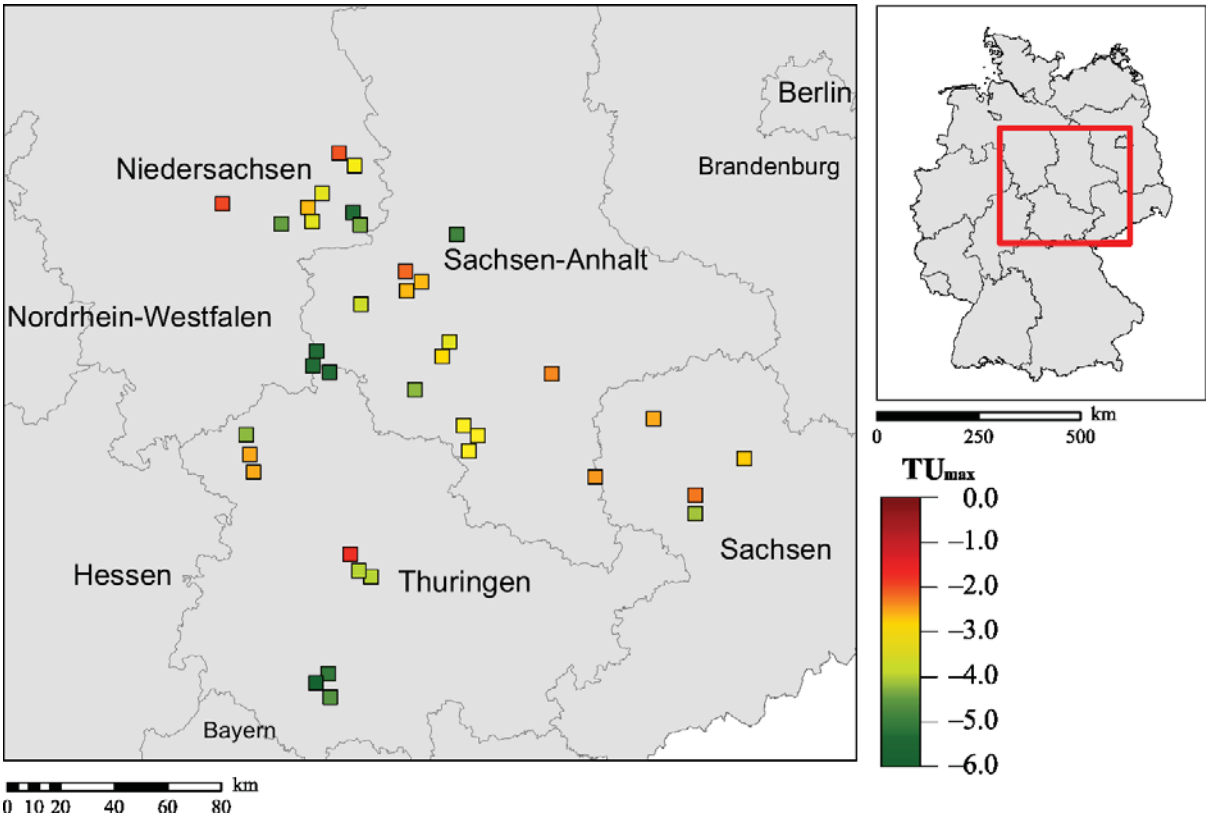

Figure C.1. Location of the sampling sites that cover a range of non-contaminated to moderately contaminated streams in central Germany. Square shapes represents the sampling sites and are coloured according to the TU_{max} values ranging from -6 (low pesticide pollution; green) to 0 (high pesticide pollution; red).

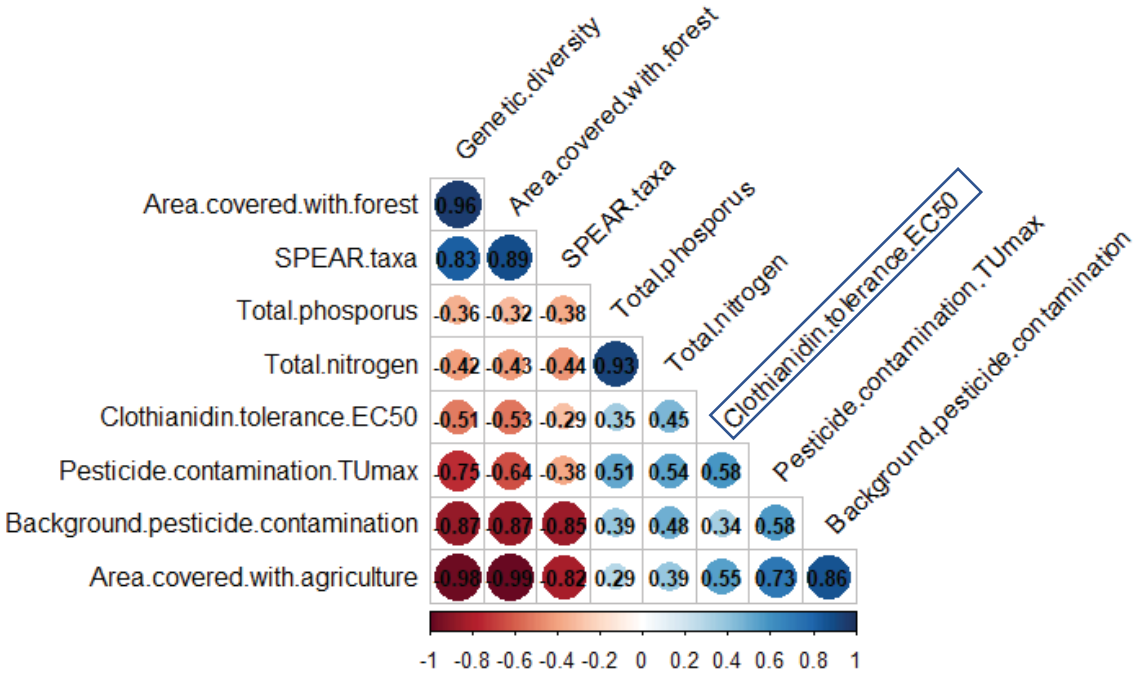

Figure C.2. Correlation plot representing correlations for different field factors determining pesticide tolerance in *G. pulex*. Genetic diversity represents within population genetic diversity, area covered with forest represents forest areas around the streams, SPEAR taxa represents proportion of sensitive species calculated by $SPEAR_{pesticide}$, total nitrogen represents sum of NO2, NO3, and NH4 in mg/L, total phosphorus represents PO4 in mg/L, pesticide contamination represents highest pesticide concentrations measured at each stream represent as TU_{max} and background contamination represents pesticide concentrations measured with grab samples. Values in the boxes represent correlation power, negative values represent negative correlations, positive values represent positive correlation. White boxes represent insignificant correlations (*p-value* > *0.05*).

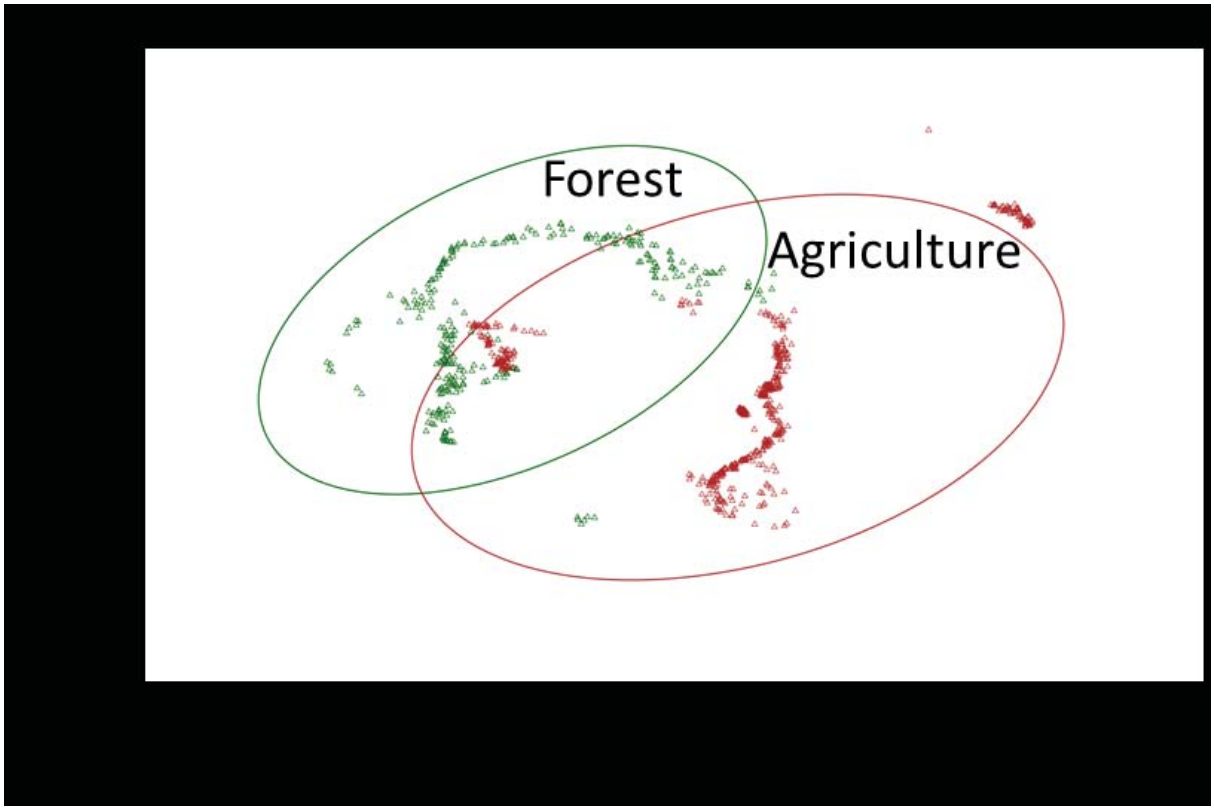

Figure C.3. Non-metric multi-dimentional scaling (NMDS). Two-dimensional ordination of populations of *G. pulex* collected from different sites in 2018, 2019 Spring and 2019 Autumn from non-metric multi-dimensional scaling (MDS) applied to a Bray-Curtis similarity matrix with 95% eclipse. Each symbol represents one individual.

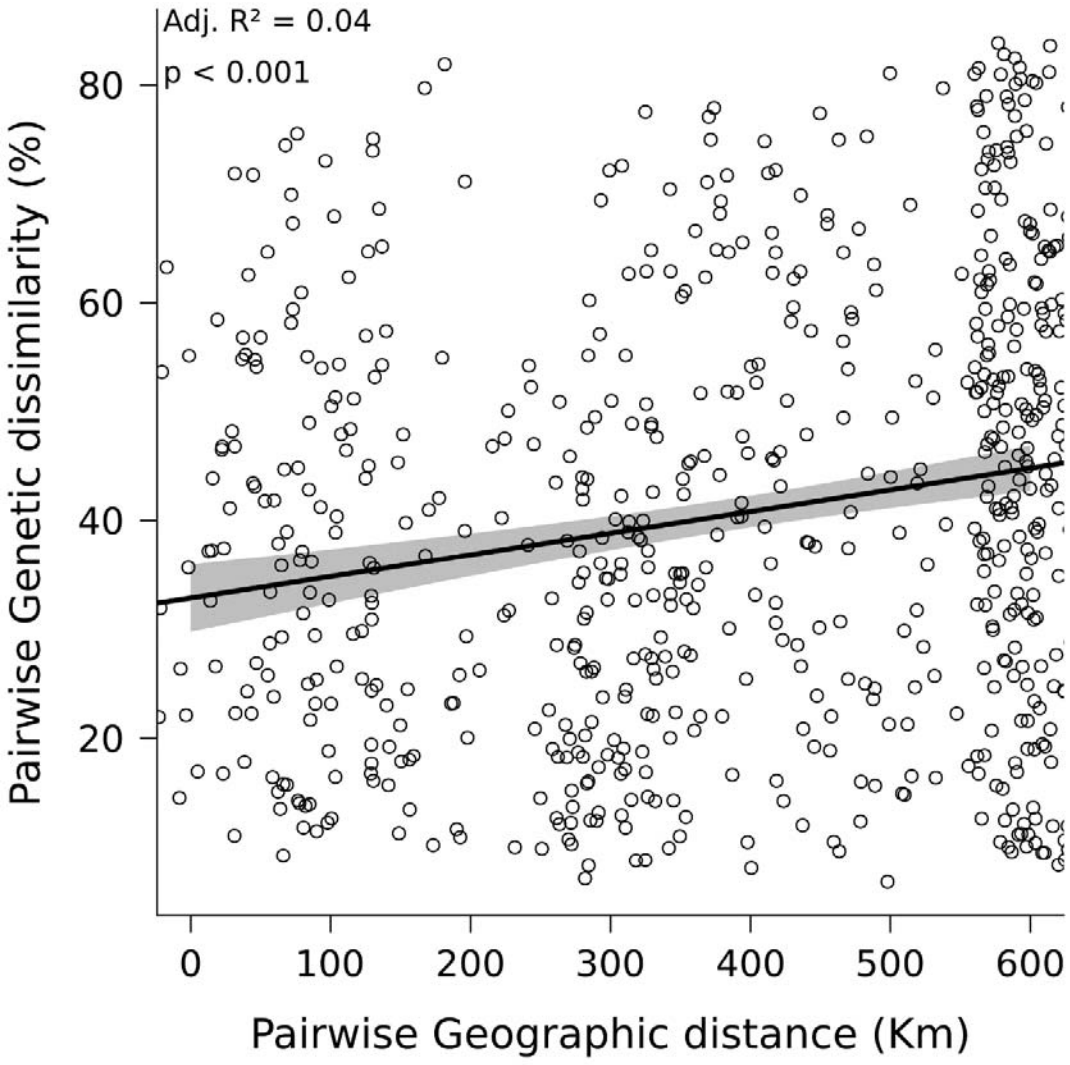

Figure C.4. Multiple linear regression between pair-wise geographic distance (waterways) and pairwise genetic dissimilarity for all populations based on Bray–Curtis similarity index (converted to diversity index) for AFLP measurements. Grey area represents ± 95% confidence intervals and dots represent data points.

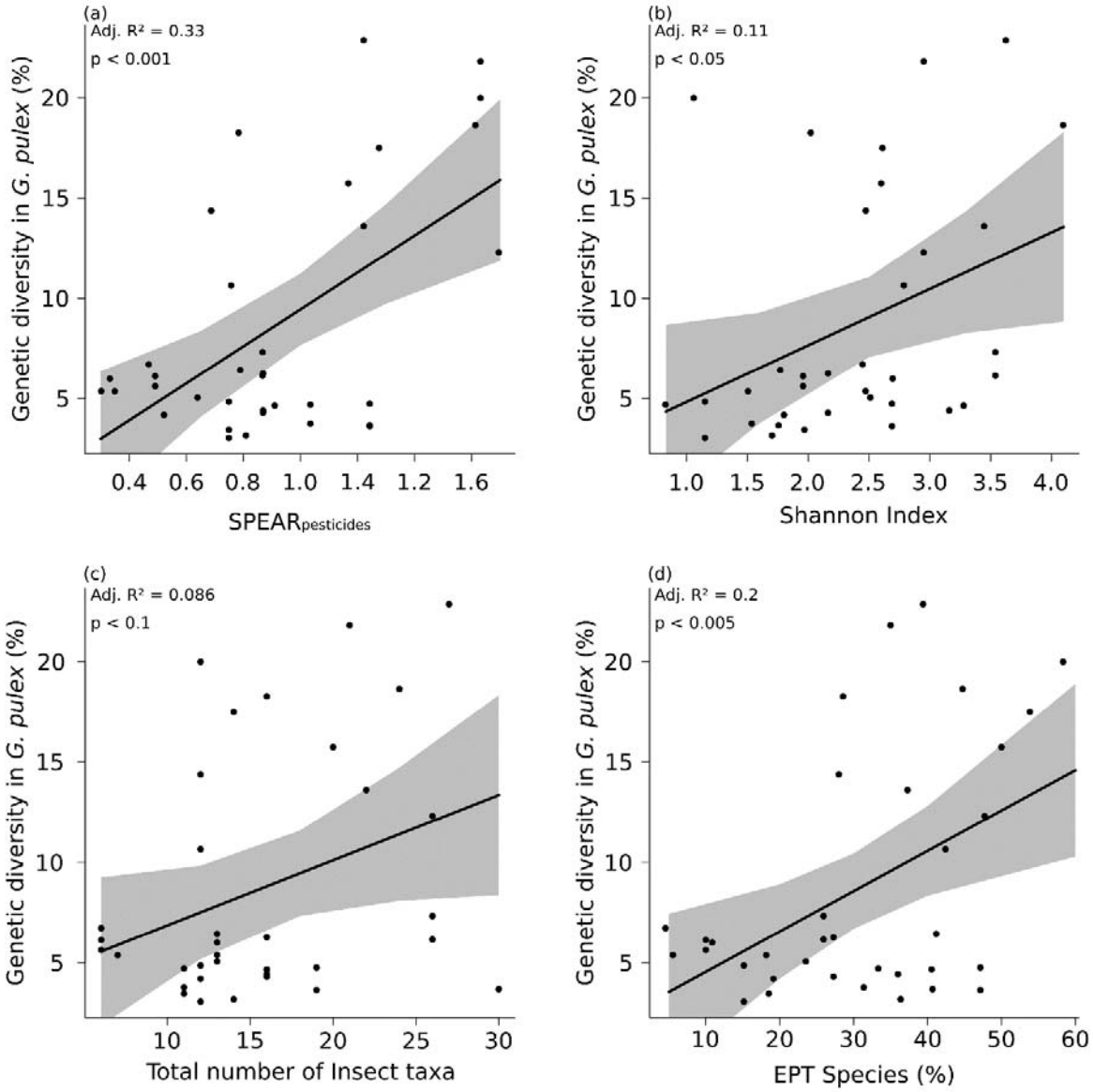

Figure C.5. Relationship between within population genetic diversity and (a) macroinvertebrate community structure expressed as $SPEAR_{Pesticides}$, (b) Species diversity expressed as Shannon index, (c) Total number of insect taxa, and (d) Percentage of sensitive species (EPT). Grey area represents ± 95 % confidence interval and dots represent data points.

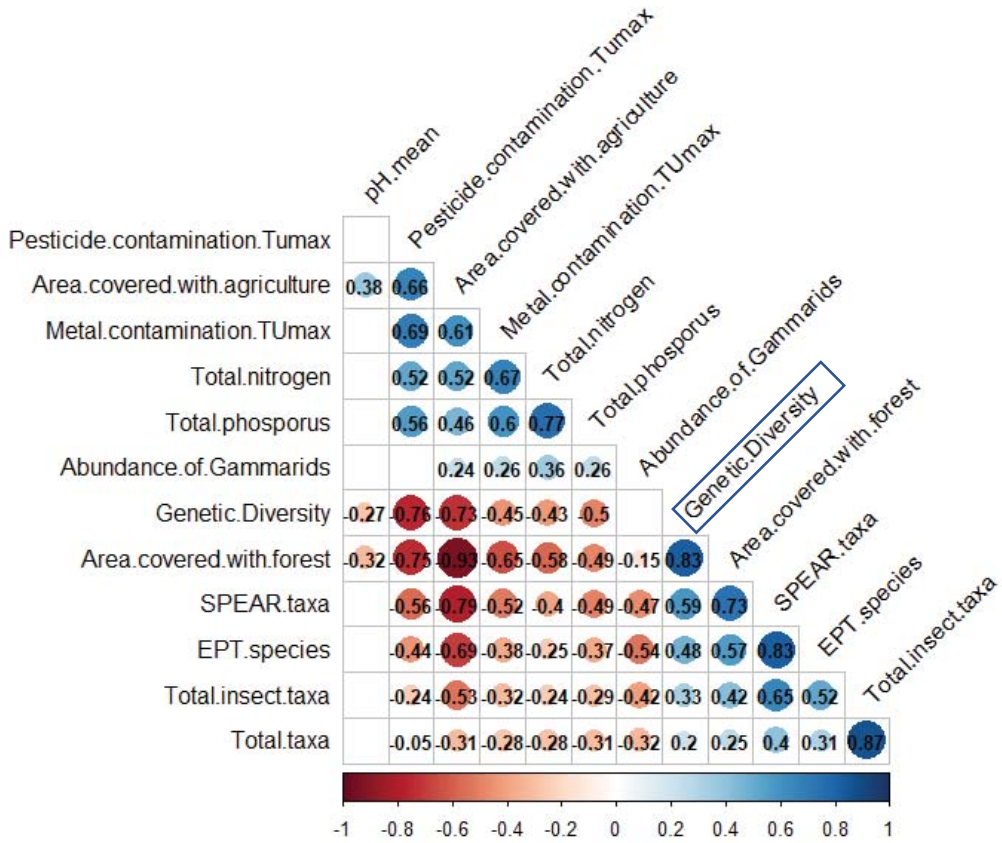

Figure C.6. Correlation plot representing correlations for different field factors affecting the genetic diversity of *G. pulex*. pH mean represents mean of pH measured continuously from April to July, pesticide contamination represents highest pesticide concentrations measured at each stream represent as TU_{max}, area covered with agriculture represents agricultural areas with pesticides around the streams,metal contamination represents maximum toxicity caused by metals represent as TU_{max}, total nitrogen represents sum of NO2, NO3, and NH4 in mg/L, total phosphorus represents PO4 in mg/L, abundance of gammarids represent a count of *G. pulex* sampled from each stream alng with other macroinvertebrates, Genetic diversity represents within population genetic diversity, area covered with forest represents forest areas around the streams, SPEAR taxa represents proportion of sensitive species calculated by $SPEAR_{pesticide}$, EPT species represent percentage of EPT species at each sites and total insect taxa represents total taxa found at each stream. Values in the boxes represent correlation power, negative values represent negative correlations, positive values represent positive correlation. White boxes represent insignificant correlations (*p-value > 0.05*)

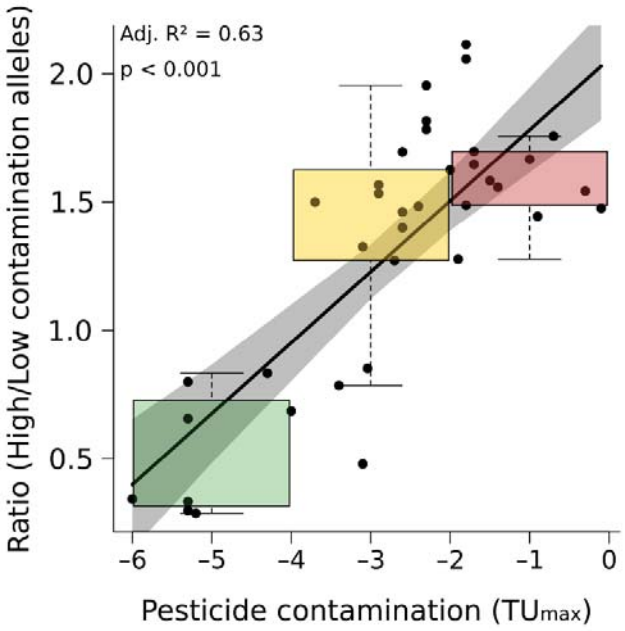

Figure C.7. Relationship between local pesticide contamination (TU_{max}) and ratio of high to low contamination alleles. Grey area represents ± 95% confidence interval and dots represent data points.

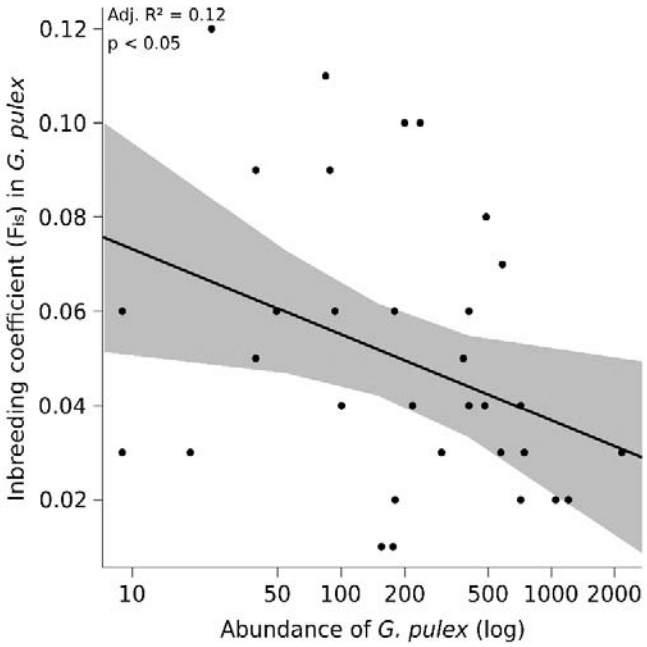

Figure C.8. Relationship between abundance of *G. pulex* and inbreeding coefficient (Fis) in the studied populations. Grey area represents ± 95% confidence interval and dots represent data points.

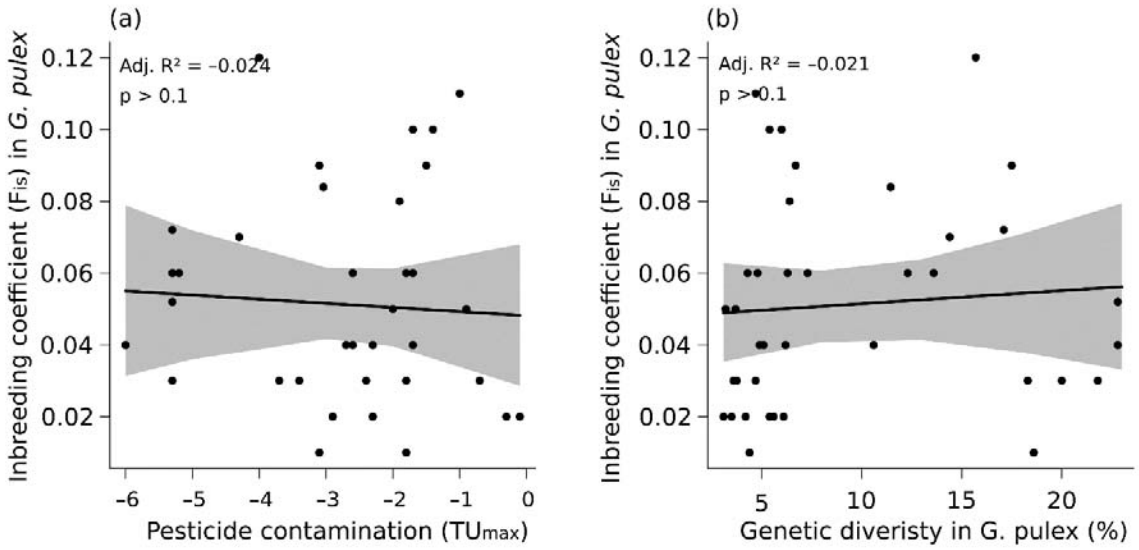

Figure C.9. Relationship between Gammarid inbreeding coefficient (F_{is}) and (a) Local pesticide contamination (TU_{max}), and (b) Genetic diversity in *G. pulex*. Grey area represents ± 95% confidence interval and dots represent data points.

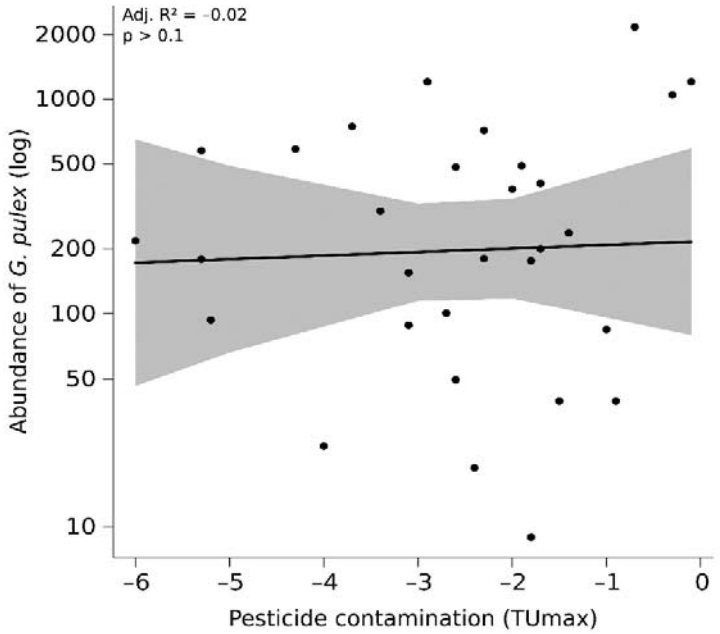

Figure C.10. Relationship between local pesticide contamination (TU_{max}) and abundance of *G. pulex* (log-transformed) in the respective streams. Grey area represents ± 95% confidence interval and dots represent data points.

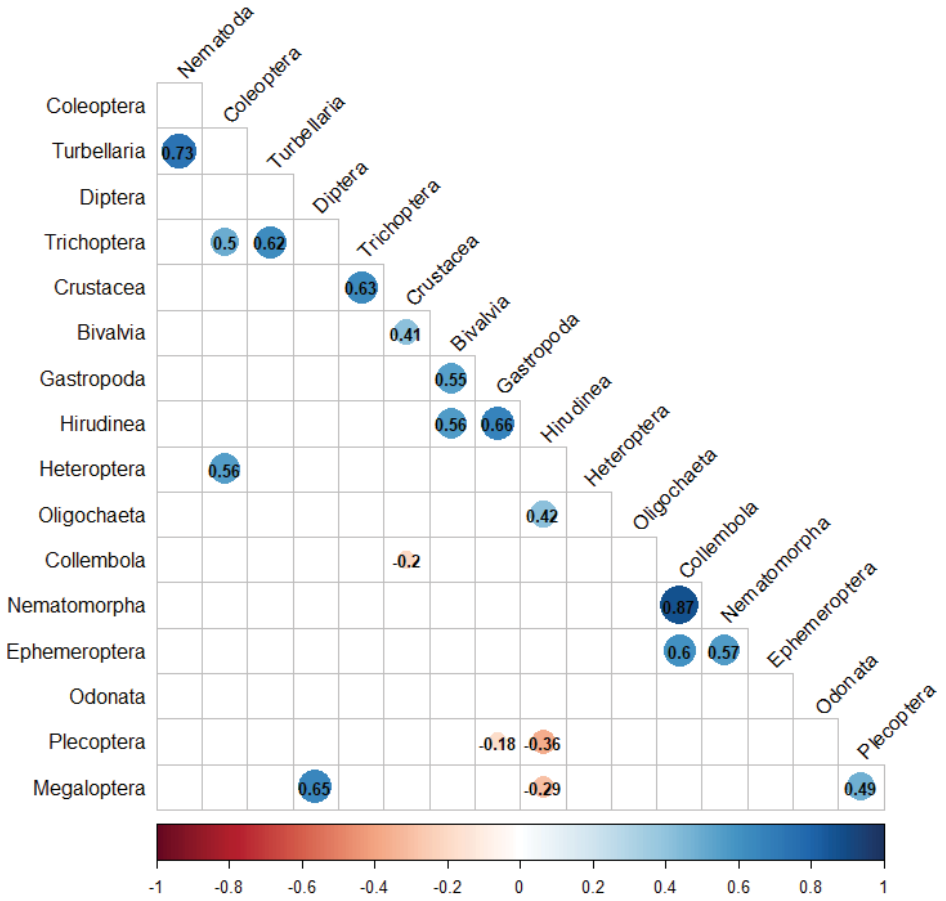

Figure C.11. Correlation plot for macroinvertebrates abundance represented here at the group level. Values in the boxes represent correlation power, negative values represent negative correlations, positive values represent positive correlation. White boxes represent insignificant correlations (*p-value* > *0.05*).

Table C.1. Physico-chemical parameters of the streams.

Site ID	Coordinates	Temp [°C]	Flow velocity [m/s]	Dissolved O_2 [mg/L]	pH	SPEAR	TU_{max} [log]
S116.1	50.623331, 10.635981	14.6	0.27	9.58	7.3	0.75	-6
S105.1	52.165597, 10.832033	18.6	0.12	8.85	7.8	1	-2.96
S33	52.148642, 10.477586	17.7	0.24	7.28	8.1	0.48	-2.62
S117	50.592508, 10.646661	21.6	0.05	7.86	7.3	0.72	-6
S73	51.616645, 11.210976	19.3	0.17	7.9	8.1	0.78	-2.62
S115.1	51.734614, 10.660653	20.3	0.32	9.84	7.9	1.04	-6
S124	51.153917, 12.700556	NA	NA	NA	7.9	0.91	-1.04
S78	52.075711, 11.31275	16	0.36	8.7	8.2	0.42	-4.21
S82	51.892306, 10.976806	20.4	0.42	6.55	8.3	0.46	-2.55
S32	52.228258, 10.166289	17.8	0.02	5.69	7.9	0.19	-1.47
S74	51.289945, 12.152368	19.4	0.25	6.81	8	0.29	-1.89
S35.1	52.175367, 10.684714	19.7	0.17	6.46	8.4	0.53	-1.83
S98.1	51.941583, 11.158639	NA	NA	NA	NA	1.24	-0.9
S76.1	51.460978, 11.471981	18.3	0.3	8.2	8.3	0.46	-2.55
S77	51.635608, 11.938936	19.5	0.31	7.14	8.3	0.21	-1.42
S68	51.492756, 12.486058	24.6	0.12	8.52	8.1	0.2	-1.53
S69	51.335278, 12.971361	16.2	0.41	8.31	7.8	0.48	-2.1
S71	51.211849, 12.7032	19.5	0.49	5.63	7.8	0.56	-1.04
S79.1	51.72064, 11.296767	20.1	0.24	5.99	8.1	0.5	-2.22
S43.1	52.277353, 10.751378	16.4	0.44	8.29	7.1	0.43	-1.58
S85.1	51.009792, 10.89115	16.1	0.12	8.49	7.9	0.32	-0.14
S116.2	50.623331, 10.635981	21.8	0.35	9.26	7.4	0.75	-3.86
S118	51.409195, 10.259272	21.6	0.23	8.3	7.4	0.99	-3.47
S115.2	51.734614, 10.660653	20.3	0.32	9.84	7.9	1.04	-6
105.2	52.165597, 10.832033	18.6	0.12	8.85	7.8	1	-2.96
S83.1	51.400363, 10.251626	14.4	0.34	8.77	7.5	0.53	-1.68
S35.2	52.175367, 10.684714	19.6	0.27	8.66	8.2	0.53	-1.48
S98.2	51.941583, 11.158639	NA	NA	NA	NA	1.24	-1.8
S76.2	51.460978, 11.471981	17.5	0.12	8.44	8.2	0.46	-1.7
S43.2	52.277353, 10.751378	16.4	0.44	8.29	7.1	0.43	-1.58
S79.2	51.72064, 11.296767	20.1	0.24	5.99	8.1	0.5	-2.22
S85.2	51.009792, 10.89115	16.1	0.12	8.49	7.9	0.32	-0.14
S115.3	51.734614, 10.660653	20.3	0.32	9.84	7.9	1.04	-6
S83.2	51.400363, 10.251626	14.4	0.34	8.77	7.5	0.53	-1.68
S98.3	51.941583, 11.158639	NA	NA	NA	NA	1.24	-1.8
S76.3	51.460978, 11.471981	17.5	0.12	8.44	8.2	0.46	-1.7
S35.3	52.175367, 10.684714	19.6	0.27	8.66	8.2	0.53	-1.48
S85.3	51.009792, 10.89115	16.1	0.12	8.49	7.9	0.32	-0.14

Table C.2. Sequences of primers and adaptors used in the AFLP procedures.

	EcoRI	**MseI**
Restriction site	5′ GAA TTC	5′ TTA A
	CTT AAG 5′	AAT T 5′
Adaptor	5′ CTC GTA GAC TGC GTA CC	5′ GAC GAT GAG TCC TGA G
	CAT CTG ACG CAT GGT TAA 5′	TA CTC AGG ACT CAT 5′
Pre-selective primer	5′ GAC TGC GTA CCA ATT C + A	5′ GAT GAG TCC TGA GTA A + C
Selective primer	5′ GAC TGC GTA CCA ATT C + ACC	5′ GAT GAG TCC TGA GTA A + CTG* – HEX

Table C.3. Populations genetic statistics based on 451 AFLP loci.

Sr. no	Site ID	No. of Bands	PLP	Within pop Genetic diversity	Between pop Genetic diversity	N_a	H_e	uH_e	Avg. PhiPT	F_{is}
1	S116.1	336	73	13.6	49	1.5	0.193	0.197	0.45	0.056
2	S105.1	378	77	20	47.1	1.6	0.229	0.235	0.30	0.032
3	S33	390	81	18.3	46.7	1.7	0.225	0.231	0.35	0.025
4	S117	362	78	15.7	44.6	1.6	0.220	0.225	0.39	0.12
5	S73	414	90	17.5	42.5	1.8	0.257	0.262	0.31	0.09
6	S115.1	337	62	12.3	40.5	1.4	0.190	0.194	0.43	0.06
7	S124	342	68	11.4	38.5	1.4	0.204	0.210	0.41	0.084
8	S78	403	85	14.4	37.7	1.7	0.229	0.236	0.31	0.07
9	S82	361	72	10.6	35.8	1.5	0.199	0.203	0.38	0.038
10	S32	306	60	5.4	32.7	1.3	0.176	0.180	0.43	0.015
11	S74	346	70	6.7	33.7	1.5	0.210	0.215	0.38	0.09
12	S35.1	257	42	4.4	34.2	1.0	0.153	0.157	0.51	0.011
13	S98.1	247	37	3.7	33.2	0.9	0.138	0.141	0.48	0.046
14	S76.1	213	35	3.5	34.4	0.8	0.132	0.135	0.49	0.02
15	S77	304	60	5.4	35.3	1.3	0.198	0.203	0.40	0.095
16	S68	309	63	6	36.7	1.3	0.212	0.219	0.39	0.096
17	S69	371	78	6.4	37.7	1.6	0.220	0.227	0.41	0.082
18	S71	303	58	4.7	36.5	1.2	0.173	0.179	0.41	0.11
19	S79.1	281	48	3.2	36.8	1.1	0.115	0.118	0.48	0.045
20	S43.1	242	41	3.8	37.7	0.9	0.156	0.160	0.45	0.025
21	S85.1	285	50	4.2	38.6	1.1	0.158	0.162	0.43	0.022
22	S116.2	341	72	22.8	68.7	1.5	0.189	0.194	0.44	0.041
23	S118	311	53	18.6	65.9	1.2	0.127	0.130	0.49	0.011
24	S115.2	350	68	22.8	61.1	1.5	0.190	0.194	0.41	0.052
25	105.2	366	72	21.8	57.6	1.5	0.186	0.189	0.42	0.027
26	S83.1	293	57	7.3	34.4	1.2	0.148	0.151	0.49	0.059
27	S35.2	289	53	6.3	34.3	1.2	0.134	0.137	0.54	0.061
28	S98.2	264	41	4.8	32.3	1.0	0.114	0.117	0.54	0.055
29	S76.2	240	38	4.9	32.9	0.9	0.111	0.114	0.55	0.041
30	S43.2	262	45	4.7	32.1	1.0	0.122	0.125	0.55	0.033
31	S79.2	298	52	5.1	31.4	1.2	0.108	0.110	0.51	0.04
32	S85.2	299	56	6.1	31.4	1.2	0.148	0.151	0.45	0.022
33	S115.3	355	69	17.1	52.44	1.5	0.179	0.183	0.43	0.072
34	S83.2	339	68	6.2	31.5	1.4	0.156	0.159	0.45	0.038
35	S98.3	275	40	3.6	30.9	1.0	0.097	0.099	0.54	0.031
36	S76.3	229	29	3.1	31.8	0.8	0.077	0.079	0.58	0.015
37	S35.3	322	58	4.3	32.3	1.3	0.121	0.123	0.54	0.06
38	S85.3	328	63	5.6	32.2	1.3	0.156	0.159	0.43	0.018

Site ID refers to sites under KgM dataset (Liess et al., 2021a). Population genetics statistics are coded as follows. Polymorphic Loci percentage (PLP), Average number of alleles per locus (N_a), Expected heterozygosity (H_e), Unbiased Heterozygosity (uH_e), Genetic differentiation (PhiPT), Inbreeding coefficient (F_{is}).

Table C.4. Pairwise geographic distance and genetic dissimilarity computed by SIMPER based on bray-curtis similarity index.

Site ID	S116.2	S118	S115.2	S105.2	S115.3	S116.1	S105.1	S33	S117	S73	S115.1	S124	S78	S82	S83.1	S35.2	S98.2	S76.2	S43.2	S79.2	S85.2	S83.2	S98.3	S76.3	S35.3	S85.3	S32	S74	S35.1	S98.1	S76.1	S77	S68	S69	S71	S79.1	S43.1	S85.1
S116.2		600	600	600	600	0	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600
S118	39		448	520	448	600	520	600	600	345	448	600	350	600	1	509	360	127	521	369	162	1	360	127	509	162	600	600	509	360	127	600	600	600	600	369	521	162
S115.2	43	36		393	0	600	393	600	600	155	0	600	117	600	447	382	95	366	385	100	416	447	95	366	382	416	600	600	382	95	366	600	600	600	600	100	385	416
S105.2	51	45	39		600	600	0	34	600	191	393	600	97	98	519	17	305	439	71	314	499	519	305	439	17	499	71	600	17	305	439	275	600	600	600	314	71	499
S115.3	50	43	29	35		600	393	600	600	155	0	600	117	600	447	382	95	366	385	100	416	447	95	366	382	416	600	600	382	95	366	600	600	600	600	100	385	416
S116.1	41	47	41	37	27		600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600
S105.1	56	53	45	37	32	26		34	600	191	393	600	97	98	519	17	305	439	71	314	499	519	305	439	17	499	71	600	17	305	439	275	600	600	600	314	71	499
S33	62	60	52	47	39	32	27		600	184	600	600	85	93	600	24	78	600	600	145	600	600	78	600	24	600	219	600	24	78	500	139	600	600	600	145	600	600
S117	60	60	53	48	40	31	29	26		600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600	600
S73	65	62	55	50	42	37	31	26	24		155	169	99	112	344	279	101	264	282	55	313	344	101	264	279	313	219	145	279	101	264	84	600	600	163	55	282	313
S115.1	66	62	54	52	41	39	33	30	26	21		600	117	600	447	382	95	366	385	100	416	447	95	366	382	416	600	600	382	95	366	600	600	600	600	100	385	416
S124	65	62	55	52	43	38	33	32	29	24	21		600	334	600	385	319	246	600	400	299	600	319	246	385	299	600	115	385	319	246	208	600	600	8	408	500	299
S78	68	65	57	53	45	41	36	33	30	25	22	18		43	350	301	29	269	303	69	320	350	29	269	301	320	121	600	301	29	269	89	600	600	600	69	303	320
S82	68	65	58	54	47	42	36	33	31	25	23	19	16		600	107	15	371	500	46	424	600	15	371	107	424	140	500	107	15	371	181	600	600	319	46	500	424
S83.1	68	63	59	53	48	43	40	39	35	32	29	24	22	19		508	359	126	520	367	161	0	359	126	508	161	600	600	508	359	126	600	600	600	600	367	520	161
S35.2	71	67	63	55	52	46	43	44	39	36	33	29	26	23	16		294	428	60	303	488	508	294	428	0	488	62	293	0	294	428	166	600	600	370	303	60	488
S98.2	69	66	61	55	51	46	42	43	39	36	33	28	26	22	17	15		279	297	46	328	359	0	279	294	328	120	223	294	0	279	102	600	600	303	46	297	328
S76.2	74	69	65	58	54	50	46	45	42	38	35	31	28	25	19	16	14		430	287	95	126	279	0	428	95	600	129	428	279	264	251	600	600	246	287	430	95
S43.2	72	69	65	59	54	50	47	45	42	38	36	31	29	26	21	19	16	14		305	491	520	297	430	60	491	600	600	60	297	430	600	600	600	600	305	0	491
S79.2	73	68	65	61	55	50	49	48	44	41	38	34	31	29	22	18	17	14	12		337	367	46	287	303	337	180	185	303	46	287	130	600	600	400	0	305	337
S85.2	75	71	67	61	57	53	49	49	48	43	39	35	33	30	25	22	18	17	16	12		161	328	95	488	0	600	182	488	328	95	304	600	600	284	337	491	0
S83.2	76	72	68	63	59	54	51	51	47	44	41	37	35	32	22	25	21	19	17	14	12		359	126	508	161	600	600	508	359	126	600	600	600	600	367	520	161
S98.3	74	71	67	63	58	54	51	51	48	45	42	37	36	33	28	26	17	21	19	16	13	11		279	294	328	120	223	294	0	279	102	600	600	303	46	297	328
S76.3	78	74	70	65	61	58	54	53	51	47	44	40	38	35	29	27	24	17	21	17	14	13	10		428	95	600	129	428	279	264	251	600	600	246	287	430	95
S35.3	78	75	72	65	63	58	55	55	52	49	46	42	40	37	32	23	25	24	22	18	17	15	12	10		488	62	293	0	294	428	166	600	600	370	303	60	488
S85.3	78	75	72	65	63	59	56	55	52	50	46	42	40	38	32	30	26	25	24	20	16	16	13	11	10		600	182	488	328	95	304	600	600	284	337	491	0
S32	79	77	73	70	64	60	57	52	52	48	45	43	40	37	35	33	30	28	25	23	21	18	16	15	14	11		600	62	120	600	88	600	600	600	180	600	600
S74	81	79	74	71	65	62	57	54	52	48	45	44	41	37	38	36	32	31	28	26	23	21	18	18	16	13	10		600	223	129	500	600	600	102	185	600	182
S35.1	80	80	75	72	66	62	58	54	53	49	46	45	43	39	39	37	33	32	29	27	25	22	20	19	18	15	11	10		294	428	166	600	600	370	303	60	488
S98.1	79	78	73	69	65	61	57	55	53	48	46	44	42	37	38	36	32	32	29	27	24	22	19	19	17	14	12	10	9		279	102	600	600	303	46	297	328
S76.1	81	80	75	72	67	63	60	56	54	51	48	46	43	40	40	38	35	33	30	28	25	23	21	20	19	16	13	11	10	8		251	600	600	246	287	430	95
S77	82	80	75	73	67	64	60	57	55	51	48	47	45	41	41	39	36	34	32	30	27	25	23	22	20	17	14	12	11	9	7		600	600	200	130	600	304
S68	84	82	78	74	69	66	62	58	57	52	50	49	46	43	43	41	37	36	33	31	29	27	24	23	22	19	16	13	12	11	9	8		600	600	600	600	600
S69	84	81	77	74	69	66	63	62	59	56	53	50	49	46	44	41	38	37	34	32	29	27	25	23	22	19	18	17	15	14	12	11	10		600	600	600	600
S71	82	80	76	73	68	65	62	59	57	54	51	48	47	44	42	40	37	35	33	31	29	27	24	23	22	19	17	16	14	12	11	10	10	9		400	600	284
S79.1	81	78	76	72	68	64	63	62	59	57	54	51	49	47	42	38	36	35	33	26	28	26	24	22	20	18	18	18	17	14	13	12	12	10	8		305	337
S43.1	82	81	77	74	69	66	63	59	58	54	52	49	48	44	44	43	39	38	34	33	31	28	26	25	24	21	18	17	15	13	12	11	10	11	9	9		491
S85.1	83	82	77	75	70	67	64	60	59	55	53	51	49	46	45	43	40	39	36	34	31	29	27	27	25	22	19	18	16	15	14	12	12	13	10	10	7	

Genetic dissimilarity is presented in percentage (increasing from red to green) and geographic distance in Km (increasing from light to dark grey). For the streams where no water connection was possible, a maximum value of 600 Km was considered.

Table C.5. Pairwise genetic differentiation and significance.

Site ID	S116.2	S118	S115.2	S105.2	S115.3	S116.1	S105.1	S33	S117	S73	S115.1	S124	S78	S82	S83.1	S35.2	S98.2	S76.2	S43.2	S79.2	S85.2	S83.2	S98.3	S76.3	S35.3	S85.3	S32	S74	S35.1	S98.1	S76.1	S77	S68	S69	S71	S79.1	S43.1	S85.1
S116.2		0.001	0.001	0.001	0.001	0.493	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S118	0.4		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S115.2	0.3	0.3		0.001	0.49	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S105.2	0.4	0.4	0.4		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S115.3	0.4	0.4	0.0	0.4		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S116.1	0.0	0.4	0.3	0.4	0.4		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S105.1	0.3	0.3	0.2	0.2	0.3	0.3		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S33	0.4	0.4	0.38	0.4	0.4	0.4	0.2		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S117	0.3	0.5	0.38	0.4	0.4	0.3	0.2	0.3		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S73	0.4	0.4	0.34	0.4	0.4	0.4	0.2	0.1	0.2		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S115.1	0.5	0.5	0.37	0.5	0.4	0.5	0.3	0.3	0.3	0.2		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S124	0.4	0.5	0.4	0.4	0.4	0.4	0.2	0.3	0.3	0.2	0.4		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S78	0.3	0.4	0.3	0.3	0.3	0.3	0.1	0.2	0.2	0.1	0.2	0.2		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S82	0.4	0.5	0.4	0.4	0.4	0.4	0.2	0.2	0.3	0.1	0.3	0.3	0.1		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S83.1	0.5	0.5	0.4	0.4	0.5	0.5	0.3	0.5	0.5	0.4	0.5	0.5	0.4	0.5		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S35.2	0.5	0.6	0.5	0.4	0.5	0.5	0.4	0.5	0.5	0.5	0.6	0.6	0.5	0.6	0.55		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S98.2	0.5	0.6	0.5	0.4	0.5	0.5	0.4	0.5	0.5	0.5	0.6	0.5	0.4	0.5	0.53	0.6		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S76.2	0.6	0.6	0.5	0.5	0.5	0.6	0.4	0.5	0.5	0.5	0.6	0.6	0.4	0.5	0.55	0.6	0.6		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S43.2	0.6	0.6	0.5	0.5	0.5	0.6	0.4	0.5	0.5	0.4	0.6	0.5	0.4	0.5	0.57	0.6	0.7	0.6		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S79.2	0.5	0.5	0.5	0.5	0.5	0.5	0.4	0.5	0.5	0.5	0.6	0.6	0.4	0.5	0.51	0.5	0.6	0.6	0.6		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.45	0.001	0.001
S85.2	0.5	0.5	0.4	0.4	0.5	0.5	0.3	0.4	0.4	0.4	0.5	0.5	0.3	0.5	0.4	0.5	0.5	0.5	0.6	0.5		0.001	0.001	0.001	0.001	0.47	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S83.2	0.4	0.5	0.4	0.4	0.4	0.5	0.3	0.4	0.4	0.4	0.5	0.5	0.4	0.5	0.0	0.5	0.5	0.5	0.5	0.5	0.4		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S98.3	0.5	0.6	0.5	0.5	0.5	0.5	0.4	0.5	0.5	0.5	0.6	0.5	0.4	0.5	0.5	0.6	0.2	0.6	0.6	0.6	0.5	0.51		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S76.3	0.6	0.6	0.5	0.5	0.6	0.6	0.5	0.5	0.6	0.5	0.6	0.6	0.5	0.6	0.6	0.6	0.7	0.2	0.7	0.6	0.5	0.5	0.7		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S35.3	0.5	0.6	0.5	0.4	0.6	0.6	0.4	0.5	0.5	0.5	0.6	0.6	0.5	0.6	0.6	0.2	0.6	0.6	0.6	0.5	0.5	0.5	0.6	0.6		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S85.3	0.4	0.4	0.4	0.3	0.4	0.4	0.3	0.4	0.4	0.4	0.5	0.4	0.3	0.4	0.4	0.5	0.5	0.5	0.5	0.4	0.0	0.4	0.5	0.5	0.5		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S32	0.4	0.5	0.4	0.5	0.4	0.5	0.3	0.2	0.4	0.2	0.4	0.4	0.3	0.3	0.5	0.6	0.6	0.6	0.5	0.5	0.5	0.5	0.6	0.6	0.6	0.5		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S74	0.4	0.5	0.4	0.4	0.4	0.4	0.2	0.2	0.3	0.1	0.3	0.3	0.2	0.2	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.6	0.5	0.4	0.3		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S35.1	0.5	0.6	0.5	0.5	0.5	0.6	0.4	0.3	0.4	0.3	0.4	0.5	0.3	0.4	0.6	0.7	0.6	0.7	0.6	0.6	0.6	0.6	0.6	0.7	0.7	0.6	0.44	0.3		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
S98.1	0.5	0.6	0.5	0.5	0.5	0.5	0.3	0.3	0.4	0.2	0.4	0.4	0.3	0.2	0.6	0.6	0.6	0.6	0.6	0.6	0.5	0.5	0.6	0.7	0.6	0.5	0.4	0.3	0.5		0.001	0.001	0.001	0.001	0.001	0.001	0.001
S76.1	0.5	0.6	0.5	0.5	0.5	0.5	0.3	0.3	0.4	0.3	0.4	0.4	0.3	0.4	0.6	0.7	0.7	0.7	0.6	0.6	0.6	0.5	0.7	0.7	0.7	0.5	0.4	0.3	0.5	0.5		0.001	0.001	0.001	0.001	0.001	0.001
S77	0.4	0.5	0.4	0.4	0.4	0.5	0.2	0.2	0.3	0.2	0.3	0.3	0.2	0.2	0.5	0.6	0.6	0.6	0.6	0.5	0.5	0.5	0.6	0.6	0.6	0.4	0.3	0.2	0.4	0.4	0.3		0.001	0.001	0.001	0.001	0.001
S68	0.4	0.5	0.4	0.4	0.4	0.5	0.2	0.1	0.3	0.1	0.3	0.3	0.2	0.2	0.5	0.6	0.6	0.6	0.6	0.5	0.6	0.5	0.6	0.6	0.6	0.5	0.3	0.2	0.4	0.4	0.4	0.2		0.001	0.001	0.001	0.001
S69	0.4	0.4	0.3	0.4	0.4	0.4	0.3	0.3	0.3	0.3	0.4	0.3	0.3	0.4	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.4	0.4	0.5	0.5	0.4	0.4	0.4	0.5	0.5	0.5	0.4	0.3		0.001	0.001	0.001
S71	0.4	0.5	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.2	0.4	0.3	0.2	0.3	0.5	0.5	0.6	0.5	0.5	0.5	0.5	0.4	0.5	0.6	0.6	0.4	0.3	0.3	0.5	0.4	0.4	0.3	0.3	0.4		0.001	0.001
S79.1	0.5	0.5	0.4	0.4	0.5	0.5	0.4	0.5	0.5	0.4	0.5	0.5	0.4	0.5	0.5	0.5	0.6	0.5	0.6	0.0	0.4	0.5	0.6	0.6	0.5	0.4	0.5	0.5	0.6	0.6	0.6	0.5	0.5	0.5	0.5		0.001	0.001
S43.1	0.5	0.6	0.5	0.5	0.5	0.5	0.3	0.2	0.4	0.2	0.4	0.4	0.2	0.3	0.6	0.6	0.6	0.6	0.6	0.6	0.5	0.5	0.6	0.7	0.6	0.5	0.4	0.3	0.4	0.4	0.4	0.3	0.3	0.4	0.4	0.6		0.001
S85.1	0.5	0.5	0.4	0.5	0.4	0.5	0.3	0.2	0.3	0.2	0.4	0.4	0.2	0.3	0.5	0.6	0.6	0.6	0.6	0.6	0.5	0.5	0.6	0.6	0.6	0.5	0.3	0.3	0.4	0.4	0.4	0.3	0.3	0.4	0.4	0.6	0.3	

Genetic differentiation is represented as PhiPT (F_{st} analog) below the diagnol (increasing from red to blue) and significance for each pair is presented above the diagonal.

D

Supporting Information for chapter 5

Naeem Shahid, **Ayesha Siddique**, Matthias Liess 2023. Predicting the combined effects of multiple stressors and individual stress adaptation (Submitted).

Table D.1: Information of the investigated streams including physicochemical parameters such as water temperature (°C), water level (cm), dissolved oxygen (DO) and pH, water toxicity in terms of TU_{max} values, and the composition of the macroinvertebrate community structure expressed as $SPEAR_{pesticides}$.

Sites ID	Temperature [°C]	Water level (cm)	DO [mg/L]	pH	Toxic Unit (TU_{max})	SPEAR
Agri 1	9.3	19	13.56	8.45	-2.5	0.65
Agri 2	5.6	19	12.7	8.07	-3.0	0.89
Agri 3	7.6	34	12.82	7.75	-2.8	0.58
Agri 4	5.3	24	11.92	7.54	-2.5	0.50
Agri 5	6.8	36	14.42	7.89	-2.0	0.40
Agri 6	5.8	10	13.93	8.61	-1.5	0.24
Agri 7	7.2	25	13	8.23	-3.0	0.63
Agri 8	6.4	30	15.88	8.34	-1.5	0.52
Non-Agri 1	7.1	12	7.59	7.77	-4.7	1.63
Non-Agri 2	7.1	20	11.7	7.89	-3.7	0.80
Non-Agri 3	4.5	18	12.66	7.88	-4.9	1.69
Non-Agri 4	6.9	18	12.11	8.1	-3.7	0.79

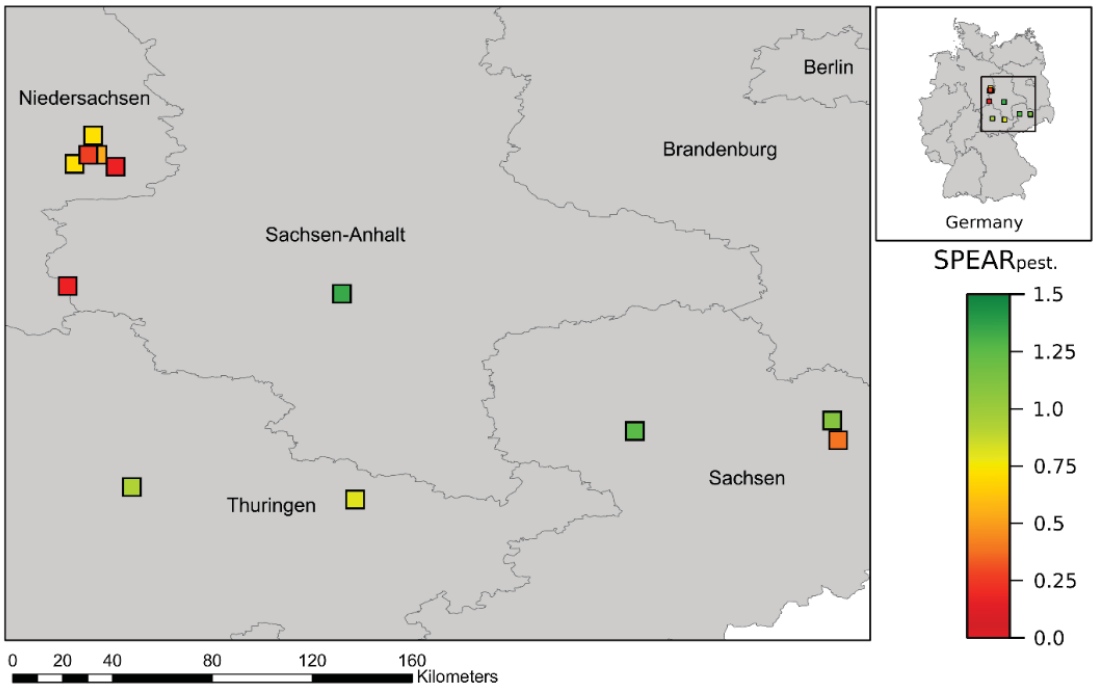

Figure D.1: Location of the sampling sites in central Germany that cover a wide range from non-contaminated to highly contaminated streams. Square shapes represent sampling sites and are coloured according to the $SPEAR_{pesticide}$ values ranging from good (green) to bad ecological status (red).

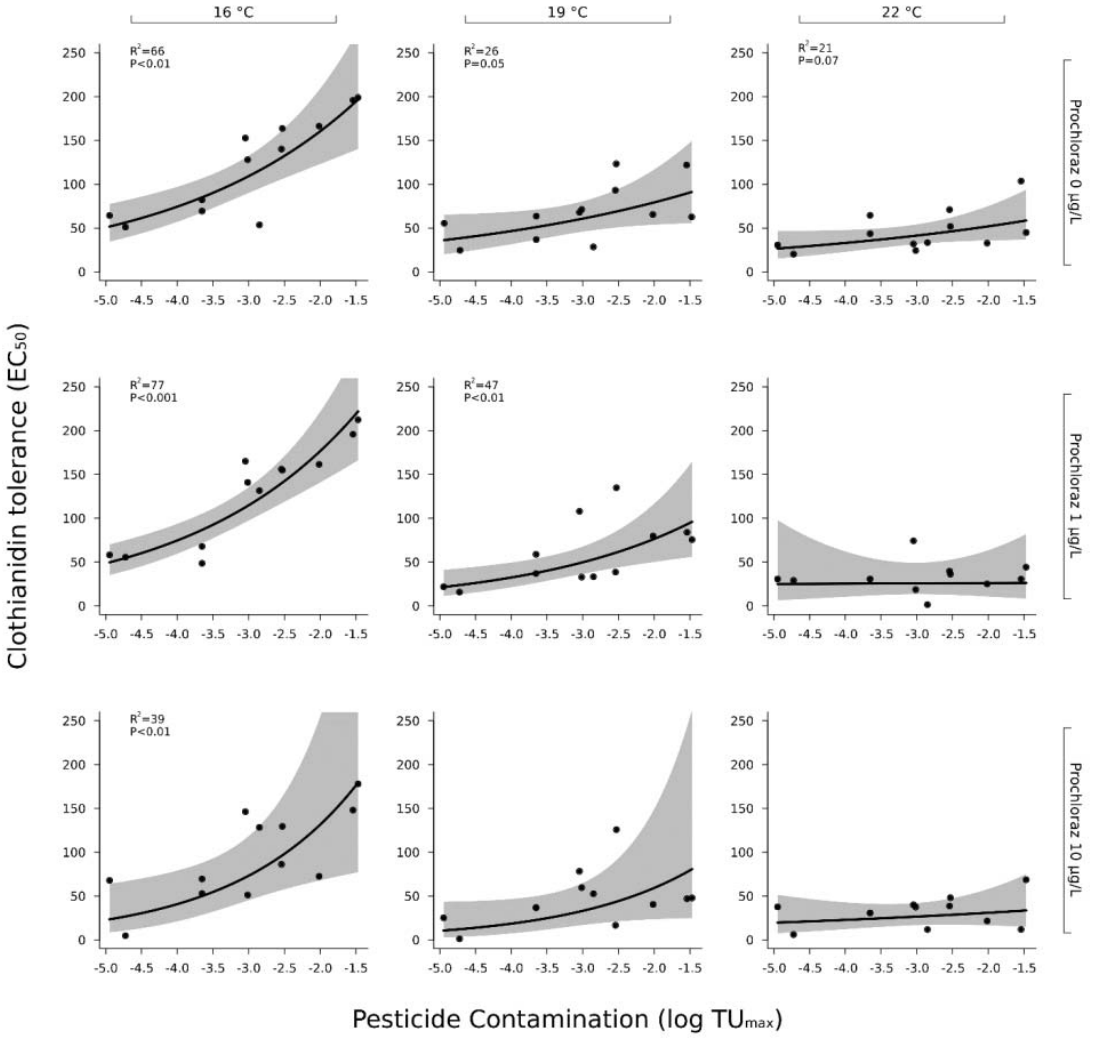

Figure D.2: Local pesticide contamination increases clothianidin tolerance of *Gammarus pulex* in small streams. Pesticide contamination is quantified as a decadic logarithm of the concentration of a compound measured in an event-driven water sample divided by its acute LC_{50} for a standard reference organism. Relationships are shown for clothianidin tolerance at different temperatures and prochloraz concentrations as additional stressors. The grey area corresponds to the 95% confidence interval.

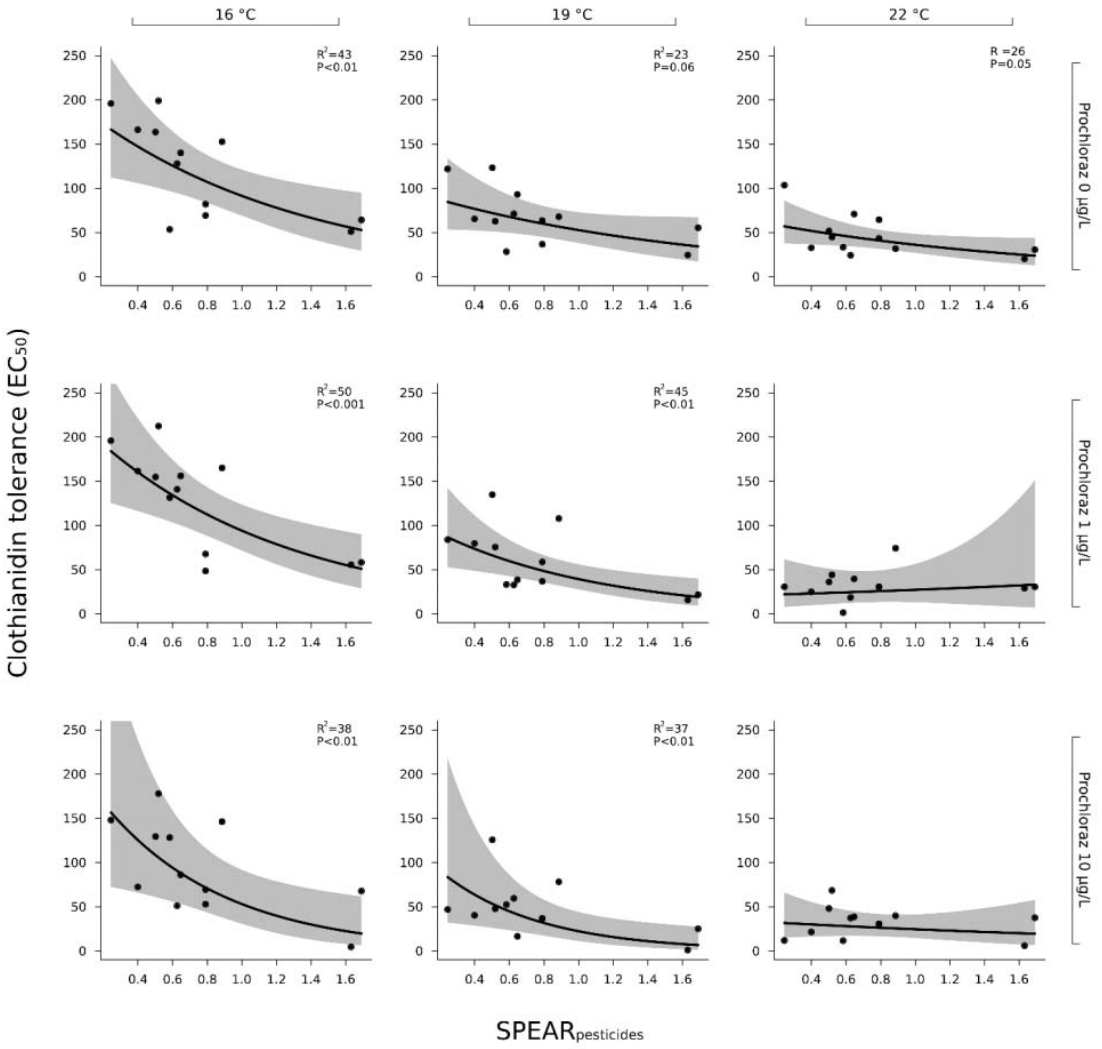

Figure D.3. Relationship between the median effect concentrations (48 h EC_{50}) of clothianidin for *G. pulex* and the community composition of macroinvertebrates expressed as $SPEAR_{pesticides}$. The grey areas correspond to the 95% confidence intervals.

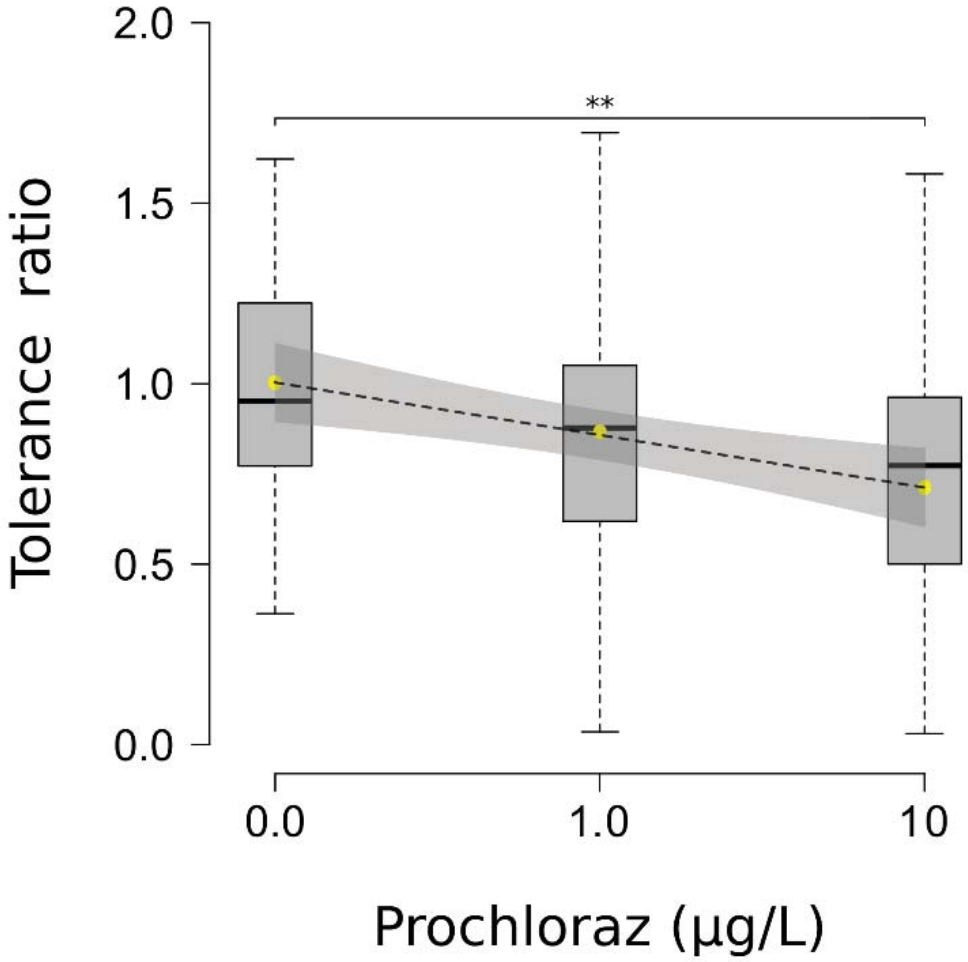

Figure D.4: Tolerance to clothianidin decreased with increasing concentration of prochloraz. The tolerance ratio was calculated by dividing LC_{50} of each population by the average LC_{50} of the respective controls without prochloraz. The lower and upper boundaries of the box represent 25th and 75th percentile, the horizontal line denotes the median, and the whiskers correspond to the lowest and highest values. Dashed lines represent fitted regression with confidence intervals displayed by shaded areas. The significance level is displayed as "**" for $p <$ 0.01.

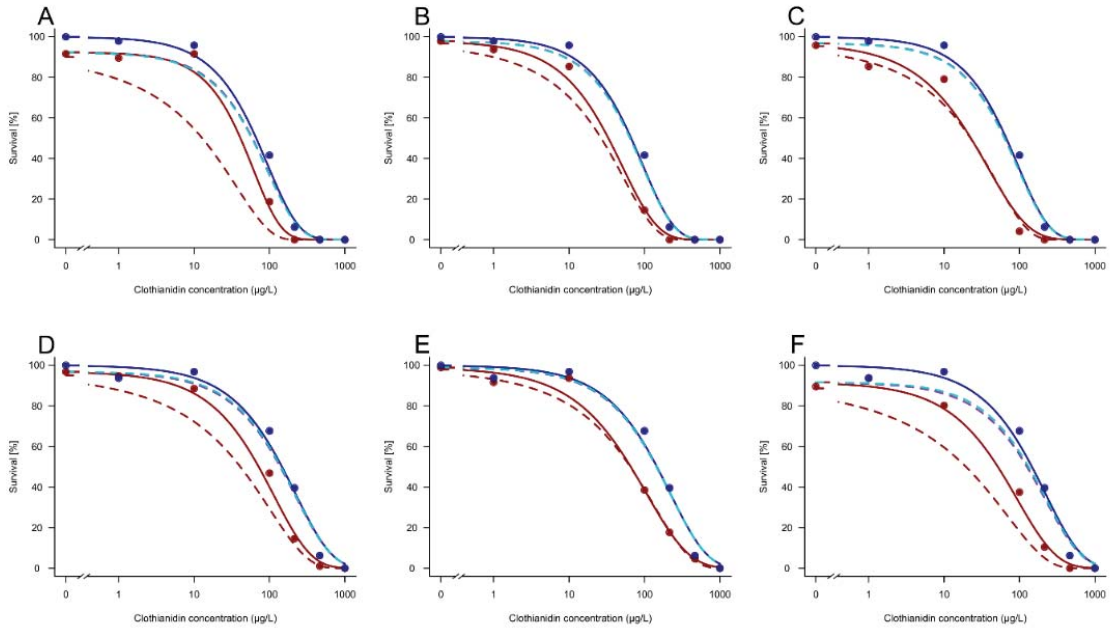

Figure D.5. Survival of *Gammarus pulex* exposed to a neonicotinoid insecticide clothianidin and an azole fungicide prochloraz at 19°C. Dose-response relationships are displayed for reference (A-C) and agricultural (D-F) populations - without additional stress at 16°C (blue points, solid line) and in combination with different prochloraz concentrations (A, D; 0µg/L, B, E; 1µg/L and C, F; 10µg/L) and elevated temperature (19°C) as additional stressors (red points, solid line). Data points represent the average survival of the populations from the respective group. The red dashed line represents the modelled concentration-response relationship under additional stress using the Stress Addition Model (SAM); whereas, violet and cyan dashed lines represent the EA and CA models respectively.

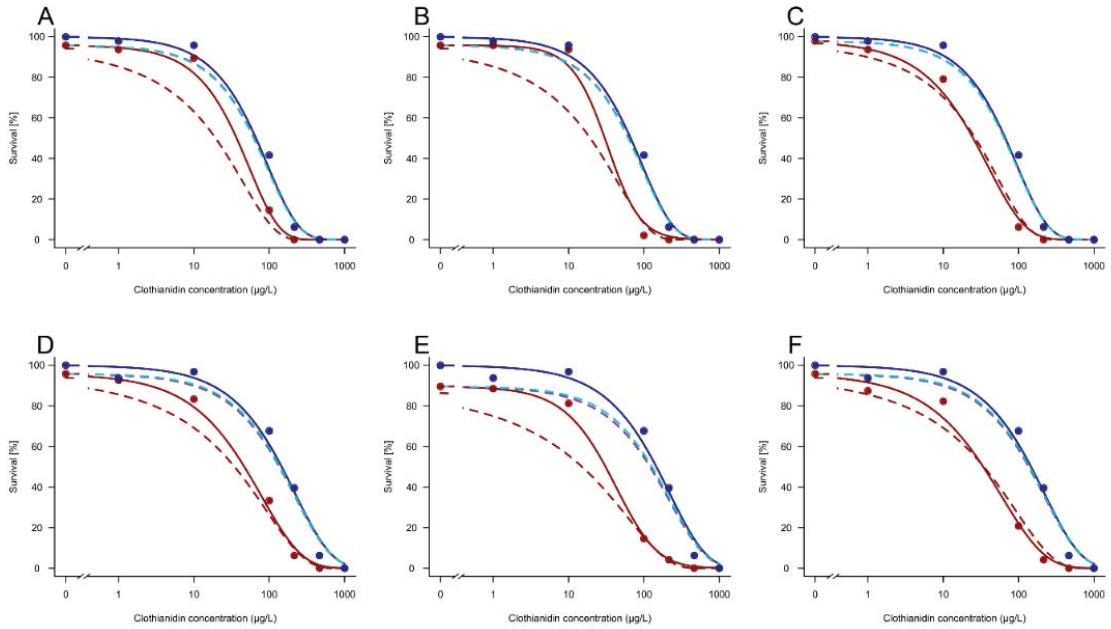

Figure D.6. Survival of *Gammarus pulex* exposed to a neonicotinoid insecticide clothianidin and an azole fungicide prochloraz at 22°C. Dose-response relationships are displayed for reference (A-C) and agricultural (D-F) populations - without additional stress at 16°C (blue points, solid line) and in combination with different prochloraz concentrations (A, D; 0µg/L, B, E; 1µg/L and C, F; 10µg/L) and elevated temperature (22°C) as additional stressors (red points, solid line). Data points represent the average survival of the populations from the respective group. The red dashed line represents the modelled concentration-response relationship under additional stress using the Stress Addition Model (SAM); whereas, violet and cyan dashed lines represent the EA and CA models respectively.

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Contributions to the published articles and chapters

Chapter 1 This chapter is written by Ayesha Siddique

Chapter 2 This chapter is based on the manuscript "Insecticides in agricultural streams exert pressure for adaptation but impair performance in *Gammarus pulex* at regulatory acceptable concentrations" published in Science of the Total Environment. Ayesha Siddique (>70%), designed the study, collected test organisms, conducted all the sensitivity tests and maintained the organisms in cultures for long-term data, analyzed the data and wrote first draft of manuscript. Matthias Liess (10%) contributed to study design and interpretation of results. Naeem Shahid (8%), contributed to data analysis and interpretation of results. Jeremias Martin Becker (12%) contributed to study design, data collection, analysis and interpretation. All authors read, improved and approved the final version of manuscript.

Chapter 3 This chapter is based on the manuscript "Multiple Stress Reduces the Advantage of Pesticide Adaptation" published in Environmental Science and Technology. Ayesha Siddique (>70%), designed the study, collected test organisms, conducted all lab experiments, analyzed the data and wrote first draft of manuscript. Naeem Shahid (10%), contributed to data analysis and interpretation of results. Matthias Liess (20%) contributed to study design, interpretation of results and supervised the study. All authors read, improved and approved the final version of manuscript.

Chapter 4 This chapter is based on the manuscript "Persistent pesticides effects favour single population dominance and reduces biodiversity of vulnerable species", Submitted. Ayesha Siddique (>70%), designed the study, collected test organisms, conducted all the lab experiments and data collection, analyzed the data and wrote first draft of manuscript. Matthias Liess (20%) contributed to study design, Extended the cognitive process of analysis, interpretation of results and supervised the study. Naeem Shahid (10%), contributed to data collection, data analysis and interpretation of results All authors read, improved and approved the final version of manuscript.

Chapter 5 This chapter is based on the manuscript "Predicting the combined effects of multiple stressors and individual stress adaptation", Submitted. Naeem Shahid (45%), Ayesha Siddique (35%), Matthias Liess (20%) conceived the research concept, contributed to interpretation of results. contributed to data analysis and interpretation of results. All authors read, improved and approved the final version of manuscript.

Chapter 6 This chapter is written by Ayesha Siddique.

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Publications

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2. **Siddique, A.**, Shahid, N., Liess, M., 2023. Persistent pesticides effects favour single population dominance and reduces biodiversity of vulnerable species. (Submitted).
3. **Siddique, A.**, Fatima. W., Shahid, N., 2023. Association of common BRCA1 variants with predisposition to breast tumors in Pakistan. Annals of Human Genetics. https://doi.org/10.1111/ahg.12511
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