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Assessing pesticide effects on macro-invertebrates under field relevant conditions

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Assessing pesticide effects on macroinvertebrates under field relevant conditions

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

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Aachen, November 09, 2020
Naeem Shahid

The thesis is based on the following scientific publications:

1. **Shahid, N.,** Becker, J. M., Krauss, M., Brack, W., and Liess, M. 2018. Adaptation of *Gammarus pulex* to agricultural insecticide contamination in streams. Science of the Total Environment, 621, 479-485. doi: https://doi.org/10.1016/j.scitotenv.2017.11.220

2. **Shahid, N.,** Becker, J. M., Krauss, M., Brack, W., Liess, M. 2018. Pesticide Body Burden of the Crustacean *Gammarus pulex* as a Measure of Toxic Pressure in Agricultural Streams. Environmental Science & Technology, 52(14), 7823-7832. doi: https://doi.org/10.1021/acs.est.8b01751

3. **Shahid, N.,** Liess, M., Knillmann, S., 2019. Environmental stress increases synergistic effects of pesticide mixtures on *Daphnia magna*. Environmental Science & Technology. doi: https://doi.org/10.1021/acs.est.9b04293

4. **Shahid, N.,** Rolle-Kampczyk, U., Siddique, A., Von Bergen, M., Liess, M. 2020. Pesticide-induced metabolic changes are amplified by environmental stress. Submitted to the Journal "Science of the Total Environment".

Abstract

Exposure to pesticides may affect non-target aquatic macroinvertebrates even below the regulatory acceptable concentrations. Similar low pesticide concentrations can force the organisms for adaptation. Aquatic organisms are often exposed to multiple stressors acting simultaneously or sequentially, including agrochemicals and suboptimal environmental conditions. However, a little is known about the pesticide effects on aquatic macroinvertebrates under field relevant conditions. In order to improve the existing risk assessment, this dissertation aims to assess important factors for pesticide effects in the field that are still not well understood. It contributes to the understanding of adaptation to pesticides, assessment of toxic pressure, interaction of mixtures, and the role of environmental stressors for the eco-toxicological effects of pesticides.

To identify environmental parameters that govern the development of increased pesticide tolerance, a field investigation was conducted **(Chapter 2)**. *Gammarus pulex* were collected from 15 sites within the central Germany that cover a wide range from un-contaminated to highly contaminated streams. Populations from contaminated streams showed almost 3-fold higher tolerance to the neonicotinoid insecticide clothianidin as compared to non-exposed populations. This tolerance of exposed populations increased from 2- to 4-fold with increasing distance to the next refuge area. Thus, distance from the refuge area and local toxic pressure were important factors that drive the development of pesticide resistance.

In the second investigation **(Chapter 3)**, pesticide body burden was applied to assess the pesticide exposure and potential effects in freshwater organisms. Body burdens of a crustacean *G. pulex* were converted into equivalent pesticide concentrations in the water, and linked with the observed ecological effects on freshwater macroinvertebrates. The toxic pressure derived from body burden was reliable to explain the effect on the macroinvertebrate community composition and the development of insecticide tolerance in *G. pulex*.

For better understanding of multiple stressors in the environment **(Chapter 4)**, interaction between food stress and a mixture of a pyrethroid esfenvalerate and prochloraz was investigated. To predict the joint effects of multiple stress, com-

monly applied models i.e. effect addition (EA), concentration addition (CA), and stress addition model (SAM) were compared. Results showed that the strength of interaction between esfenvalerate and prochloraz was increased with an increasing concentration of prochloraz. The combination of both pesticides and food stress caused highly synergistic effects even at 1 µg/L of prochloraz. Moreover, synergistic effects of pesticides and food stress were predicted best with the SAM model.

The fourth investigation contributed to understand the mechanisms behind delayed effects at very low pesticide exposure in the field **(Chapter 5)**. The metabolic response of *Daphnia magna* exposed to a pyrethroid esfenvalerate under suboptimal food supply was investigated. Metabolomic effects were observed at ultra-low concentrations, and were more pronounced under low food conditions. Interaction between food- and chemical stress was mainly responsible for extreme stress, and thereby strong down-regulation of different metabolites.

Zusammenfassung

Die Verunreinigung von Gewässern mit Pestiziden kann aquatische Makroinvertebraten unterhalb der gesetzlich zulässigen Konzentrationen beeinträchtigen. Zugleich können niedrige Pestizidkonzentrationen die Anpassung von Organismen forcieren. Aquatische Organismen sind oft mehreren Stressoren ausgesetzt, die gleichzeitig oder nacheinander wirken, darunter Agrochemikalien und suboptimale Umweltbedingungen. Über die Auswirkungen von Pestiziden auf aquatische Makroinvertebraten unter feldrelevanten Bedingungen ist bisher nur wenig bekannt. Um die bestehende Risikobewertung zu verbessern, sollen in dieser Dissertation bedeutsame Faktoren für Pestizidwirkungen im Feld bewertet werden, die noch nicht hinreichend verstanden wurden. Die Dissertation trägt außerdem zum Verständnis der Anpassung an Pestizide sowie der Bewertung des toxischen Drucks auf Markoinvertebratengemeinschaften bei. Sie erweitert die Erkenntnisse in Bezug auf Interaktionen in Mixturen und die Rolle von Umweltstressoren für die ökotoxikologischen Wirkungen von Pestiziden.

Um Umweltparameter zu identifizieren, die die Entwicklung der Pestizidtoleranz beeinflussen, wurde eine Felduntersuchung durchgeführt **(Kapitel 2)**. *Gammarus pulex* wurden an 15 Standorten in Mitteldeutschland gesammelt, welche ein breites Spektrum von unkontaminierten bis hochkontaminierten Flüssen abdeckten. Populationen aus kontaminierten Bächen zeigten im Vergleich zu nicht exponierten Populationen eine dreimal höhere Toleranz gegenüber dem Insektizid Clothianidin. Diese Toleranz stieg mit zunehmender Entfernung zum nächstgelegenen nicht kontaminierten Schutzgebiet vom 2- auf das 4-fache. Demnach wurden der lokale toxische Druck und die Entfernung vom Schutzgebiet als wichtige Faktoren, die die Entwicklung von Pestizidresistenzen förderten, identifiziert.

In der zweiten Untersuchung **(Kapitel 3)** wurde die Pestizid-Körperbelastung untersucht, um die Pestizid-Exposition und die möglichen Auswirkungen auf Süßwasserorganismen zu bewerten. Die Körperbelastungen des Krustentieres *G. pulex* wurden in äquivalente Pestizidkonzentrationen des Wassers umgerechnet und mit den beobachteten ökologischen Auswirkungen auf Süßwasser-Makroinvertebraten in Verbindung gebracht. Der von der Körperbelastung

abgeleitete toxische Druck war erwies sich als zuverlässiges Instrument, um die Pestizidwirkung auf die Zusammensetzung der Makroinvertebratengemeinschaft und die Entwicklung der Insektizidtoleranz bei *G. pulex* zu erklären.

Zum besseren Verständnis der Wirkung von multiplen Stressoren in der Umwelt **(Kapitel 4)** wurde die Wechselwirkung zwischen Nahrungsstress und einer Mischung aus einem Pyrethroid Esfenvalerat und Prochloraz untersucht. Zur Vorhersage der gemeinsamen Auswirkungen von multiplen Stressfaktoren wurden gängige Modelle, d.h. Effektaddition (EA), Konzentrationsaddition (CA) und Stressadditionsmodell (SAM), verglichen. Die Ergebnisse zeigten, dass die Stärke der Wechselwirkung zwischen Esfenvalerat und Prochloraz mit zunehmender Konzentration von Prochloraz zunahm. Die Kombination beider Pestizide mit zusätzlichem Nahrungsmittelstress verursachte selbst bei 1 µg/L Prochloraz stark synergistische Effekte. Darüber hinaus wurden die synergistischen Effekte von Pestiziden und Lebensmittelstress am besten mit dem SAM-Modell vorhergesagt.

Die vierte Untersuchung trug zum Verständnis der zugrundeliegenden Mechanismen von verzögerten Wirkungen bei sehr geringer Pestizidbelastung im Feld bei **(Kapitel 5)**. Die metabolische Reaktion von Daphnia magna, die bei suboptimaler Nahrungszufuhr einem Pyrethroid, Esfenvalerat, ausgesetzt war, wurde untersucht. Metabolischen Wirkungen wurden bereits bei extrem niedrigen Konzentrationen beobachtet und waren bei geringer Nahrungszufuhr noch stärker ausgeprägt. Hauptursächlich für extreme Stressbedingungen war die Wechselwirkung zwischen Nahrungsmangel und toxischem Druck, wodurch eine starke Reduktion verschiedener Metabolite verursacht wurde.

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List of Abbreviations

AChE	Acetylcholinesterase
ADaM	Artificial Daphnia medium
AOP	Adverse outcome pathways
CA	Concentration Addition
C_{ew}	Equilibrium water concentrations
$C_{w,eq}$	Estimated equilibrium water concentrations
DMSO	Dimethyl sulfoxide
DO	Dissolved oxygen
EA	Effect Addition
EU	European Union
EC	Electrical conductivity
ECx	Effective concentration
EDSs	Event-driven water samplers
GCA	Generalized concentration addition model
KOC	Soil organic carbon-water partition coefficient
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
nAChRs	Nicotinic acetylcholine receptors
NOEC	No observed-effect concentration
OECD	Organisation of economic cooperation and development
OMPs	Organic micro pollutants
POCIS	Polar organic chemical integrative samplers
PPDB	Pesticide properties database
PPPs	Plant protection products
RAC	Regulatory acceptable concentration
SAM	Stress addition model

SPEAR	Species at risk
SPMD	Semipermeable membrane devices
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TU	Toxic Unit
$\mathrm{TU}_{\mathrm{max}}$	Toxic unit value representing maximum toxicity
$\mathrm{TU}_{\mathrm{sum}}$	Toxic unit value representing the sum toxicity of chemicals
UVR	Ultraviolet radiation
WWTP	Waste water treatment plant

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Background

1.1 Use of pesticides

During the past 50 years, pesticides are extensively applied to enhance the crop production worldwide (Kohler and Triebskorn, 2013; Zhang et al., 2011). They are commonly used in agriculture to protect the crops from pests, weeds and plant diseases. Globally, about 4 million tons of chemical pesticides have been applied every year (FAO, 2019), of which only 1% is effective on their targets (Pimentel and Levitan, 1986) and the remaining 99% act as environmental pollutants (Pimentel, 1995; Zhang et al., 2011).

In 1940s, a revolution in the field of pesticides took place and chemical industry started producing synthetic compounds to control insects (insecticides), animal pests (rodenticides), unwanted weeds (herbicides), and fungal diseases (fungicides) (Casida and Quistad, 1998). Currently in modern agriculture, a wide range of plant protection products (PPPs) have been used frequently. However, the distribution of pesticide application throughout the world is quite uneven (Pimentel, 1996). On the basis of total pesticide consumption, the continent Asia holds a top position (53.2%) followed by America (29.4%) and Europe (14%) (Figure 1.1).

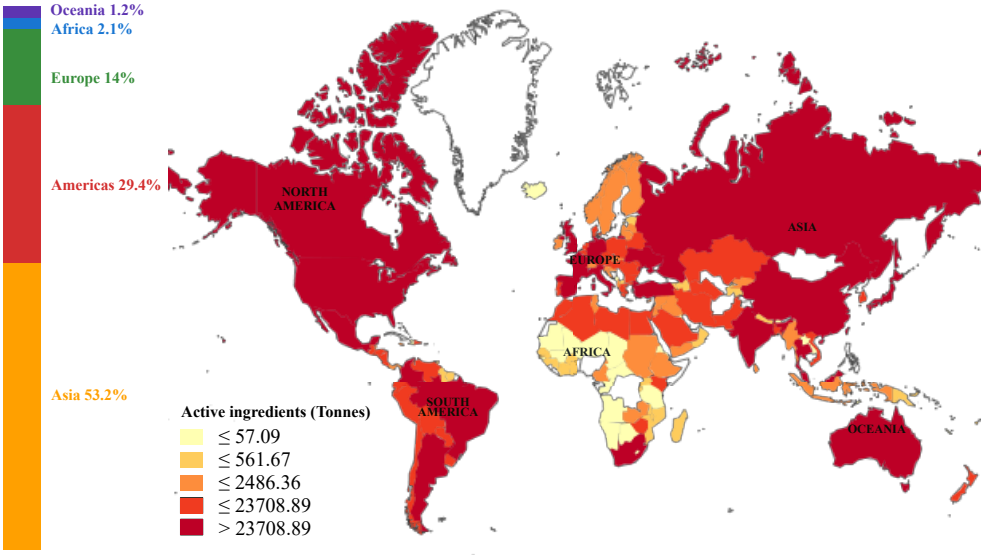

Figure 1.1: Average annual use of pesticides (1990-2016) in different countries is given in tones (http://www.fao.org/faostat/en/#data/RP/visualize) retrieved on: 21 January, 2020.

More than half of the land in Germany (51.1%) is occupied by agriculture, which is used for cultivation of crops or permanent grassland for meadows. In agriculture, farming methods are significantly important for the protection of environment. Although the Federal Government is trying to increase the share of organic farming up to 20%, so far it accounts only 7.5% of total agricultural land. The conventional farming is predominant form of agriculture and consumes a lot of active ingredients (Figure 1.2). For the last 20 years, the demand of pesticides has been dramatically increasing. In Europe, Germany is among the top five countries that are using massive amounts of pesticides. Germany, Italy, France, and Spain collectively shares over 50% of the total EU 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 various forms (e.g., liquid, solid and gaseous), and can be applied through a number of methods (spraying, with water or incorporated in soil) (Miller, 2002). The time of application and selection of pesticides is directly associated with crop type, crop stage, application method, intended target, chemical formulation of the product and weather conditions (Leonard, 1988). In the post-application scenario, distribution and fate of pesticides also depends on multiple factors including application method, physicochemical characteristics

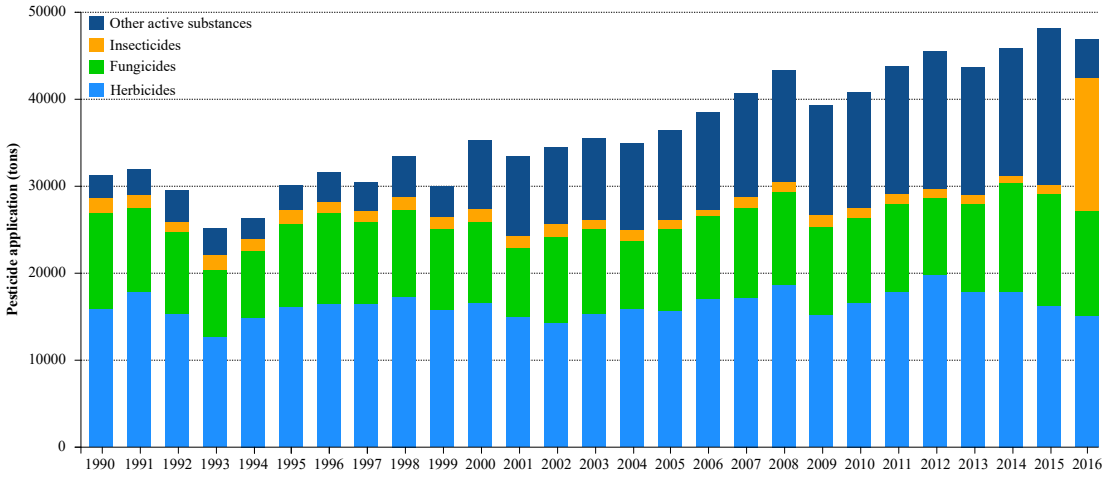

Figure 1.2: Annual use of plant protection products in Germany since 1990 until 2016 (http://www.fao.org/faostat/en/data/RP) retrieved on 21 January, 2020.

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 different biotic (microbial degradation) and abiotic (chemical degradation in soil, sorption, photo-degradation, hydrolysis) distribution 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. Different transport pathways and fates of pesticides are shown in Figure 1.3.

Method of application is not only important for the efficiency of pesticides (Van Timmeren et al., 2011) but it also affect the fate and potential water pollution (Davis et al., 1996). Briefly, for soil incorporated pesticides, loss of pesticides occurs mainly through microbial transformation instead of volatilization and surface runoff. In contrast, for pesticides sprayed directly on crops or bare soils, volatilization and field runoff events mainly contribute to the pesticide loss (Davis et al., 1996; Kronvang et al., 2004). Similarly, chemical properties of pesticides influence their reactivities (Chaplain et al., 2011). 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 easily disappear in the atmosphere after application. Sorption is another major process that determines 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, degradation of pesticides involves both chemical and biological processes, i.e. biogeochemical cycles.

Plant protection products enter into surface waters via point and non-point (diffuse) sources. The point source pathways include farmyard runoff, wastewater treatment plant effluents, and spillages whereas diffuse pesticide input comes from air-borne (i.e., spray drift, volatilization, and atmospheric deposition) and water-driven transport processes (i.e., surface runoff, drain flow, leaching through the soil, and groundwater discharge). After entering into a water course, behavior and fate of a pesticide is driven by its chemical properties (e.g., water solubility and persistence) and the characteristics of the stream (e.g., current velocity, concentration of suspended particles, and constituents).

Although 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 that comprises almost 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 im-

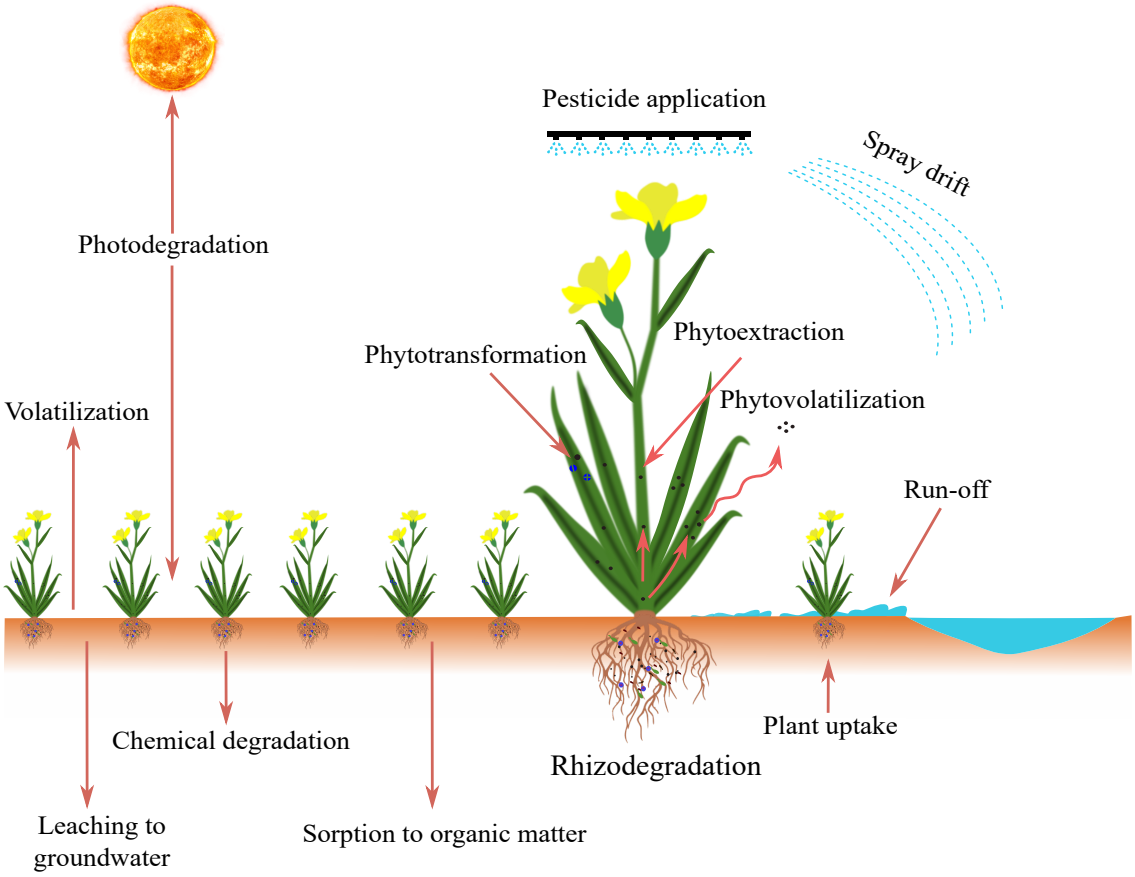

Figure 1.3: Fate and transport of pesticides: processes responsible for the fate of applied pesticides in the environment.

portant 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 andother organic micropollutants (OMPs) (Schwarzenbach et al., 2006). In agricultural areas, pesticide contamination is a well-known stressor to 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 considerably affects the species composition of freshwater macroinvertebrates (Beketov et al., 2013; Shahid et al., 2018a). For example, 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 ($\mathrm{TU}_{\mathrm{max}} \leq$–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. In agricultural landscapes, pesticides are mainly responsible for the decline in invertebrate populations (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 pesticide exposure

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 and Schulz, 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; Shahid et al., 2018a).

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). Although, ceramic dosimeter, Semipermeable membrane devices (SPMD), Chemcatchers and Polar organic chemical integrative samplers (POCIS) are commonly used passive samplers for monitoring of organic compounds (Escher et al., 2006; Huckins et al., 2006; Thomatou et al., 2011), the use of body burden provides a low-cost alternative approach for quantification of pesticide exposure in the field.

Pesticide concentrations in water bodies can erratically change over time, while the internal body burden of exposed organisms provides a better time-integrated measure of the pesticide exposure and adverse effects in aquatic ecosystems. Due to the lipophilic nature of most pesticides, organisms can accumulate these compounds from the water phase and can serve as biological passive-samplers (Shahid et al., 2018b). Additionally, pesticide effects can be better related to internal concentrations (at the site of action) within an organism, as compared to external concentrations detected in water, because the intake is affected by various behavioral and physico-chemical processes (Escher and Hermens, 2002).

1.4 Biological monitoring of pesticide effects

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 more 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., 2018a), hydromorphological 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 Ohe (2005) developed a trait-based approach $\mathrm{SPEAR}_{\mathrm{pesticides}}$ (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).

$$SPEAR_{pesticides} = \frac{\sum_{i=1}^{n} log(x_i + 1)y}{\sum_{i=1}^{n} log(x_i + 1)} \times 100 \tag{1.1}$$

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.

1.5 Resistance development

Pesticides can have extensive lethal and non-lethal impacts on non-target aquatic species, including development of pesticide tolerance (Landis et al., 2003; Liess

and Schulz, 1999). Tolerance is the ability of an organism to cope with a stress resulting from unwanted natural or anthropogenic environmental changes such as chemicals' inputs and/or physicochemical variations like change in temperature, food availability, pH and dissolved oxygen etc. Organisms adapt different strategies to cope with environmental pollution such as exclusion, removal and detoxification of toxicants followed by excretion, and repair of damages caused by contaminants. Increased tolerance has been reported in several species including bacteria, fungi, phytoplankton, terrestrial plants, macroinvertebrates and vertebrates like amphibians. Increased tolerance can be due to physiological acclimation (temporary) or genetic adaptation (long-term).

Some less sensitive species may tolerate a contaminant through physiological acclimation mechanisms. During pesticide exposure, many types of contaminants can induce detoxification processes that may result in increased tolerance (physiological acclimation) (Di Giulio et al., 1995). Eventually, pre-exposed organisms become less sensitive to the future exposures of the contaminants. However, after release from pesticide exposure, populations may lose the acquired tolerance. Such kind of non-genetic changes are present especially under irregular pesticide exposure. For instance, Hua et al. (2013) exposed wood frogs to sub-lethal concentrations of an insecticide carbaryl and found induced tolerance to subsequent lethal exposures in the same generation. Similarly, Poupardin et al. (2008) exposed mosquito larvae to sub-lethal concentrations of temephos and permethrin insecticides, and found increased tolerance in pre-exposed larvae during subsequent insecticide exposures. In both cases, the induced higher tolerance was most likely due to inconsistent and sub-lethal pesticide exposure. In contrast, the consistent pesticide exposure over multiple generations leads to prevail genetic adaptation through natural selection (Lopes et al., 2008).

Different molecular mechanisms related to genetics, epigenetics and maternal transfers can explain the increased tolerance in offspring. Overall, genetic resistance occurs more often and has been reported in several pests (Bass et al., 2011; Karunker et al., 2008; Yang et al., 2013) and non-target aquatic species (Shahid et al., 2018a; Weston et al., 2013). Genetic adaptation can be lost in the absence of toxic pressure of the contaminant, again by natural selection. Presence of genetically adapted population is a direct evidence of the ecotoxicologically relevant contamination in the local environment. Although, adapted populations can reduce the effects of pesticide induced trophic cascades (Bendis and Relyea, 2016), the increased tolerance can be associated with fitness costs (Siddique et al., 2020), and/or reduced genetic diversity (Agra et al., 2011; Xie and Klerks, 2004). Generally organisms respond to stress by allocating a larger portion of energy to maintain regular metabolism, or to activate the defense mechanisms (Spann et al., 2011), which consequently may lead to impaired growth and reproduction (Connon et al., 2008; Spann et al., 2011). Similarly, the production of mucus in amphibians can reduce uptake of contaminants but it requires a substantial

metabolic cost and leads to less growth of individuals (Calow, 1991; Forbes and Calow, 1996). Depending on the type of exposure, organisms may experience trade-off costs (in terms of survival, growth and reproduction) for a short time or during their entire life; and thus can affect the population dynamics in the field (Forbes and Calow, 1996; Sulmon et al., 2015). Additionally, evolution of genetic adaptation against specific stressors can reduce overall genetic variation that results in maladaptation to other stressors. Hence, adaptation may have negative effects in long run. For example, numerous laboratory investigations with fish lines have reported that the increased tolerance to chemicals has a higher cost, as higher sensitivity to other environmental stressors, such as ultraviolet radiations (UVB), elevated temperature and natural hypoxia (Meyer and Di Giulio, 2003; Xie and Klerks, 2004).

There are several other environmental factors that may affect the development of tolerance. For instance, Becker and Liess (2017) reported that the species diversity hinders adaptation to pesticides in *Gammarus pulex*. Similarly, Shahid et al. (2018a) 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 alter the evolution of resistance (Becker and Liess, 2017; Becker et al., 2020).

1.6 Pesticides mixtures and risk assessment

Concurrent- and sequential application of pesticides is a very common practice in agriculture. Several cereal crops such as corn and wheat are sequentially treated with different types of pesticides (Baghestani et al., 2008; Garthwaite et al., 2015). Vegetables, fruits and cut-flowers receive even higher pesticide concentrations and a wide variety of active ingredients (Garthwaite et al., 2015). Intermingling crops also increase the potential pesticide mixing in the field. In agriculture, where fruits are cultivated as major products, several other crops may be planted in a watershed, each with different requirement of pesticides that eventually cause contamination with pesticide mixtures. Additionally, several pesticides are often applied together in the form of tank mixtures. After rainfall events, these pesticides enter freshwater through runoff and co-occur in aquatic environments (Martin et al., 2003; Riise et al., 2004; Werner et al., 2004). Several authors have reported multiple pesticide residues including insecticides, fungicides, herbicides and pharmaceuticals in aquatic environment (Inostroza et al., 2016; Münze et al., 2017; Shahid et al., 2018a).

In freshwater ecosystems, organisms are often exposed to a multitude of chemical mixtures; therefore, the toxic effects are highly complex, difficult to quantify, and is a key challenge for ecologists (Laetz et al., 2009). Broadly, pesticides can be clas-

sified on the basis of targets (i.e., insecticides, fungicides and herbicides), chemical structure and mode of action (Casida, 2009). The mode of action is defined as a process of pesticide interaction with a specific target site in an organism to cause potential effects. For example, organophosphates insecticides such as chlorpyrifos, temephos, malathion, dimethoate and diazinon contain phosphorus, and inhibit the enzyme acetylcholinesterase (AChE), which is a class of enzymes that hydrolyze the neurotransmitting agent acetylcholine (Carlock et al., 1999; Fukuto, 1990). Inhibition of AChE causes acetylcholine accumulation, that overstimulates neurological activity and potentially affect the survival and fitness of the exposed individuals (Beauvais et al., 2000; Sismeiro-Vivas et al., 2007). Carbamates such as alanycarb, carbofuran and carbaryl also inhibit AChE, enzymes like organophosphates (Fukuto, 1990). Although, pyrethroids such as esfenvalerate, deltamethrin and cypermethrin also cause neurological damages, but their target site is different (Leahey, 1985). Voltage-dependent sodium channels are primary targets of synthetic pyrethroids (Casida, 2009; Fukuto, 1990). In contrast, neonicotinoids are systemic compounds, widely applied to protect the crops from pest insects (Nauen and Denholm, 2005). This class of insecticides is well known substitute of carbamate and organophosphate insecticides, registered in over 120 different countries (Morrissey et al., 2015). The neonicotinoid insecticides interfere with nervous system; specifically act agonistically on nicotinic acetylcholine receptors (nAChRs) (Casida and Durkin, 2013; Jeschke et al., 2010). These receptors are 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., 2015).

Pesticide mixtures can cause synergistic effects especially if they include substances with different mode of actions. Well studied pesticides that cause synergistic effects in mixtures include for example Lambdacyhalothrin and Propiconazol (Pilling and Jepson, 1993), Chlorpyrifos and Imidacloprid (Svendsen et al., 2010), Atrazin and organophosphate Methylparathion (Anderson and Lydy, 2002), Atrazin and Chlorpyrifos (Anderson and Lydy, 2002), Alfa-cypermethrin and Epoxiconazol (Nørgaard and Cedergreen, 2010), and several other contaminants reviewed by Cedergreen (2014). 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 (Warne and Hawker, 1995). While pesticide mixtures in the field may contain several toxicants including insecticides, fungicides and herbicides (Münze et al., 2017; Shahid et al., 2018b) and are rarely equitoxic. Thus, pronounced interactions might be even more likely to occur in real ecosystems when few toxicants dominate the overall toxicity (Alabaster and Lloyd, 1980; Cedergreen, 2014).

Existing field studies in small freshwater streams show that pesticide effects on macroinvertebrate communities are significantly related to the most toxic pesti-

cide concentrations (maximum toxic unit - $\mathrm{TU_{max}}$) and the sum of toxicities (sum of toxic unit - $\mathrm{TU_{sum}}$) from all pesticides did not increase the explained variance of biological effects based on the assumption of additivity (Schäfer et al., 2012; Shahid et al., 2018a). In addition, these studies report significant change in macroinvertebrate community composition at concentrations three to four orders of magnitude below the acute $\mathrm{LC_{50}}$. Hence, reasons for these low effect concentrations and the unexplained variance of field effects on non-target invertebrates include an increased sensitivity of individuals to pesticides under multiple stress conditions (Liess et al., 2016), the effect culmination of sequential pesticide exposures (Liess et al., 2013) and the underestimation of exposure (Schäfer et al., 2012; Xing et al., 2013). Multiple stress conditions might also include synergistic mixtures, combined effects of chemical and environmental stressors (Liess et al., 2016) as well as the combination of both.

Generally, the traditional methods for risk assessment of chemical mixtures rely on the toxicity of individual chemicals and predict the overall mixture-toxicity. However, in case of interactions, the combined effects of pesticide mixtures could be significantly underestimated (Chen et al., 2015; Shahid et al., 2019). The risk assessments based on such inaccurate effect-predictions are not protective for the environment. Therefore, accurate risk assessment of pesticide mixtures is vital for the protection of non-target aquatic organisms.

Experimentally, it is not possible to consider all potential mixtures. However, existing approaches on mixture toxicity may help the prediction of combined effects of chemical mixtures from the toxicity data of a single substance. In this regard, two reference models namely 'effect addition' (EA, Bliss (1939); Eq. 1.2) and 'concentration addition' (CA, Loewe and Muischnek (1926); Eq. 1.3) are commonly applied for prediction of the combined toxicity effects, and usually CA is considered as the most conservative approach (Belden et al., 2007; Bjergager et al., 2017). It is assumed that the chemicals with similar mode of action can be predicted using the concept concentration addition, whereas dissimilarly acting chemicals can be predicted by effect addition or independent action. However, there is no model to quantify the synergy of pesticide mixtures.

$$E(C_{mix}) = 1 - \prod_{i=1}^{n}(1 - E(Ci)) \tag{1.2}$$

where $\mathrm{E(C_{mix})}$ is the total effect of all stressors $\mathrm{E(C_i)}$. According to the concept of CA, prediction is based on the following equation (Eq. 1.3):

$$ECx_{mix} = \sum_{i=1}^{n} \frac{p_i}{ECx_i} \tag{1.3}$$

where $\mathrm{ECx_{mix}}$ is the total concentration of the mixture, *pi* indicates the proportion

of component *i* in mixture, and $\mathrm{EC_i}$ is the concentration of component *i* producing effect.

In the last decade, additional toxicological approaches such as Generalized Concentration Addition model (GCA) (Schafer and Piggott, 2018; Tanaka and Tada, 2017) and Stress Addition Model (SAM) (Liess et al., 2016) have been developed. The GCA model extends underlying concepts of CA and can predict the cumulative response curve that is independent of the response functions for each component individually. In contrast, the SAM is an appropriate approach to quantitatively predict the synergistic effects of different stressor combinations like contaminants and environmental stressors. Up until now, SAM is the only approach that considers sensitivity distribution within a population, and predicts a certain range of effects for chemicals under different levels of environmental stress.

1.7 Environmental stressors

In natural ecosystems, organisms commonly experience sub-optimal conditions and have to cope with environmental stress (Holmstrup et al., 2010). Generally, sub-optimal environmental conditions can be due to physical, chemical and biological stressors (Lydy et al., 2004). Different stressors including agrochemicals, elevated temperatures, salinity and food limitation may interact in a variety of ways (Dinh et al., 2016; Heugens et al., 2001; Pieters et al., 2005). For example, environmental parameters such as pH, temperature, or ultraviolet radiation (UVR) and food stress may strongly affect the bioavailability of toxicants, toxicokinetics and physiological state of an organism (Clements et al., 2008; Franklin et al., 2000; Kashian et al., 2004). Pettis et al. (2012) reported increased mortality of wild honey bee colonies as a result of interactions between pesticides and environmental stressors, such as pathogens. In another investigation, pathogens increased the effect of agrochemicals on amphibians (Rohr et al., 2008). Similarly, environmental stress from the food limitation and UV-B radiations increased the sensitivity of marine crustaceans to copper (Liess et al., 2001). Several other environmental stressors such as high temperature, salinity, and eutrophication are also known to interact with agrochemicals and increase the toxic effects of contaminants (Beermann et al., 2018; Matthaei et al., 2010). Recently, Delnat et al. (2019) investigated the effect of daily temperature variation on the combined toxicity of an organophosphate chlorpyrifos and a biopesticide *Bacillus thuringiensis* var towards vector mosquito *Culex pipiens*. A high variation in daily temperature changed the interaction between both pesticides from additive to synergistic. Although the so-called mixture-toxicity has been discussed for a long time, a little effort was undertaken to understand the interactive effects of multiple stressors that is of great interest for the management of aquatic ecosystems.

1.8 Metabolomic responses

Frequently occurring negative effects of pesticides in the field indicate that the current risk assessments of pesticides are failing to derive protective thresholds of risk. Current guidelines for regulatory toxicology are actually based on the phenotypical end points such as survival, mobility, reproduction and behavioral responses (Ankley et al., 2007; De Coen and Janssen, 2003). However, these standard toxicity experiments are unable to represent a realistic environmental exposure scenario and therefore are failing to derive enough protective regulatory acceptable concentrations for ecological effects. Hence, new advances in ecotoxicology are needed to address open questions such as low-dose effects. Over the last two decades, several biochemical biomarkers including metabolomics have been developed for analyzing sub-lethal effects of environmental stressors (Jemec et al., 2010), yet their application in regulatory practices is limited (van Ravenzwaay et al., 2014).

Metabolomics is one of the high-throughput omics techniques that has been widely employed to understand the organismal responses to stress conditions. Numerous scientific studies have looked at the metabolic responses to chemical and physiological stresses (Bundy et al., 2008; Viant et al., 2003). Moreover, metabolomics has been applied for a limited number of regulatory purposes such as chemical grouping based on mode of action (MOA) and characterizing the adverse outcome pathways (AOP) (Tralau et al., 2015; Viant et al., 2019).

Metabolomics technique is being applied in both lab investigations (mostly for toxicity testing) and field investigations (mostly for environmental monitoring). Several investigations have reported detrimental effects of pesticides and other toxic compounds on the aquatic organisms (Jones et al., 2012; Maity et al., 2012; Nagato et al., 2013), and environmental stressors (Garreta-Lara et al., 2018; Kullgren et al., 2013; Warne et al., 2001). Although, metabolomic techniques play significant role in risk assessment, its application in regulatory toxicology is currently under debate (Ankley et al., 2007; Viant et al., 2019).

1.9 Objectives and outline of the thesis

In agricultural landscapes, abundance of sensitive species is decreasing drastically, whereas some species are reported to undergo adaptation. Furthermore, impact of insecticides may be altered in the form of complex mixtures with different types of pesticides and environmental stressors. Therefore, the goal of this doctoral research was to assess important factors for pesticide effects in the field that are still not well understood. Objectives of the study were addressed through

a series of activities, including field research, microcosm and laboratory studies. More specifically, this study involved activities to answer the following settled up research questions:

1. What environmental factors are involved in the development of pesticide tolerance?
2. How the pesticide body burden can be used to quantify the pesticide exposure and potential effects in aquatic organisms?
3. How environmental stressors increase the combined effect of mixture toxicity?
4. What are the underlying mechanisms of pesticide effects at ultra-low concentrations in the field?

In order to achieve these objectives, first of all, changes in community structure and resistance development in non-target invertebrates to pesticides were assessed experimentally using a systemic insecticide clothianidin and widely distributed crustacean *Gammarus pulex* (CHAPTER 2). Additionally, linear solvation energy relationship (LSER) was applied to derive equivalent pesticide concentrations in stream water from the body burden of gammarids collected from streams; and results were compared with water samples and ecological effects of pesticides in terms of $\mathrm{SPEAR}_{\mathrm{pesticides}}$ (CHAPTER 3). Further investigations focused on the mixture toxicity under different food conditions (i.e. high and low) and prediction of combined effects using traditional approaches for toxicant mixtures (i.e. concentration addition, and effect addition) and stress addition model (SAM) (CHAPTER 4). Since, pesticides may have potential effects on the non-target organisms even at 1/ 1000 of the acute LC_{50}, the underlying mechanism was studied through a metabolomic investigation (CHAPTER 5). From this dissertation, CHAPTER 2 was published in the journal Science of the Total Environment, CHAPTER 3 and CHAPTER 4 were published in Environmental Science and Technology and CHAPTER 5 will be submitted to Nature communications. Finally, a brief synthesis of obtained results, conclusions and outlook for future research are given in CHAPTER 6.

2

Adaptation of *Gammarus pulex* to insecticides

2.1 Abstract

Exposure to pesticides affects non-target aquatic communities, with substantial consequences on ecosystem services. Adaptation of exposed populations may reduce the effects of pesticides. However, it is not known under which conditions adaptation occurs when only a low toxic pressure from pesticides is present. Here, we show that *Gammarus pulex*, a dominant macroinvertebrate species in many agricultural streams, acquires increased tolerance to pesticides when recolonization from non-contaminated refuge areas is low. Populations in the field that were exposed to pesticides at concentrations several orders of magnitude below considerable acute effects showed almost 3-fold higher tolerance to the neonicotinoid insecticide clothianidin (mean EC_{50} 218 µg/L) compared with non-exposed populations (mean EC_{50} 81 µg/L). This tolerance of exposed populations increased from 2- to 4-fold with increasing distance to the next refuge area (0 to 10 km). We conclude that the development of tolerance for non-target species may occur at very low concentrations, much below those affecting sensitive test organisms and also lower than those predicted to be safe by governmental risk assessment frameworks.

2.2 Introduction

Exposure to pesticides may affect the structure and function of freshwater non-target communities (Liess and Von der Ohe, 2005; Münze et al., 2017). Beketov et al. (2013) reported that pesticide pollution has significant effects on the species and family richness of macroinvertebrates in Australia and Europe, with losses of approximately one third of the taxonomic pools.

The repeated occurrence of toxic pressure may result in the weakening of exposed individuals (Russo et al., 2018), but also in the acquisition of increased tolerance towards pesticides by physiological acclimation or genetic adaptation (Becker and Liess, Becker and Liess, 2017; Klerks and Weis, 1987; Vigneron et al., 2015; Weston et al., 2013). Although the principles of adaptation to various pesticides are well-known, the roles of the magnitude of the toxic pressure and the prevailing environmental factors in the development of tolerance are still under debate. Developing a greater understanding of the relationship between environmental factors and tolerance to insecticides is of high relevance for the management of non-target species because the development of tolerance may have significant implications for ecology and conservation (Hua et al., 2013). For example, pesticides can decrease genetic variation at the population level (Bijlsma and Loeschcke, 2012), which may reduce the ability to adapt to upcoming environmental changes (Bach and Dahllöf, 2012; McMillan et al., 2006). However, resistant non-target populations can minimize the effects of pesticide-induced trophic cascades (Bendis and Relyea, 2016).

In addition to the local toxic pressure, non-contaminated refuge area is an important factor that drives the development of pesticide resistance. Recolonization of sensitive individuals from refuges can partially compensate the selection for pesticide resistance in agricultural fields (Gassmann et al., 2009). In the same way, the recolonization of sensitive species from upstream refuges can partially compensate the effects of pesticides on the macroinvertebrate community at downstream sections (Bunzel et al., 2014; Orlinskiy et al., 2015; Von der Ohe and Goedkoop, 2013). However, the impact of refuges on the resistance development in non-target species is still unclear, in spite of the relevance for the risk assessment of pesticides and the planning of mitigation measures.

The aim of this investigation is to reveal the extent to which low pesticide contamination induces adaptation in aquatic non-target species. Additionally, we aim to assess the environmental parameters that govern the development of increased pesticide tolerance. For this purpose, we selected *Gammarus pulex* (Linnaeus, 1758), a benthic macroinvertebrate as test organism because of its ecological relevance in aquatic ecosystems. *Gammarus pulex* is one of the most common freshwater macroinvertebrates and widely distributed in Europe. It plays

a central role in the degradation of organic matter (Foucreau et al., 2013; Maltby et al., 2002; Mora Gómez, 2014) and constitutes an imperative element in the food web (Macneil et al., 1999). Numerous investigations in the laboratory have reported detrimental effects of pesticides on reproduction, feeding behavior and survival in *Gammarus pulex* and related crustaceans that results in reduced leaf litter degradation (Agatz et al., 2014; Baudy et al., 2017; Cold and Forbes, 2004; Nyman et al., 2013). *Gammarus pulex* is sensitive to a wide range of chemicals and has been frequently used for risk assessment (Adam et al., 2009; Agatz et al., 2014; Maltby et al., 2002; Vigneron et al., 2015), However, field studies suggested that the species is able to recover well from pesticide exposure through reproduction and recolonization, and therefore considered it not at high risk (Liess and Von der Ohe, 2005; Rasmussen et al., 2012; Schäfer et al., 2012).

2.3 Materials and methods

2.3.1 Description of investigated streams

In total, 15 sites were investigated in 2015 and 2016 within central Germany that cover a wide range from uncontaminated to highly contaminated streams (Figure A1). In order to exclude the contaminants other than pesticides, it was ensured that the investigated sites had no wastewater treatment plants, industrial facilities, or mining drainage upstream. Sampling sites were characterized by soft- and hard-bottom substrates in different proportions. Major crops in the study area were wheat, rapeseed, sugar beets, corn and barley. Of the 15 sites, six were located in less contaminated forested areas and used as control sites. Populations from forested streams are generally not contaminated in Germany with the exception of rare accidents (Zwick, 1992). In contrast, streams with an agricultural catchment were not protected from pesticide contamination and most likely to experience higher pesticide contents.

During the sampling, parameters such as the water level, electrical conductivity (EC), pH, temperature and dissolved oxygen level (DO) were measured. Additionally, undisturbed forested stream sections that may serve as refuge areas were identified. The distance to the closest undisturbed forested stream section was measured using Google Maps. We considered both upstream and downstream refuge sections because *Gammarus pulex* can migrate in both directions.

2.3.2 Characterization of pesticide contamination

Sampling

Water samples were collected from all the selected sites using event-driven water samplers (EDSs) Liess and Von der Ohe (2005) in summer 2016, during the period of pesticide application. For this purpose, two glass bottles were installed at heights of 5 and 15 cm from the level of the stream water to collect rainfall-induced short-term maximum pesticide contamination. The bottles were collected within 24 hours after rainfall events and transported to the laboratory at 4°C. After settlement of the particles, 1 mL aliquots from the top 1 cm of the bottle were extracted and placed into 2 mL autosampler vials and then stored at –20°C until analysis.

Chemicals and reagents

For the liquid chromatography–high-resolution mass spectrometry (LC-HRMS) analyses, we used methanol, water and formic acid of LC-MS grade (Chromasolv, Sigma-Aldrich, Germany). Stock solutions of the target analytes were prepared in methanol at 1 mg/mL and stored in amber glass vials (20 mL) at –20°C in the dark. Mixed solutions of 10 µg/mL were prepared in methanol and used for identification and calibration.

LC-HRMS target screening

The water sample aliquots (1 mL in 2 mL autosampler vials) received 25 µL of an internal standard solution (40 ng/mL of isotope-labeled compounds in methanol), 25 µL of methanol and 10 µL of a 2 M NH_4-formate buffer (pH 3.5).

For the analysis, a Thermo Ultimate 3000 LC system (consisting of a ternary pump, auto sampler and column oven) was coupled to a quadrupole-orbitrap instrument (Thermo QExactive Plus) via a heated electrospray ionization (ESI) source. LC separation was performed on a Kinetex C18 EVO column (50×2.1mm, 2.6 µm particle size) using a gradient elution with 0.1% of formic acid (eluent A) and methanol containing 0.1% formic acid (eluent B) at a flow rate of 300 µL/min. After 1 min of 5% B, the fraction of B was linearly increased to 100% within 12 min, and 100% B was maintained for 11 min. The eluent flow was diverted to waste, and the column was rinsed for 2 min using a mixture of isopropanol + acetone (50:50)/eluent B/eluent A (85%/10%/5%) to remove hydrophobic matrix constituents from the column. Finally, the column was re-equilibrated to ini-

tial conditions for 5.7 min. The injection volume was 100 µL, and the column was operated at 40°C. The heated ESI source and the transfer capillary were both operated at 300°C, the spray voltage was 3.8 κV (pos. mode) or 3.5 κV (neg. mode), the sheath gas flow rate was 45 a.u., and the auxiliary gas flow rate was 1 a.u. Separate runs were conducted in positive and negative ion mode by combining a full scan experiment (100-1000 m/z) at a nominal resolving power of 70,000 (referenced to m/z 200) and data-independent MS/MS experiments at a nominal resolving power of 35,000. For the latter, we acquired the data using broad isolation windows of approximately 50 (i.e., m/z ranges 97-147, 144-194, 191-241, 238-288, 285-335, 332-382, 379-429, 426-476) and 280 (i.e., m/z ranges 460-740, 730-1010).

The calibration standards at levels of 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, and 2000 ng/L were prepared via the same method applied to the samples using 1 mL of pristine stream water, 25 µL of a methanolic analyte stock solution of the appropriate concentration, 25 µL of an internal standard solution and 10 µL of a 2 M NH_4-formate buffer (pH 3.5). The software Trace Finder 3.2 (Thermo) was used for the data evaluation, and the internal calibration with the isotope-labeled compound with the closest retention time for each analyte was used. The full scan extracted ion chromatogram (7 ppm window) was used for quantification, and one or two diagnostic MS/MS fragments as well as isotope patterns were used for confirmation.

2.3.3 Exposure calculation

The sum of the toxic units found at a given sampling site ($\mathrm{TU_{sum}}$) was used to determine the pesticide-induced water toxicity at each site (Sprague, 1970) (Eq. (2.1));

$$TU_{sum} = log\left[\sum_{i=1}^{n}\left(\frac{C_i}{LC_{50i}}\right)\right] \tag{2.1}$$

where $\mathrm{TU_{sum}}$ is the sum of the effect of n pesticides detected at each sampling site, C_i is the concentration (µg/L) of the respective pesticide i, and $\mathrm{LC_{50}}$i is the median lethal concentration (48 h, µg/L) of that pesticide for the preferred reference organisms. Here, we used $\mathrm{LC_{50}}$ 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 organism was selected as the reference.

To identify the pesticide responsible for the highest effect at each site, the maximum toxic unit ($\mathrm{TU_{max}}$) was calculated (Liess and Von der Ohe, 2005). For illustration purposes, a log-transformation was performed for both formulas (i.e.,

formulas 2.1 and 2.2);

$$TU_{max} = Max_{i=1}^{n} \left[log \left(\frac{C_i}{LC_{50i}} \right) \right] \quad (2.2)$$

where $\mathrm{TU_{max}}$ is the highest value of n pesticides at each sampling site, C_i is the concentration (µg/L) of pesticide *i*, and $\mathrm{LC_{50i}}$ is the median lethal concentration (48 h, µg/L) of that pesticide for the reference organism. The $\mathrm{TU_{max}}$ and $\mathrm{TU_{sum}}$ generated the same results; therefore, we preferred to use the commonly applied $\mathrm{TU_{max}}$ for further analysis (Liess and Von der Ohe, 2005; Münze et al., 2017; Orlinskiy et al., 2015).

2.3.4 Sampling of test organisms

For the sensitivity tests, *G. pulex* was sampled from December 2015 to January 2016 before pesticide application (low contamination), and again at the same sites from May to July 2016 during the peak period for pesticide application in the field (high contamination) (Huber et al., 2000; Liess et al., 1999) (see Figure A2). The populations sampled in winter had not been exposed to high pesticide contamination because they belong to the autumn generation born from August to September after the period of maximum pesticide application; however, the populations sampled in summer had been exposed to high pesticide contamination (May to July). We hypothesized that if the tolerance was caused by physiological acclimation, then both generations would show different levels of sensitivity; whereas, in case of similar sensitivity, we can assume resistance development.

2.3.5 Characterizing the ecological effects of pesticide contamination

The toxic pressure of pesticides was identified by applying the bio-indicator "$\mathrm{SPEAR_{pesticides}}$" (SPEcies At Risk) (Liess and Von der Ohe, 2005). Generally, the SPEAR index categorizes aquatic taxa into "SPEcies At Risk" (sensitive species) and "SPEcies not At Risk" (tolerant species) and is performed based on four ecological traits: (1) physiological sensitivity to organic compounds; (2) generation time; (3) pesticide exposure potential; and (4) recolonization ability. The $\mathrm{SPEAR_{pesticides}}$ value was calculated according to formula 3 using the SPEAR calculator 0.10.0 (System-Ecotoxicology, 2005; Liess and Von der Ohe, 2005).

$$SPEAR_{pesticides} = \frac{\sum_{i=1}^{n} log(x_i + 1)y}{\sum_{i=1}^{n} log(x_i + 1)} \times 100 \quad (2.3)$$

where *n* is the number of taxa; *xi* is the richness of taxon *i*; and y is 1 if taxon i is classified as "at risk"; otherwise, it is 0.

Stream macroinvertebrates were sampled during the main season of pesticide application (May to July). Organisms were collected using a 25×25 cm Surber sampler with a mesh size of 500 µm. 10 subsamples from each site were collected along a stream section of approximately 50 m in length. The organisms were filtered through a set of four sieves (mesh sizes: 8 mm, 4 mm, 2 mm, and 500 µm; Retsch GmbH, Haan, Germany) and transferred into water containing deep white trays. Taxa were identified in the laboratory with a stereo-microscope (Carl Zeiss, Oberkochen, Germany) to the family level using identification keys for *Amphipoda*, *Bivalvia*, *Gastropoda*, *Hirudinea*, *Isopoda*, *Oligochaeta* and *Phyllopoda* (Stresemann et al., 1992), *Coleoptera* (Bouchard et al., 2004), *Diptera* (Sundermann and Lohse, 2004), *Ephemeroptera* (Elliott and Humpesch, 2010), *Heteroptera* (Savage, 1989), *Odonata* (Theischinger and Endersby, 2009), *Plecoptera* (Nilsson, 1996) and *Trichoptera* (Wallace et al., 2003).

2.3.6 Acute toxicity tests

We tested the acute sensitivity of *G. pulex* to the neonicotinoid insecticide clothianidin that represents the most commonly applied class of insecticides in agriculture for more than a decade (Simon-Delso et al., 2015). The acute toxicity assessment was conducted following the OECD guidelines for the testing of chemicals and the rapid testing used for community-level risk assessments (Kefford, 2013; OECD, 2004). A 500 mg/L stock solution of the neonicotinoid insecticide clothianidin was prepared with DANTOP® (Spiess-Urania Chemical GmbH, Germany) in aqua dest (distilled water) with stirring for 12 h. The stock solution was further diluted in ADaM (Artificial Daphnia medium) (Klüttgen et al., 1994) to the required test concentrations. *G. pulex* was exposed to seven clothianidin concentrations i.e., 0, 5, 15, 45, 135, 405 and 1215 µg/L. Briefly, 20 organisms were submitted to each concentration for a period of 48 hours to determine the effect of the toxicant. The immobility of the organisms was checked after 24 and 48 hours. Individuals were considered immobile when they did not move their bodies within 30 seconds of undisturbed observation or after probing with a rod (fanning of gills and antenna did not count for body movement).

2.3.7 Statistical analysis

Analyses were conducted using RStudio for Windows (version 1.0.44) and R for Windows (version 3.0.3). From the acute toxicity test, the median effect concentrations (EC_{50}) and their confidence intervals (95%) were calculated using a generalized linear model with a quasi-binomial error distribution and a logit link function. For the comparison of EC_{50} values among both seasons (winter vs.

summer), Wilcoxon's rank sum test was applied. As the water samples for pesticide analysis were collected only in summer, only the EC_{50} values from the summer season were used in the further analysis. Linear regression was applied to analyze the association between the $SPEAR_{pesticides}$ index and the toxic pressure exerted by pesticides (TU_{max}), EC_{50} and TU_{max}, and EC_{50} and distance from the recovery site. Prior to the analysis, homoscedasticity and the normal distribution of residuals were confirmed by a visual inspection using normal-Q-Q plots and plots of residuals vs. fitted values. To obtain a normal distribution of the EC_{50}, the data were ln(x) transformed prior to the analysis.

On the basis of $SPEAR_{pesticides}$, sampling sites were categorized into two groups by their ecological status according to the toxic pressure of pesticides. Streams with SPEAR values > 33 were considered as uncontaminated; whereas the streams with < 33 were assumed to be contaminated (Beketov et al., 2009). For comparison of different parameters among both groups, 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. In case the data were not normally distributed, Wilcoxon's rank sum test was used.

2.4 Results

2.4.1 Pesticide exposure

A total of 50 chemicals out of 55 targeted substances (see table A1) were detected in the water samples collected from 15 streams. More than half of the agricultural streams were contaminated with at least 35 chemicals, including insecticides, fungicides and herbicides. The maximum toxic unit (TU_{max}, see methods) ranged from –3.2 to –1.1 in agricultural streams, with a median TU of –1.8 for a single compound. Hence, all agricultural streams were considered to be substantially exposed to pesticides. The most toxic compounds were as follows: thiamethoxam (TU_{max} at 5 sampling sites; mean TU = –1.62), clothianidin (TU_{max} at 2 sampling sites; mean TU = –1.87), pirimiphos-methyl (TU_{max} at 1 sampling site; TU = –1.92) and diflufenican (TU_{max} at 1 sampling site; TU = –3.18). Accordingly, the maximum toxicity obtained by the different classes of pesticides in decreasing order of relevance are as follows: insecticides (TU_{max} = –1.1), herbicides (TU_{max} = –2.2) and fungicides (TU_{max} = –3.18). With thiamethoxam and clothianidin, neonicotinoid insecticides that were used in our sensitivity tests and share a distinct mode of action made up for the two most toxic compounds in the streams. In contrast, the forested stream sections that served as controls were contaminated only to a minor extent. Here, the maximum toxic unit ranged from –5 to –3.3, with a median TU_{max} of –3.7 which is considered not to be of ecotoxicological

relevance (Liess and Von der Ohe, 2005) (see Table 2.1).

Table 2.1: Water toxicity in terms of the $\mathrm{TU}_{\mathrm{sum}}$ and $\mathrm{TU}_{\mathrm{max}}$ values calculated from field data, the compounds responsible for the highest toxicity, and the composition of the macroinvertebrate community (expressed as $\mathrm{SPEAR}_{\mathrm{pesticides}}$). H: herbicide; I: insecticide

Site ID	$\mathrm{TU}_{\mathrm{sum}}$	$\mathrm{TU}_{\mathrm{max}}$	Most toxic compound	Pesticide type	SPEAR
Agri-1	–1.5	–1.8	Thiamethoxam	I	22.3
Agri-2	–2.9	–3.2	Diflufenican	H	24.4
Agri-3	–1.9	–1.9	Pirimiphos-methyl	I	10.2
Agri-4	–1.8	–1.8	Thiamethoxam	I	17.0
Agri-5	–1.9	–1.9	Clothianidin	I	32.4
Agri-6	–1.0	–1.1	Thiamethoxam	I	7.0
Agri-7	–1.4	–1.7	Thiamethoxam	I	14.7
Agri-8	–1.4	–1.7	Thiamethoxam	I	25.0
Agri-9	–1.7	–1.9	Clothianidin	I	15.6
Control-1	–3.5	–3.6	Clothianidin	I	49.7
Control-2	–4.4	–4.8	Prosulfocarb	H	37.7
Control-3	–3.2	–3.3	Clothianidin	I	48.4
Control-4	–3.4	–3.4	Clothianidin	I	34.4
Control-5	–3.8	–3.8	Clothianidin	I	37.2
Control-6	–5.0	–5.0	–	–	50.3

2.4.2 Effects on community structure

We identified the toxic pressure exerted by the pesticides on the invertebrate communities by quantifying their effects with the $\mathrm{SPEAR}_{\mathrm{pesticides}}$ indicator. Sites from agricultural areas showed lower $\mathrm{SPEAR}_{\mathrm{pesticides}}$ values (i.e., 7 to 32; median 17) and were categorized as low (< 11) to moderate (< 33) with respect to their ecological status (Beketov et al., 2009). In contrast, control sites from non-agricultural streams showed higher $\mathrm{SPEAR}_{\mathrm{pesticides}}$ values (i.e., 34 to 50; median 43) and their ecological status was categorized as good ($\geq$ 33) to high (> 44) (Table 2.1). Agricultural- and control streams did not differ significantly in terms of depth, pH, EC and dissolved oxygen (Welch two-sample t-tests, Table A2); however, agricultural streams showed significantly higher temperatures (p-value = 0.002, Table A2). Of all the environmental parameters, the change in $\mathrm{SPEAR}_{\mathrm{pesticides}}$ depended only on the $\mathrm{TU}_{\mathrm{max}}$ (linear regression, p-value < 0.001, adjusted $\mathrm{R2}$ = 0.64; Fig. 2.1). Control streams ($\mathrm{TU}_{\mathrm{max}}$ < –3.2) were characterized by a significantly higher SPEAR value as compared to the contaminated agricultural streams characterized by higher $\mathrm{TU}_{\mathrm{max}}$ values (Wilcoxon's rank sum test, W = 54, p-value < 0.001).

2.4.3 Adaptation of *Gammarus pulex* to pesticides

G. pulex populations from agricultural streams ($\mathrm{TU}_{\mathrm{max}} \geq -3.2$) were 3-fold more tolerant to the insecticide clothianidin compared with those from uncontaminated control streams (Fig. 2.2, table A3). Significant seasonal variation in the sensitivity (i.e., winter vs. summer) was not observed among exposed (Wilcoxon's rank sum test, W = 36, *p*-value = 0.73) or non-exposed populations (W = 25, *p*-value = 0.31).

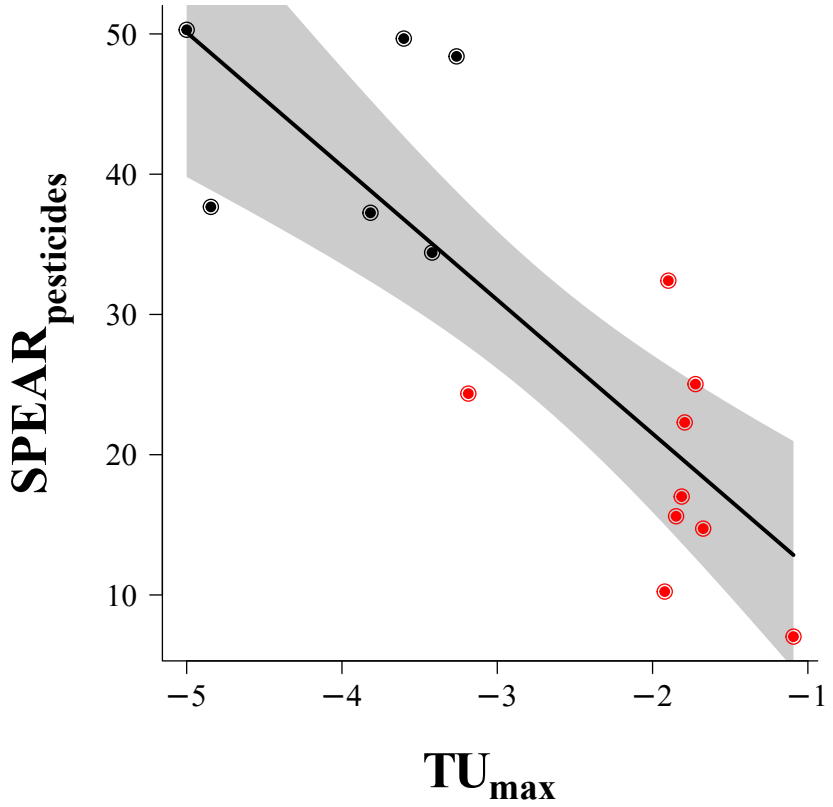

Figure 2.1: Relationship between the community compositions of macroinvertebrates expressed as $\mathrm{SPEAR}_{\mathrm{pesticides}}$ and water contamination in terms of toxic units ($\mathrm{TU}_{\mathrm{max}}$). Control sites are represented with black dots, agricultural streams with red dots. Grey area corresponds to the 95% confidence interval. R^2 = 0.67, adjusted R^2 = 0.64, F = 26.28, residual d.f. = 13, $p < 0.001$.

Among all environmental factors measured, the toxic pressure of pesticides, $\mathrm{TU}_{\mathrm{max}}$, showed the strongest effect on the sensitivity of *G. pulex* to clothianidin (linear regression, *p*-value = 0.0013, adjusted $\mathrm{R2}$ = 0.53; Fig. 2.3). This occurred at concentrations that were in the range of 1/100 to 1/1000 compared to the acute LC_{50} of the most sensitive standard test organism and accordingly also lower than those predicted to be safe by governmental risk assessment frameworks.

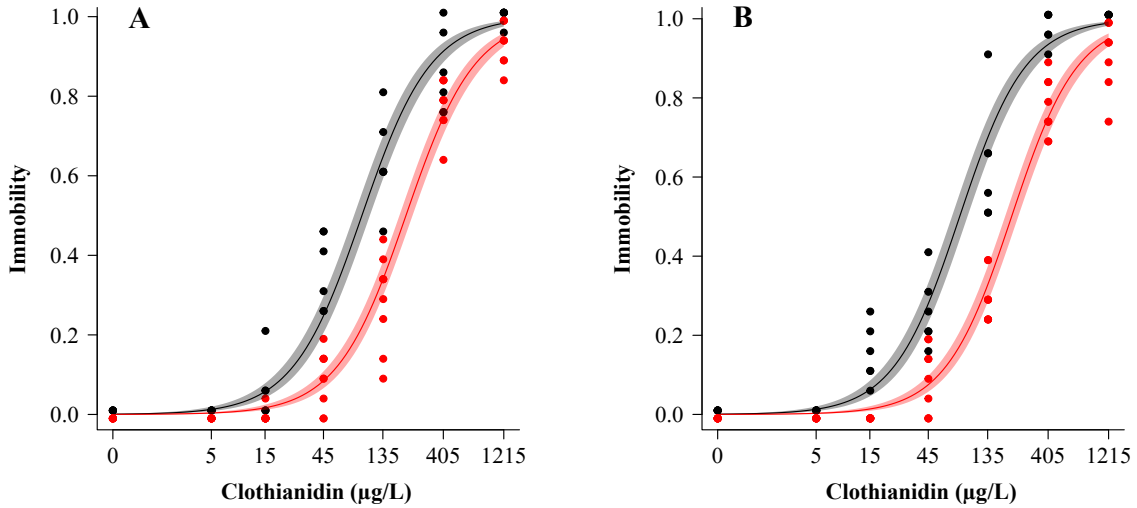

Figure 2.2: Dose response curves for *Gammarus pulex* exposed to clothianidin in laboratory test systems. Figure "a" and "b" represent winter and summer seasons respectively. In both figures, black curves denote the response of organisms from non-agricultural streams whereas red curves show the response of organisms from agricultural streams. Shaded areas correspond to the 95% confidence intervals.

Additionally, when regarding only the contaminated agricultural streams, the increase in clothianidin tolerance was best explained by an increasing distance to the next refuge area (linear regression, p-value < 0.001, adjusted $R2$ = 0.86; Fig. 2.4). TU_{max} and the distance from a refuge area were not correlated (p-value > 0.05, adjusted $R2$ = 0.07). The results indicate that the development of tolerance is determined collectively by the local contamination and the distance to non-contaminated refuge sections.

2.5 Discussion

2.5.1 Pesticide exposure and effects on community structure

In the present study, the toxicity (TU_{max}) of insecticides in agricultural streams ranged from –3.2 to –1.1. According to Ockleford (2013) and the European regulation framework, acute exposure to concentrations that are $\leq 1/100$ times of the LC_{50} of a sensitive reference organism (TU= –2) is generally considered as safe. Furthermore, Brock et al. (2000) concluded that a TU < –1 generally does not show any toxic effects in mesocosms. In contrast to these considerations, the insecticides we found in agricultural streams showed strong effects on macroinvertebrate community composition already at TU_{max} levels below –1. These effects

on the macroinvertebrate community quantified with $\mathrm{SPEAR}_{\mathrm{pesticides}}$ were similar to the findings of previous studies that observed ecological effects already at concentrations above a $\mathrm{TU}_{\mathrm{max}}$ of –3 (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; Schäfer et al., 2012). However, these studies did not include neonicotinoids which have become the most commonly applied insecticides worldwide for the last 10 years (Jeschke et al., 2010; Simon-Delso et al., 2015). Indeed, although numerous studies have shown adverse impacts of neonicotinoids on aquatic organisms in test systems (Morrissey et al., 2015; Pisa et al., 2015), there are only two recent studies that showed their effects on the aquatic community structure in the field (Becker and Liess, 2017; Münze et al., 2017). These investigations revealed that in our study area, insecticide toxicity in small agricultural streams and outlets of waste water treatment plants were mainly attributed to neonicotinoids, together with pyrethroids. In our study, we again

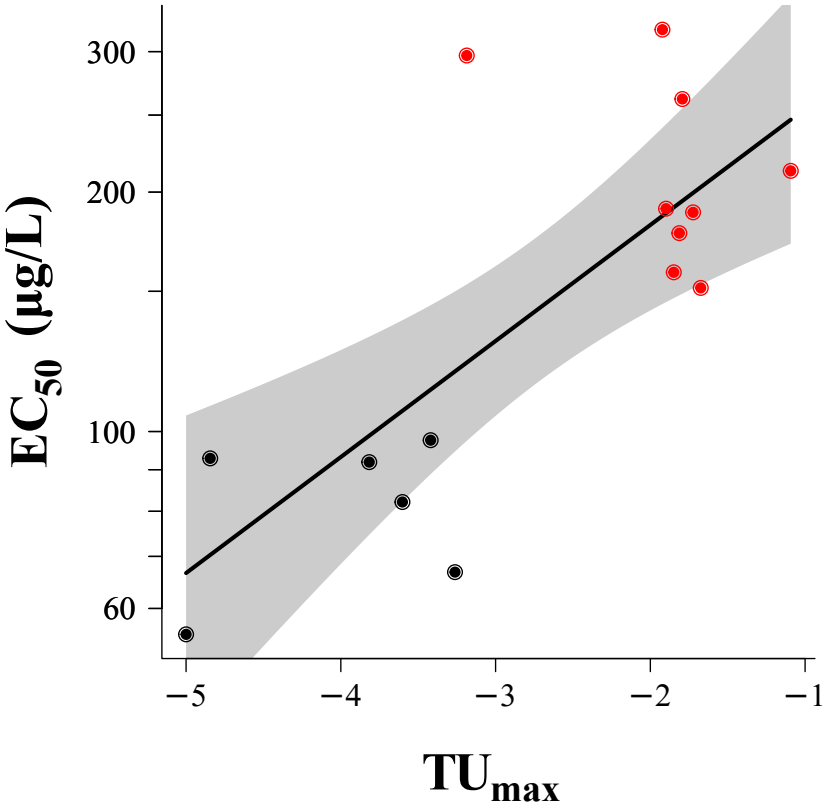

Figure 2.3: Relationship between the mean effect concentrations (48 h EC_{50}) of *Gammarus pulex* collected from streams with different pesticide pollution levels and the water contamination in terms of toxic units ($\mathrm{TU}_{\mathrm{max}}$). Grey area corresponds to the 95% confidence interval. R^2 = 0.56, adjusted R^2 = 0.53, F = 16.65, residual d.f. = 13, *p*-value = 0.001.

used $\mathrm{TU}_{\mathrm{max}}$ rather than $\mathrm{TU}_{\mathrm{sum}}$ to link the toxic pressure of pesticides with community effects ($\mathrm{SPEAR}_{\mathrm{pesticides}}$) as the variance explained by $\mathrm{TU}_{\mathrm{max}}$ and $\mathrm{TU}_{\mathrm{sum}}$

only differed negligibly. Obviously, the effects are typically caused by the most toxic compound (Schäfer et al., 2013; Verbruggen and Van den Brink, 2010). Accordingly, $\mathrm{TU}_{\mathrm{max}}$ is a good proxy to predict the toxic pressure on aquatic macroinvertebrate communities.

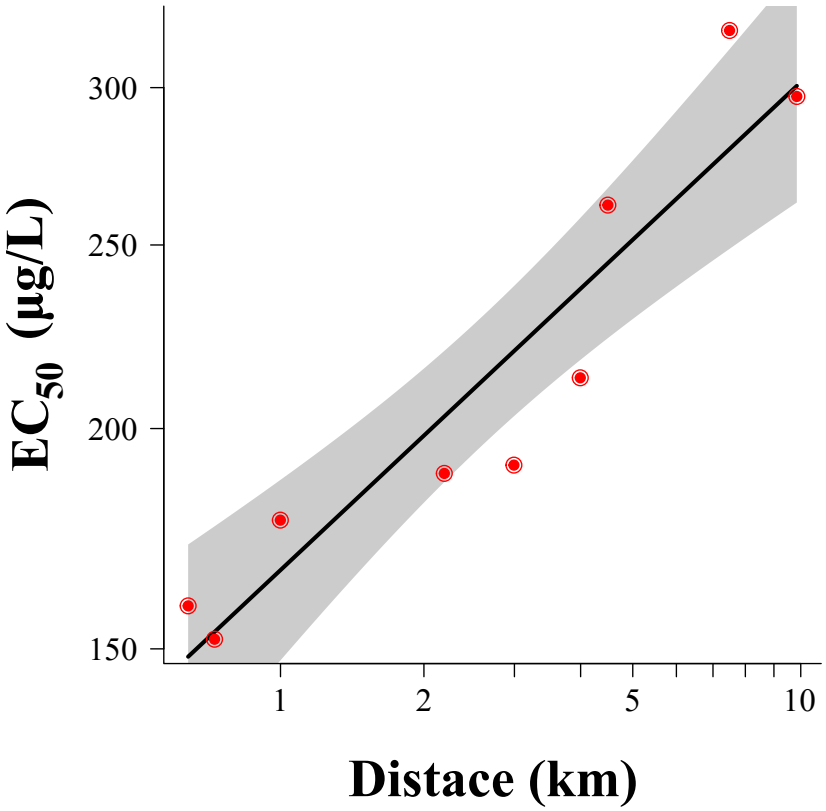

Figure 2.4: Relationship between the mean effect concentrations (48 h EC_{50}) of *Gammarus pulex* collected from streams with different pesticide pollution levels and the water contamination in terms of toxic units ($\mathrm{TU}_{\mathrm{max}}$). Grey area corresponds to the 95% confidence interval. R^2 = 0.56, adjusted R^2 = 0.53, F = 16.65, residual d.f. = 13, *p*-value = 0.001.

2.5.2 Resistance acquisition

Our findings indicated that *G. pulex* populations from highly contaminated streams were more tolerant to clothianidin compared with populations from less contaminated stream sections. This higher pesticide tolerance may be related to physiological acclimation or resistance development. Non-genetic changes (physiological acclimation), such as induced enzyme activity that increases detoxification have been detected so far only in few cases and are considered to prevail when pesticide exposure is rare and irregular (Hua et al., 2013;

Poupardin et al., 2008). In contrast, the evolution of resistance (genetic adaptation) is considered to occur more frequently. Numerous pest species (Bass et al., 2011; Jones et al., 2011; Karatolos et al., 2010; Karunker et al., 2008; Yang et al., 2013) as well as a number of non-target species (Bendis and Relyea, 2014; Clark et al., 2015; Vigneron et al., 2015; Weston et al., 2013) have been shown to genetically adapt to toxicant exposure in the field. Resistance is considered to prevail particularly when pesticide exposure occurs repeatedly and regularly (Hua et al., 2013), as it is likely the case for *G. pulex* in agricultural streams over several decades (Burdick et al., 1964; Butler, 1966; Cottam and Higgins, 1946; Herzel, 1972; Miles and Harris, 1973; Moore, 1967). Therefore, we expect that the observed adaptation in *G. pulex* might have a predominant genetic component. An observation supporting this conclusion was the similar level of sensitivity to clothianidin observed in the populations throughout the year regardless of the seasonal variation in contamination. In winter, when pesticide contamination was low, individuals of the early autumn generation showed the same level of sensitivity to clothianidin as did the individuals sampled in summer, when pesticide contamination was high.

Refuge areas were defined here as stream sections serving as a source of unexposed individuals, and they represent an additional factor that influenced the development of resistance at the contaminated sites. *G. pulex* populations inhabiting stream sections far from refuge areas showed lower sensitivity to clothianidin possibly resulting from the development of resistance. Reports have established that forested undisturbed upstream zones positively influence the quality of downstream habitats and partly compensate for the effects of pesticides via recolonization of sensitive species (Bunzel et al., 2014; Liess and Von der Ohe, 2005; Orlinskiy et al., 2015; Schäfer et al., 2007; Von der Ohe and Goedkoop, 2013). Additionally, sensitive conspecifics from non-contaminated sections can migrate to the downstream contaminated sites, which may slow the local evolution of resistance to pesticides. To our knowledge, this is the first study to reveal that uncontaminated stream sections reduced the evolution of resistance to pesticides in freshwater non-target species.

In the present study, the increased tolerance in *Gammarus pulex* against neonicotinoids is moderate as compared to those observed in target pest species (Khan et al., 2015; Longhurst et al., 2013; Szendrei et al., 2011). Also some non-target species such as black flies and Hyalella azteca showed much higher resistance against pyrethroid insecticides (Clark et al., 2015; Montagna et al., 2012; Weston et al., 2013). However, these populations were exposed to high concentrations of pesticides, lethal to standard test organisms, and possibly had a lower ability to exchange conspecifics through immigration from non-contaminated sites. Such a low exchange rate is likely for pond populations of Hyalella azteca and for black fly populations breeding in a channel network that manages water for fruit production (Montagna et al., 2012).

2.6 Conclusions

The development of tolerance for non-target species may occur at very low concentrations, much below those predicted to be safe by governmental risk assessment frameworks. Such an adaptation may reduce the ability of non-target species to compensate environmental stress such as climate change. On the other hand pest species may acquire resistance already at low toxic pressure. Accordingly, the consequences of our findings for risk assessment and also resistance management of pesticides needs to be identified.

2.7 Supporting Information

A single file of supporting information is provided that includes a map of selected study sites, information regarding pesticide application in 2015, a description of the water quality parameters of investigated streams, and a list of chemicals considered for water analysis and list of acute EC_{50} values of insecticide clothianidin for *Gammarus pulex* in both winter and summer seasons.

2.8 Acknowledgements

We thank Oliver Kaske, Stefanie Lippmann, Klaus Seyfarth and Ingrid Ränker from the Department of System-Ecotoxicology, Helmholtz Centre for Environmental Research GmbH – UFZ, for their support in the collection, identification and testing of the study organisms. Funding: This work was supported by German Academic Exchange Service (Deutscher Akademischer Austauschdienst, DAAD).

3

Pesticide body burden as a measure of toxic pressure

3.1 Abstract

Risk assessments of toxicants in aquatic environments are typically based on the evaluation of concentrations in water or sediment. However, concentrations in water are highly variable, while the body burden may provide a better time-integrated measure of pesticide exposure and potential effects in aquatic organisms. Here, we quantified pesticide body burdens in a dominant invertebrate species from agricultural streams, *Gammarus pulex*, compared them with pesticide concentrations in water samples, and linked the pesticide contamination with observed ecological effects on macroinvertebrate communities.

In total, 19 of 61 targeted analytes were found in the organisms, ranging from 0.037 to 93.94 ng/g (wet weight). Neonicotinoids caused the highest toxic pressure among the pesticides detected in *G. pulex*. Using linear solvation energy relationships (LSERs), we derived equivalent pesticide concentrations in stream water based on the body burden. These equivalent concentrations correlated with the concentrations in water samples collected after run-off (65% of variance explained).

Pesticide pressure significantly affected the aquatic macroinvertebrate community structure, expressed as $\mathrm{SPEAR}_{\mathrm{pesticides}}$, and caused, on average, threefold in-

creased insecticide tolerance in *G. pulex* as a result of adaptation. The toxic pressure derived from body burden and from water samples similarly explained the change in community structure (68% and 64%). However, the increased tolerance of *G. pulex* to pesticides was better explained by the toxicity derived from body burden (70%) than by the toxicity from water samples (53%). We conclude that the internal body burden of macroinvertebrates is suitable to assess the overall pesticide exposure and effects in agricultural streams.

3.2 Introduction

Pesticides in agricultural streams considerably affect the diversity and species composition of aquatic macroinvertebrates (Beketov et al., 2013; Liess and Von der Ohe, 2005) and consequently, related ecosystem functions such as leaf litter degradation (Bowmer, 2013; Münze et al., 2015; Peters et al., 2013; Schäfer et al., 2007). Sensitive species show significant reduction even at concentrations more than 2 orders of magnitude below the acute median lethal concentration (LC_{50}) that kills 50% of the individuals 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., 2016) and the culmination of effects from sequential exposure (Liess et al., 2013). The same low concentrations exert a considerable pressure for adaptation, which results in increased pesticide tolerance in exposed non-target species, with unknown ecological consequences on their general fitness (Becker and Liess, 2017; Shahid et al., 2018a). To reduce effects of pesticides in the environment, local management measures regarding the protection from pesticide contamination are necessary. The decision base for such measures is a profound quantification and localisation of pesticide exposure and effects through monitoring in agricultural streams.

One relevant pathway of pesticides to enter agricultural streams is through run-off from arable land after heavy rainfall. Accordingly, aquatic organisms are typically exposed to short pulses of high pesticide concentrations (Liess et al., 1999). Thus, relevant measurements of pesticide exposure must include run-off events; as such peak concentrations are related to the long-term effects on the macroinvertebrate community much better than the low concentrations found in event-independent samples (Liess and Schulz, 1999; Liess and Von der Ohe, 2005). However, measuring these short-term pesticide pulses is challenging as the peak concentrations depend on the timing and intensity of both the pesticide application and the subsequent run-off event, which can vary widely within a spraying season (Liess et al., 1999; Liess and Schulz, 1999).

Currently, a range of passive samplers have been suggested for integrative sam-

pling of various pollutants in surface waters (Allan et al., 2009; Gunold et al., 2008; Schäfer et al., 2008) and have also been used to capture short-term pollution events (Fernandez et al., 2014; Greenwood et al., 2007). Generally, pesticide concentrations determined by passive samplers are highly suitable for linking local toxic pressure to ecological effects on macroinvertebrate communities (Leonard et al., 2000; Münze et al., 2015; Schäfer et al., 2008). However, the installation of passive samplers is costly and time-consuming. Toxic pressure was defined here as a stress exerted by pesticides in streams, using Toxic Unit (Sprague, 1970) modified by applying the highest Toxic Unit – $\mathrm{TU}_{\mathrm{max}}$ as a measure for the ecological relevant toxic pressure (Liess and Von der Ohe, 2005). In the present study, we additionally used an alternative quantification of pesticide exposure based on pesticide residues in macroinvertebrates sampled from agricultural streams. Organisms accumulate pesticides in lipids and proteins from the water phase according to the compound properties and therefore, may serve as natural passive samplers (Huckins et al., 1993). Their body burden may provide a better time-integrated picture of the overall pesticide exposure than do short-term measurements from the water phase. Additionally, an assessment of pesticide exposure based on internal concentrations may be more representative because it is the concentration at the site of action within organisms, rather than the external concentration in water, that triggers adverse effects (Escher and Hermens, 2002).

We used pesticide residues in the common freshwater shrimp *G. pulex* to predict the time integrated pesticide concentrations in stream water based on equilibrium partitioning (Inostroza et al., 2017) with a linear solvation energy relationship (LSER) (Ulrich et al., 2017) model. This model estimates the partitioning of pesticides into different compartments such as storage and membrane lipids and proteins and considers polar and H-bonding interactions rather than hydrophobicity only. The predicted equilibrium concentrations in water (Cw,eq) were compared with the pesticide peak concentrations in water samples from the same streams following rain events. Additionally, we evaluated the ecological effect of the toxic pressure estimated from body burden and from water samples on the macroinvertebrate community composition according to the SPEAR approach (Species at Risk) (Liess and Von der Ohe, 2005) and on the pesticide tolerance in *G. pulex* as a consequence of adaptation.

3.3 Materials and methods

3.3.1 Description of the study sites

The investigated streams were located in central Germany, including 9 sites from intense agricultural landscapes and 6 sites from either undisturbed forested sec-

tions or from streams with considerable buffer strips and non-agricultural upstream reaches (Supporting Information, Figure B1). The second group was considered non-agricultural streams and used as reference sites. During macroinvertebrate sampling, different environmental parameters such as water level, pH, water temperature, dissolved oxygen and electrical conductivity were measured (Supporting Information, Table B1).

3.3.2 Sampling of *Gammarus pulex* and water

Thirty individuals of *G. pulex* (wet weight 900mg) per site were sampled for the analysis of pesticide body burden in May 2016 during the maximum pesticide application period in the study area (Huber et al., 2000; Liess et al., 1999). Organisms of different sizes were collected to avoid any bias regarding greater bioaccumulation and exposure time. After collection, individuals were transferred to the lab and stored at –20°C.

Water samples from all streams were collected using event-driven samplers (EDS) (Liess and Von der Ohe, 2005) from May to July 2016 during the maximum pesticide application period. The number of samples from each site varied from 1 to 5, depending on rainfall events. Briefly, two brown glass bottles, each with a capacity of 1 L, were installed at each sampling site. Both bottles were attached to a stainless rod at heights of 5 and 15 cm from the regular level of the stream water that filled when the water level had risen due to heavy rainfall (see Supporting Information, Figure B2 for illustration of event-driven samplers). After rainfall events, bottles were collected within 24 h, kept in a cool box at 4°C and transported to the laboratory. Subsequently, 1 mL aliquots were transferred into 2 mL autosampler vials and stored at –20°C for analysis. Results of pesticide concentrations in water samples have already been published elsewhere (Shahid et al., 2018a). In the present study we compared water concentrations (external concentrations) with body burden (internal concentrations) to measure the pesticide exposure and potential effects in aquatic organisms.

3.3.3 Sample preparation and extraction

Target compounds were extracted from gammarids using the method of Inostroza et al. (2016) (pulverized liquid extraction and a QuEChERS approach with an additional hexane phase). Frozen gammarids were transferred to a 10 mL centrifuge tube; 1 mL of LC-MS grade acetonitrile, 1 mL of LC-MS grade water and 0.5 mL of LC grade hexane were added; then the sample was homogenized using an Ultra-Turrax (IKA, Staufen, Germany) homogenizer. Subsequently, 400

mg of $MgSO_4$ and 100 mg of NaCl were added to induce phase separation between water and acetonitrile. The top-layer hexane fraction was taken for the analysis of pyrethroids via gas chromatography-tandem mass spectrometry (GC-MS/MS), evaporated to dryness in a nitrogen stream, reconstituted in 500 µL of ethyl acetate and filtered using a PTFE syringe filter (pore size 0.45 µm) into a 2 mL autosampler vial. Permethrin$-D_6$ was added as an injection standard. The acetonitrile phase was cleaned up via dispersive solid-phase extraction using primary-secondary amine and transferred into a 2 mL glass vial. The sample was evaporated to dryness and reconstituted in 450 µL of methanol:water (70:30) for liquid chromatography-high-resolution mass spectrometry (LC-HRMS) analysis. A volume of 50 µL of internal standard solution containing 40 isotope-labelled compounds (1 µg/mL) was added.

3.3.4 GC-MS/MS analysis

The pyrethroid analysis was conducted via GC-MS/MS using an Agilent GC 7890-MS/MS 7000 system with negative-mode chemical ionization. For separation, we used a HP-5ms UltraInert column (30 m $\times$ 0.25 mm, 0.25 µm film thickness) equipped with a guard column (5 m $\times$ 0.25 mm). The column oven program started at 100°C, was held for 1 min, increased at 30°C/min to 150°C, then at 6°C/min to 186°C and at 10°C/min to 300°C, and was then held for 2 min. The transfer line temperature was 300°C. Splitless injection of 1 µL was used at an injector temperature of 280°C. Pyrethroids were quantified with MassHunter software (Agilent) using one quantifier and up to two qualifier MS/MS transitions. A method-matched calibration was prepared using water spiked at seven concentration levels ranging from 0.028 to 28 ng/(g gammarids wet weight), for which the same sample preparation protocol was used as for the samples.

3.3.5 LC-HRMS analysis

Polar pesticides were analysed using LC-HRMS in positive ion mode (Ultimate 3000 LC system coupled to a QExactive Plus MS equipped with a heated electrospray ionization (ESI) source, all from Thermo Scientific). Separation was conducted using a methanol:water gradient (eluent A and B, respectively, both containing 0.1% formic acid) on a Kinetex C18 EVO column (50 x 2.1 mm, 2.6 µm particle size, Phenomenex) at a flow rate of 300 µL/min. After eluting for 1 min with 5% B, the fraction of B was linearly increased to 100% within 12 min and kept for 11 min. Then, the flow was diverted to waste, and the column was rinsed for 2 min using isopropanol:acetone 50:50/eluent B/eluent A (85%/10%/5%). Finally, the column was re-equilibrated to the starting conditions for 5.7 min. The

heated ESI source and the transfer capillary were operated at $300°C$, the spray voltage was 3.8 κV, the sheath gas flow rate was 45 a.u., and the auxiliary gas flow rate was 1 a.u. The runs combined a full scan experiment (100–1000 m/z) at a nominal resolving power of 70,000 (referenced to m/z 200) and data-independent MS/MS experiments at a nominal resolving power of 35,000. For the latter, we acquired the data using isolation windows of approximately 50 mass units (i.e., m/z ranges 97–147, 144–194, 191–241, 238–288, 285–335, 332–382, 379–429, 426–476) and 280 mass units (i.e., m/z ranges 460–740, 730–1010). Eight method-matched calibration standards corresponding to levels of 0.056 to 27.8 ng/(g gammarids wet weight) were prepared the same way as the samples using 1 mL of spiked water instead of gammarids. For water analysis, the calibration standards at concentrations from 1 to 2000 ng/L were prepared using the same method as that applied to the samples using 1 mL of pristine stream water, 25 µL of a methanolic analyte stock solution of the appropriate concentration, 25 µL of an internal standard solution and 10 µL of a 2 M NH_4-formate buffer (pH 3.5).

For data evaluation, the software Trace Finder 3.2 (Thermo) was used, employing internal calibration with the isotope-labelled compound with the closest retention time of each analyte. For quantification, we used the full scan extracted ion chromatogram (7 ppm window), while for confirmation, one or two diagnostic MS/MS fragments and isotope patterns were compared with those of reference standards. Table B2 provides an overview of all analysed pesticides, their physicochemical properties, method detection limits and recoveries for the gammarid extraction method and Table B3 contains detected pesticides at all study sites.

3.3.6 Calculation of chemical activity and partition coefficients

We assumed proteins and lipids as the relevant phases for the absorption of organic pollutants and calculated estimated equilibrium concentrations (Cw,eq) in body tissues of *G. pulex* (Eq. 3.1).

$$C_{w,eq} = \frac{C_{b,m}}{f_{SL}K_{SLW} + f_{ML}K_{MLW} + f_P K_{PW} \times 0.2175} \tag{3.1}$$

where $C_{b,m}$ is the total measured concentration of contaminants in body tissues, f_{SL} is the storage lipid fraction, K_{SLW} represents the storage lipid-water partitioning coefficient, f_{ML} is the membrane lipid fraction, K_{MLW} represents the membrane lipid-water partition coefficient, f_P is the protein fraction, and K_{PW} is the protein-water partitioning coefficient. To calculate gammarid-water partition coefficients, the composition of *G. pulex* (per dry weight) was assumed as 47% protein (Fredrickson and Reid, 1988), 4.5% storage lipid and 1.5% membrane lipid (Ashauer et al., 2010). The factor 0.2175 (Nitchals, 2014) was used for the conversion of wet weight to dry weight. This assumption is well in agreement with

typical values from more in-depth studies on the composition of *Gammarus pulex* (Gee, 1988), although ignoring variability between sexes, seasons and individuals. The present experimental setting did not allow for measuring individual lipid contents in the specimens used in the experiments. Partition coefficients were calculated using linear solvation energy relationships (LSERs, Eq. 3.2) for (1) protein (muscle)–water, (2) storage lipid–water and (3) membrane lipid–water. The system parameters (v, e, s, a, b, and c) used for the respective systems were taken from the open-access UFZ-LSER database (Ulrich et al., 2017). The compound descriptors (V, E, S, A and B) were calculated for all compounds using the software ACD/Percepta (ACD labs, v2016; Supporting Information, Table B4).

$$logK_i = vV + eE + sS + aA + bB + c \tag{3.2}$$

3.3.7 Calculation of pesticide exposure

Measured and estimated equilibrium water concentrations of pesticides were converted into toxic units (TU) representing the decadic logarithm of the concentration of a compound divided by its LC_{50} in a 48 h acute toxicity test with a standard reference organism. We estimated the overall pesticide-induced toxic pressure in a stream as the maximum toxic unit of all compounds ($\mathrm{TU}_{\mathrm{max}}$; Eq. (3.3)(Liess and Von der Ohe, 2005)), quantified either from internal body burdens ($\mathrm{TU}_{\mathrm{max-Int}}$) or from external water samples ($\mathrm{TU}_{\mathrm{max-Ext}}$). $\mathrm{TU}_{\mathrm{max}}$ works very well for agricultural streams but may not for other ecosystems that receive contamination from many different sources (Massei et al., 2018).

$$TU_{max} = Max_{i=1}^{n} \left[log \left(\frac{C_i}{LC_{50i}} \right) \right] \tag{3.3}$$

where $\mathrm{TU}_{\mathrm{max}}$ is the highest toxic unit of n pesticides at a given site, $\mathrm{C_i}$ is the freely dissolved pesticide concentration (µg/L), and $\mathrm{LC}_{50\mathrm{i}}$ is the median lethal concentration (µg/L) of that pesticide for a reference organism after a 48 h exposure (Supporting Information, Table B5). We used *Daphnia magna*, *Chironomus tentans*, *Chironomus riparius* and *Hyalella azteca* as reference organisms; the LC_{50} values were obtained from the ECOTOX database (USEPA, 2014) and the Pesticide Properties Database PPDB (PPDB, 2014). When data from several organisms were available for the same compound, the most sensitive organism was used. For instance, *Chironomus riparius* and *Hyalella azteca* showed much higher sensitivity to neonicotinoid insecticides as compared to *Daphnia magna* and were therefore considered as reference organisms. In few cases, the name of the reference species was not provided in the database and therefore considered unknown (Supporting Information, Table B5).

3.3.8 Characterizing effects of pesticides on the macroinvertebrate community

At each sampling site, we quantified the long-term effects of pesticides on the macroinvertebrate community composition using the bio-indicator $\mathrm{SPEAR}_{\mathrm{pesticides}}$ (Liess and Von der Ohe, 2005). The SPEAR index categorizes aquatic macroinvertebrates into vulnerable and non-vulnerable species on the basis of four ecological traits: (1) potential for pesticide exposure, (2) physiological sensitivity, (3) generation time (pace of recovery), and (4) migration ability (recolonization). We calculated SPEAR using the desktop application SPEAR Calculator 0.10.0 (System-Ecotoxicology, 2005; Liess and Von der Ohe, 2005).

Stream macroinvertebrates were sampled from May to July 2016 along with the collection of *G. pulex*. At each site, ten subsamples were collected from different habitats along a 50 m stream section. Macroinvertebrates were collected using a 25×25 cm kick-net with a 500 µm mesh size. The content of the Surber sampler was filtered through a set of four sieves (mesh sizes: 8 mm, 4 mm, 2 mm, and 500 µm; Retsch GmbH, Haan, Germany) and transferred into plastic trays for the identification of taxa (family level) and determination of abundance. When the family of an organism could not be identified, the individuals were preserved in ethanol and identified in the laboratory with a stereo-microscope (Zeiss Discovery V20; Oberkochen, Germany). The abundance of each family was divided by the sampling area to calculate population densities per m^2 used for the calculation of the $\mathrm{SPEAR}_{\mathrm{pesticides}}$ values.

3.3.9 Identification of insecticide tolerance in *Gammarus pulex*

At each study site, 170 individuals of *G. pulex* within a size range of 6–10 mm were collected together with the individuals used for the body burden analysis, using a 25×25 cm kick-net with a 500 µm mesh size. The individuals were caught with a pipette and transferred into aerated and cooled plastic boxes filled with stream water. On the day of collection, the organisms were transported to the laboratory and left to acclimate in white trays filled with a mixture of aerated ADaM (Artificial Daphnia medium) (Klüttgen et al., 1994) and steam water at 15°C for 24 h.

Insecticide sensitivity was assessed in acute toxicity tests following the OECD guidelines for the testing of chemicals and the guidelines for rapid tests for community-level risk assessment (Kefford, 2013; OECD, 2004). We selected the neonicotinoid insecticide clothianidin for sensitivity testing that represents the most commonly applied class of insecticides in agriculture for more than a decade (Simon-Delso et al., 2015). A 500 mg/L stock solution of the neonicotinoid insecti-

cide clothianidin was prepared with DANTOP® (Spiess-Urania Chemical GmbH, Germany) in distilled water while stirring for 12 h and further diluted in ADaM to obtain the required test concentrations. The test organisms were exposed to seven different concentrations i.e., 0, 5, 15, 45, 135, 405 and 1215 µg/L for a period of 48 h. Only undamaged and active individuals without visible infection with parasites were selected for pesticide exposure. Five organisms were placed in a stainless tea strainer, and four tea strainers per concentration were placed in a glass beaker containing 1000 mL of the test solution. During contamination, the beakers were constantly aerated and the temperature was maintained at 15°C. After exposure for 48 h, the organisms were checked for immobility. Organisms were considered immobile when they neither moved their bodies within 30 seconds of undisturbed observation nor after probing with a rod (fanning of gills and antenna was not considered body movement).

3.3.10 Data analysis

All data analyses were conducted using the open-source application RStudio version 1.1.383 for Windows and the basic R version 3.4.3 for Windows. To compare the insecticide tolerance in different populations, we calculated the EC_{50} (concentration that immobilized 50% of the test individuals) and the 95% confidence intervals from the acute toxicity tests using a generalized linear model with a quasi-binomial error distribution and a logit link function. The tolerance of *G. pulex* populations from each site was quantified as the ratio of the local EC_{50}/mean EC_{50} of all populations from non-contaminated streams ($\mathrm{TU}_{\mathrm{max-Int}}$ < –3). This EC_{50} ratio allowed us to directly compare the tolerance of different populations to neonicotinoids and thus to quantify adaptation in contaminated streams.

Linear regression was applied to analyse the relationships between the toxic pressure calculated from pesticide body burden and water concentration ($\mathrm{TU}_{\mathrm{max-Int}}$ and $\mathrm{TU}_{\mathrm{max-Ext}}$, respectively) and the change in community structure or the neonicotinoid tolerance. To identify the additional effect of the local species diversity on the increase in neonicotinoid tolerance, we applied multiple linear regression model. The normal distribution and homoscedasticity of residuals were confirmed using normal-Q-Q plots, histograms, plots of residuals vs. fitted values, and statistical tests for deviations from the normal distribution and from variance homogeneity. To obtain a normal distribution, the EC_{50} ratio was ln(x) transformed before analysis. Agricultural and non-agricultural streams were compared with respect to the SPEAR index, EC_{50} and physicochemical parameters using two-sample t-tests and Welch's t-tests. In cases in which the data were not normally distributed, Wilcoxon's rank sum test was used.

3.4 Results

3.4.1 Pesticide residues in individuals of *Gammarus pulex*

A total of 19 chemicals of 61 targeted compounds (see Supporting Information, Table B2) were quantified in all organisms collected (Supporting Information, Table B3). Concentrations ranged from 0.037 to 14.28 ng/g (wet weight) for insecticides, 0.08 to 93.94 ng/g for herbicides and 0.06 to 12.74 ng/g for fungicides. The fungicide prothioconazole-desthio was the most frequently detected chemical across all sites. The highest concentration was found for the herbicide diflufenican with 93.94 ng/g (Supporting Information, Table B3).

Generally, insecticides were detected only in organisms from agricultural streams. The only exceptions were low concentrations of the pyrethroid insecticides cyfluthrin and cypermethrin in organisms from a single non-agricultural stream (Non–Agri 6). Additionally, organisms from non-agricultural streams showed only very low concentrations of the fungicide prothioconazole-desthio (0.17–0.28 ng/g) and the herbicides diflufenican (2.58 ng/g) and prosulfocarb (2.18 ng/g).

3.4.2 Pesticides in the water samples

A total of 50 chemicals of 55 targeted analytes were detected in the water samples collected from the streams. All the agricultural sites were contaminated with most of the analytes, including insecticides, fungicides and herbicides. The fungicide prothioconazole-desthio and herbicides ethofumesate and terbuthylazine were the most frequently detected chemicals in water samples across all the streams. The highest concentration was found for the herbicide ethofumesate with 84.70 µg/L. In agricultural streams, the most commonly detected insecticides were clothianidin, thiamethoxam and thiacloprid. The highest concentration was determined for thiamethoxam (2.83 µg/L). In most non-agricultural streams, only certain herbicides and fungicides were detected. Four of these sites were slightly contaminated (3.4 to 12 ng/L) with the neonicotinoid insecticide clothianidin (Supporting Information, Table B6).

3.4.3 Estimation of aquatic pesticide exposure

The toxicity of the pesticide mixture measured in water samples after runoff events was expressed in toxic units of the most toxic compound ($TU_{max-Ext}$, see

Table 3.1: Pesticide-induced toxic pressure in the investigated streams, expressed as the toxic unit of the most toxic compound (TU_{max}). The toxic units were calculated from pesticide concentrations in water samples collected after runoff events ($TU_{max-Ext}$); they were also derived from internal pesticide concentrations detected in *G. pulex*, which were converted to equivalent concentrations in water ($TU_{max-Int}$). Additionally, the toxic pressure was estimated using the $SPEAR_{pesticides}$ bio-indicator based on the macroinvertebrate community composition (SPEAR), and using the increase in tolerance of *G. pulex* (EC_{50}) to the neonicotinoid insecticide clothianidin as a result of adaptation.

Site ID	TU_{max}		Most toxic compound		SPEAR	EC_{50} (95% CI)
	Ext	*Int*	*In water*	*In organisms*		
Agri-1	–1.8	0.4	Thiamethoxam (I)	Imidacloprid (I)	22.3	263 (228–302)
Agri-2	–3.2	0.0	Diflufenican (H)	Thiacloprid (I)	24.4	298 (227–388)
Agri-3	–1.9	–0.6	Pirimiphos-methyl (I)	Thiacloprid (I)	10.2	321 (203–506)
Agri-4	–1.8	0.4	Thiamethoxam (I)	Imidacloprid (I)	17.0	178 (148–213)
Agri-5	–1.9	–1.3	Clothianidin (I)	Thiacloprid (I)	32.4	191 (135–270)
Agri-6	–1.1	0.5	Thiamethoxam (I)	Imidacloprid (I)	7.0	213 (171–265)
Agri-7	–1.7	0.4	Thiamethoxam (I)	Imidacloprid (I)	14.7	153 (121–192)
Agri-8	–1.7	0.7	Thiamethoxam (I)	Imidacloprid (I)	25.0	190 (144–249)
Agri-9	–1.9	–1.3	Clothianidin (I)	Thiacloprid (I)	15.6	160 (121–210)
Non-Agri-1	–3.6	–6.2	Pirimicarb (I)	Prothioconazole-desthio (F)	49.7	83 (62–110)
Non-Agri-2	–4.8	–6.1	Prosulfocarb (H)	Prothioconazole-desthio (F)	37.7	94 (66–132)
Non-Agri-3	–3.3	–6.0	Clothianidin (I)	Prothioconazole-desthio (F)	48.4	67 (37–120)
Non-Agri-4	–3.4	–6.1	Clothianidin (I)	Prothioconazole-desthio (F)	34.4	98 (85–111)
Non-Agri-5	–3.8	–4.3	Clothianidin (I)	Diflufenican (H)	37.2	93 (65–130)
Non-Agri-6	–5.0	–4.4	-	Cyfluthrin (I)	50.3	57 (38–83)

materials and methods). To derive the pesticide exposure alternatively from the body burden of *G. pulex*, we converted the internal pesticide concentrations to estimated equilibrium concentrations in water and calculated the maximum toxic unit ($TU_{max-Int}$) for invertebrates. The results of both methods are compared in Table 3.1.

In agricultural streams, the $TU_{max-Int}$ ranged from –1.3 to 0.7 with a median value of 0.4. In all of the agricultural sites, neonicotinoids represented the most toxic compound. The highest toxicity was caused by imidacloprid ($TU_{max-Int}$ at 5 sampling sites; mean TU = 0.6), followed by thiacloprid ($TU_{max-Int}$ at 4 sampling sites; mean TU = –0.8). In contrast, the non-agricultural streams were contaminated only to a minor extent. In those streams, the $TU_{max-Int}$ ranged from –6.2 to –4.3 with a median value of –6.0.

The $TU_{max-Ext}$ in agricultural streams ranged from –3.2 to –1.1 and was always lower than the $TU_{max-Int}$, with a mean difference of 1.8 TU. In non-agricultural streams, by contrast, the $TU_{max-Ext}$ ranged from –3.3 to –5.0 and was always higher than the $TU_{max-Int}$ (mean difference: 1.5 TU), except for one stream in which insecticides were detected in the organisms (Non-Agri 6). However, $TU_{max-Int}$ and $TU_{max-Ext}$ were significantly correlated with one another (adjusted

R^2 = 0.65, $p < 0.001$; Fig. 3.1). Both $TU_{max-Int}$ and $TU_{max-Ext}$ showed a clear separation of streams from intense agricultural areas and from reference streams. However, within these groups the $TU_{max-Int}$ was not clearly associated with the $TU_{max-Ext}$, indicating a relevant amount of residual variation.

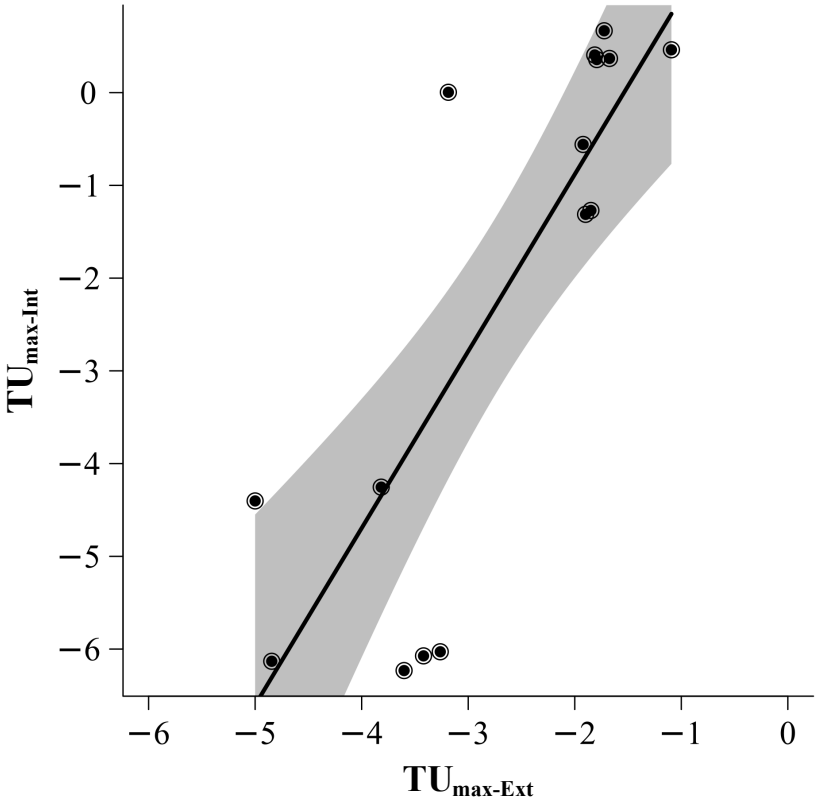

Figure 3.1: Relationship between $TU_{max-Int}$ (TU_{max} calculated using equivalent pesticide concentrations in water derived from internal pesticide concentrations of *G. pulex*) and $TU_{max-Ext}$ (TU_{max} calculated using pesticide concentrations measured in water). The grey area corresponds to the 95% confidence interval. R^2 = 0.67, adjusted R^2 = 0.65, F = 26.5, residual d.f. = 13, $p < 0.001$.

3.4.4 Ecological effect assessment

To compare how both estimations of pesticide exposure ($TU_{max-Ext}$ and $TU_{max-Int}$) could explain the observed ecological effects of pesticides on macroinvertebrate communities, we applied the $SPEAR_{pesticides}$ indicator (Liess and Von der Ohe, 2005). Calculation was performed using the INDICATE tool (http://www.systemecology.eu/indicate/). Agricultural streams showed low to medium $SPEAR_{pesticides}$ values from 7 to 32.4, which corresponded to a range from bad (< 11) to moderate (< 33) ecological status as defined by Beketov

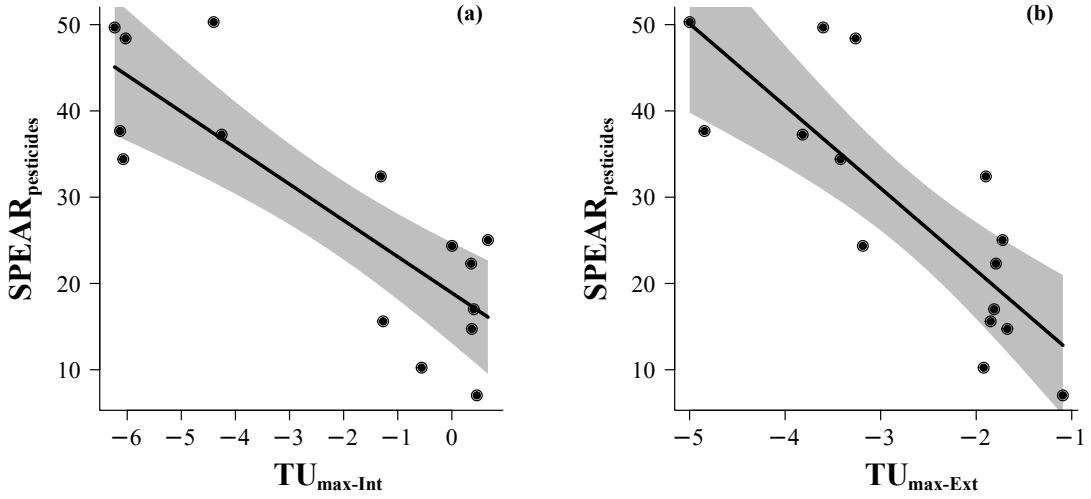

Figure 3.2: Relationship between the community composition of macroinvertebrates, expressed as $\mathrm{SPEAR}_{\mathrm{pesticides}}$, and water contamination, expressed as toxic units. The grey areas correspond to the 95% confidence interval. (a) The calculation of $\mathrm{TU}_{\mathrm{max-Int}}$ was based on the equivalent pesticide concentrations in water derived from internal pesticide concentrations in *G. pulex*; R^2 = 0.70, adjusted R^2 = 0.68, F = 30.89, regression d.f. = 1, residual d.f. = 13, $p < 0.0001$. (b) The calculation of $\mathrm{TU}_{\mathrm{max-Ext}}$ was based on pesticide concentrations in water samples collected from streams after run-off events; R^2 = 0.67, adjusted R^2 = 0.64, F = 26.28, regression d.f. = 1, residual d.f. = 13, $p < 0.0001$.

et al. (2009). In contrast, non-agricultural streams showed significantly higher $\mathrm{SPEAR}_{\mathrm{pesticides}}$ values (Wilcoxon's rank sum test, W = 54, *p*-value < 0.001; Supporting Information, Figure B3) that ranged from 34 to 50 and corresponded to a good ($\geq$ 33) or high (> 44) ecological status (Table 3.1). Of all environmental parameters measured, $\mathrm{SPEAR}_{\mathrm{pesticides}}$ depended only on pesticide exposure and was equally well explained by the toxicity calculated from body burdens ($\mathrm{TU}_{\mathrm{max-Int}}$, Fig. 3.2a) and by the toxicity in water samples ($\mathrm{TU}_{\mathrm{max-Ext}}$, Fig. 3.2b).

In the next step, we compared the calculated $\mathrm{TU}_{\mathrm{max}}$ values and $\mathrm{SPEAR}_{\mathrm{pesticides}}$ with a second indicator of pesticide effects, the insecticide tolerance of *G. pulex* from agricultural and non-agricultural sites. On average, populations from agricultural streams showed 3-fold higher tolerance to the neonicotinoid insecticide clothianidin than those from non-agricultural control streams (Welch two-sample t-test, $p < 0.001$, t = –6.39, d.f. = 9.67; Fig. 3.3).

The observed increase in insecticide tolerance was better explained by the pesticide toxicity estimated from internal body burden ($\mathrm{TU}_{\mathrm{max-Int}}$, adjusted $\mathrm{R2}$ = 0.70, Fig. 3.4a) than that from pesticide concentrations in water samples ($\mathrm{TU}_{\mathrm{max-Ext}}$, adjusted $\mathrm{R2}$ = 0.53, Fig. 3.4b). The increase in insecticide tolerance with $\mathrm{TU}_{\mathrm{max-Int}}$ tended to be stronger when the local species diversity was below average, but the

difference was not significant (adjusted R^2 = 0.72, F = 3.28, d.f. = 1, residual d.f. = 11, p= 0.097 for the interaction toxic unit:Shannon; Supporting Information, Figure B4). The two indicators of pesticide effects, $SPEAR_{pesticides}$ and the insecticide tolerance in *G. pulex*, were closely correlated (adjusted R^2 = 0.67; Fig. 3.5).

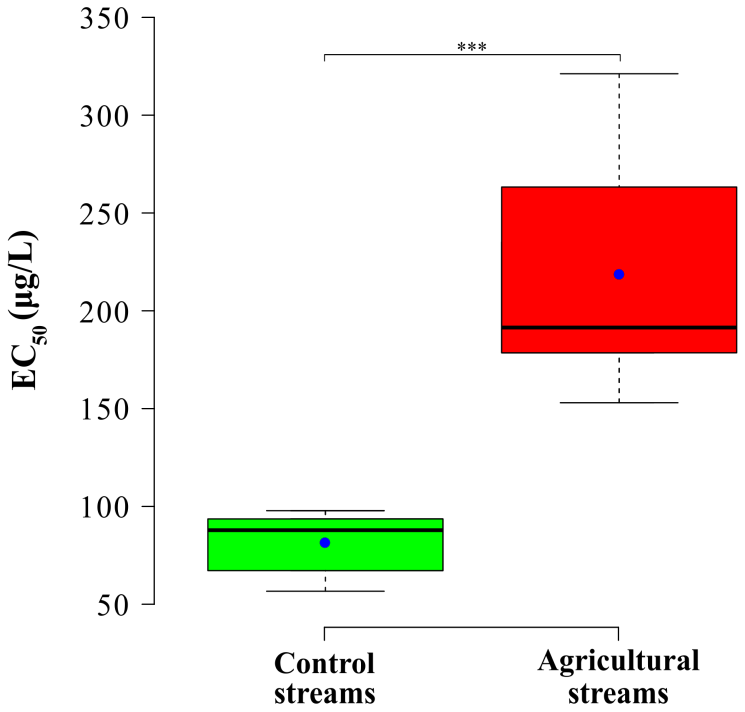

Figure 3.3: Comparison of the median effective concentrations (EC_{50}) of *G. pulex* collected from agricultural (red) and non-agricultural streams (green) after 48 h exposure to the neonicotinoid insecticide clothianidin in the laboratory. The boundaries of the central box are the 25th and 75th percentiles; the horizontal line is the median; the blue dot indicates the mean; and whiskers of the boxplot represent the minimum and maximum values. The significance level for the observations is displayed as: *** = $p < 0.001$.

3.5 Discussion

3.5.1 Pesticide exposure derived from water samples and body burden

Both the pesticide exposure derived from water samples after runoff ($TU_{max-Ext}$) and from body burden in *G. pulex* ($TU_{max-Int}$) showed that the agricultural and

the non-agricultural streams differed in the level of pesticide contamination (Table 3.1). All agricultural streams showed a $TU_{max-Int} \geq -1.3$ and a $TU_{max-Ext} \geq -3.2$, whereas all non-agricultural streams showed a $TU_{max-Int} \leq -4.3$ and a $TU_{max-Ext} \leq -3.3$. According to Liess and Von der Ohe (2005), a pesticide contamination of $TU_{max} > -3$ results in considerable chronic effects on macroinvertebrates, and such an effect was confirmed by an increased dominance of tolerant macroinvertebrate taxa (low $SPEAR_{pesticides}$ values) and an increased insecticide tolerance of *G. pulex* in agricultural streams. However, within the agricultural and the non-agricultural streams, no clear correlation was observed between $TU_{max-Int}$ and $TU_{max-Ext}$, which presumably results from the uncertainty associated with both methods. The neonicotinoid insecticides provided the highest

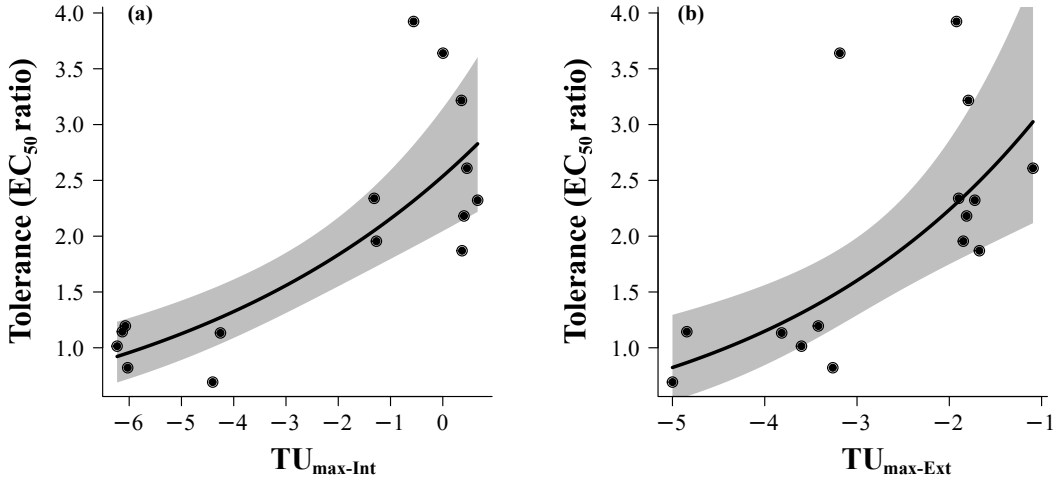

Figure 3.4: The freshwater shrimp *G. pulex* adapted to pesticide exposure, resulting in increased tolerance to the neonicotinoid insecticide clothianidin. Pesticide exposure (in toxic units) were calculated from equivalent pesticide concentrations in water derived from the internal body burden of *G. pulex* (a) or from pesticide concentrations measured in water samples (b). The increase in tolerance was quantified as the ratio of the local EC_{50}/mean EC_{50} of all populations from non-contaminated streams ($TU_{max-Int} < -3$). The grey areas correspond to the 95% confidence interval. (a) $R^2 = 0.72$, adjusted $R^2 = 0.70$, F = 33.95, residual d.f. = 13, $p < 0.001$. (b) $R^2 = 0.56$, adjusted $R^2 = 0.53$, F = 16.58, residual d.f. = 13, $p < 0.001$.

toxicity (both in organisms and in the stream water), confirming previous pesticide measurements from water samples in the study area (Becker and Liess, 2017; Münze et al., 2015). Inostroza et al. (2016) measured the pesticide body burden in *G. pulex* from the nearby Holtemme River and identified neonicotinoids as the most toxic compounds. Neonicotinoids represent the class of insecticides that is currently sold in the largest quantities in the study area (BVL, 2015) and worldwide (Jeschke et al., 2010). Although Inostroza et al. (2016) reported no pesticide measurements from water samples, the derived toxicity from body burden ($TU_{max-Int}$ = –2.7 to –0.07) was generally lower than that in our study. This

difference might result from the fact that the highest pesticide concentration is typically found in the small upper reaches of streams due to the dilution effects in larger rivers (Schulz, 2004; Szöcs et al., 2017).

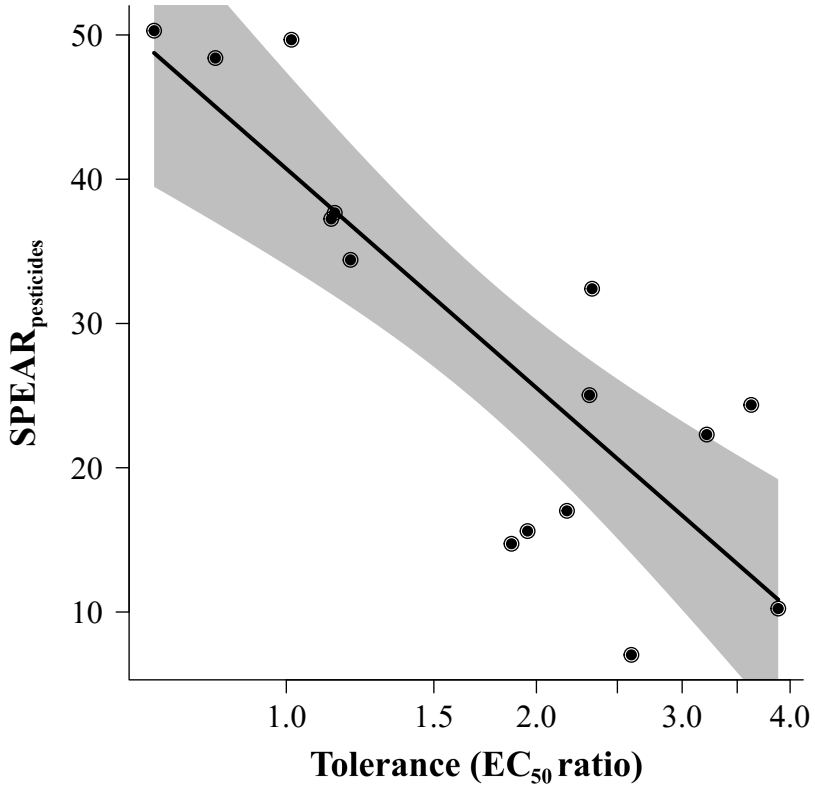

Figure 3.5: Relationship between the community composition of macroinvertebrates, expressed as $\mathrm{SPEAR}_{\mathrm{pesticides}}$, and the increase in tolerance of *G. pulex* to the insecticide clothianidin, expressed as ratio of the local EC_{50}/mean EC_{50} of all populations from non-contaminated streams ($\mathrm{TU}_{\mathrm{max-Int}} < -3$). The grey areas correspond to the 95% confidence interval. R^2 = 0.70, adjusted R^2 = 0.67, F = 29.74, d.f. = 13, $p < 0.001$.

In the present study, we found that in agricultural streams, the $\mathrm{TU}_{\mathrm{max-Int}}$ calculated from estimated equilibrium water concentrations derived from body burden was approximately one order of magnitude higher than the $\mathrm{TU}_{\mathrm{max-Ext}}$ derived from measured water concentrations (Table 3.1). This difference was most likely due to the uncertainty of calculated partition coefficients between *G. pulex* and the water phase but might also reflect disequilibrium at highly dynamic water concentrations during a peak event. As shown previously, neonicotinoids have a tendency to exhibit a higher bioaccumulation than that estimated with existing partitioning models Inostroza et al. (2016). The availability of effect concentrations and thus TUs based on internal concentrations in *G. pulex* would largely remove this uncertainty and provide a better basis for the estimation of effects on

populations and communities. However, good correlation between body burden and community effect (SPEAR) shows that even with uncertainties resulting from back calculation to water concentrations, internal concentrations are a useful and robust approach in the assessment of pesticide effects.

3.5.2 Comparison of pesticide exposure with pesticide effects

The effects of pesticide exposure on the macroinvertebrate community composition ($\mathrm{SPEAR_{pesticides}}$) were similar to the findings of previous studies in which pesticide exposure was quantified from water and sediment samples (Hunt et al., 2017; Münze et al., 2015; Schäfer et al., 2012). The toxicity derived from body burden ($\mathrm{TU_{max-Int}}$) explained the changes in the community composition equally as effectively as the toxicity derived from water samples ($\mathrm{TU_{max-Ext}}$). These results suggest that pesticide concentrations in both water samples after run-off and macroinvertebrates can be used for an adequate assessment of long-term pesticide exposure in streams.

In contrast, the $\mathrm{TU_{max-Int}}$ explained the increase in tolerance to the insecticide clothianidin in *G. pulex* considerably better (adj. $\mathrm{R^2}$ = 0.70) than the $\mathrm{TU_{max-Ext}}$ (adj. $\mathrm{R^2}$ = 0.53). This difference resulted primarily from a particularly low $\mathrm{TU_{max-Ext}}$ in a single agricultural stream (Agri-2, Table 3.1), whereas $\mathrm{TU_{max-Int}}$, $\mathrm{SPEAR_{pesticides}}$ and the insecticide tolerance consistently indicated a high pesticide exposure in this stream. This observation suggests that assessing the pesticide exposure of a stream from water samples was in our case less reliable than the assessment based on body burdens or on observable effects on macroinvertebrates. Pesticide peaks in the stream water may be more subject to unpredictable short-term variation caused by the erratic combination of rainfall and pesticide applications than to the other exposure indicators that rely on longer-term processes. However, peak concentrations in the water still provided an adequate measure of pesticide exposure as the difference from the other indicators became obvious only in one of 15 streams (Fig. 3.2b, Fig. 3.4b).

Consistent with an earlier study (Becker and Liess, 2017), we observed that the increase in insecticide tolerance of *G. pulex* with $\mathrm{TU_{max-Int}}$ was less strong when the local species diversity was high. However, this effect was not significant, probably due to the limited number of study sites compared with Becker and Liess (2017). In contrast, Russo et al. (2018) even reported increased sensitivity to esfenvalerate in *G. pulex* populations collected from agricultural streams. Pesticides may weaken exposed organisms (Ashauer et al., 2007), rendering them more sensitive to subsequent exposure unless they can adapt to the toxicant. Refuge sections can serve as a source of immigrating sensitive organisms and increase the local species diversity, both of which undermine the local adaptation to pesticides

(Becker and Liess, 2017; Gassmann et al., 2009; Shahid et al., 2018a). The higher availability of refuge sections at the sites studied by Russo et al. (2018) may therefore explain the increased sensitivity instead of adaptation, as opposed to the populations investigated in (Shahid et al., 2018a) and the present study. Another study also reported increased sensitivity (Zubrod et al., 2017) to thiacloprid in *G. pulex* populations experiencing pre-exposure to wastewater. Organisms subjected to press disturbance are more likely less tolerant to superimposed pulse disturbances (Parkyn and Collier, 2004). Interestingly, with increasing duration of exposure, gammarids subjected to pure wastewater showed a slight increase in EC_{50} between 4 to 6 weeks. It might be an indication towards evolution of co-tolerance (Lopes et al., 2005). The increase in the insecticide tolerance of *G. pulex* in agricultural streams was closely correlated with changes in the macroinvertebrate community composition. Both effects of pesticide exposure likely represent a selection process for more tolerant organisms that acts simultaneously on the population and the community levels (Grant, 2002). Therefore, as previously suggested by Luoma (1977), the local increase in pesticide tolerance of a rather tolerant species such as *G. pulex* (Liess and Von der Ohe, 2005) can be used as an approximation of long-term effects on the macroinvertebrate community composition and vice versa.

3.6 Conclusions

We conclude that pesticide residues in macroinvertebrates are suitable to assess the overall pesticide exposure and effects in agricultural streams. This method appears to be at least as reliable as the assessment based on pesticide concentrations in stream water after run-off events. Water concentrations underestimated the high toxicity in one stream that was indicated by body burdens and by the adaptation of macroinvertebrates to insecticides at the species and community levels. In contrast, the back-calculation from body burden to water concentration tended to overestimate the pesticide toxicity in highly exposed streams. However, both assessment methods generally provided adequate results that were confirmed by the observed pesticide effects on macroinvertebrates. We suggest that the comparison between body burden and synthetic passive samplers is an important area for future investigation.

3.7 Acknowledgements

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3.8 Author Contributions

N.S. collected the water samples and the invertebrates for the chemical analysis and SPEAR calculation, conducted the sensitivity tests, analyzed the data and wrote the first draft of the manuscript. J.M.B. contributed to the data analyses, interpretation of results and writing of the manuscript. M.K. and W.B. performed the chemical analyses and contributed to the writing of the manuscript. M.L. conceived the concept of research and contributed to the interpretation of results and writing of the manuscript.

3.9 Supporting Information

Figures showing the study map, illustration of event-driven water sampler (EDS), comparison of the ecological status of streams, and effect of species diversity on the adaptation to pesticides. Tables showing physicochemical properties of investigated streams, compound descriptors, list of median lethal concentrations and reference organisms used in TU calculation, and lists of pesticides analysed and detected in water and *G. pulex*.

4

Environmental stress increases synergistic effects of pesticide mixtures

4.1 Abstract

Some widely used pesticide mixtures produce more than additive effects according to conventional combined effect models. However, synergistic effects have been so far generally observed at unrealistically high pesticide concentrations. Here, we used *Daphnia magna* as a test organism and investigated how food limitation – a common ecological stressor – affects the mixture toxicity of a pyrethroid insecticide and an azole fungicide. We also compared three models regarding the prediction of mixture effects including concentration addition (CA), effect addition (EA) and stress addition model (SAM). We revealed that especially under low food, the strength of synergism between esfenvalerate and prochloraz increased with an increasing concentration of prochloraz independent of the null model. Under high food conditions and at concentrations of prochloraz $\geq$ 32 µg/L, we observed a marginal synergistic effect with an MDR = 2.1 at 32 µg/L prochloraz and 2.2 at 100 µg/L prochloraz when using CA as null model. In contrast, the combination of both pesticides and food stress caused synergistic effects shown by an MDR = 10.9 even at 1 µg/L of prochloraz that is frequently detected in the environment. The combined effects of pesticides and food stress could be predicted best with the stress addition model (SAM) that showed the

lowest mean deviation between effect observation and prediction (mean deviation SAM = 16 [SD = 28], EA = 1072 [2105], CA = 1345 [2644]). We conclude that common environmental stressors can strongly increase the synergistic effects of toxicants. This knowledge is especially relevant considering current efforts to include the additional risk of pesticide mixtures and environmental stressors into the environmental risk assessment of pesticides.

4.2 Introduction

Over the last few decades, pesticide contamination originating from intensive agricultural land use has been observed to cause negative impacts on the structure of freshwater communities (Knillmann et al., 2018; Liess and Von der Ohe, 2005; Schäfer et al., 2012) and ecosystem functions (Beketov et al., 2013; Münze et al., 2015; Rasmussen et al., 2012; Schäfer et al., 2012). Other studies have further discussed the decline in aquatic invertebrate biodiversity (Beketov et al., 2013) or decline in terrestrial biomass (Benton et al., 2002; Fox, 2013) due to pesticides.

The frequent occurrence of negative effects of pesticides on non-target organisms in the field shows that the current environmental risk assessments of pesticides fail to determine protective thresholds of risk. This scenario mainly occurs due to (i) an error prone estimation of pesticide exposure (Knabel et al., 2013, 2012) and (ii) because pesticides are commonly evaluated as single products without considering realistic environmental stress and exposure conditions (Liess et al., 2016). In agricultural practice, pesticides are often applied together as tank mixtures in spray series and hence co-occur in the environment. For example, high loads of pesticide mixtures can be found in streams, especially after run-off events (Knillmann et al., 2018; Liess et al., 1999; Liess and Von der Ohe, 2005; Martin et al., 2003; Riise et al., 2004; Schäfer et al., 2007; Werner et al., 2004).

Especially, azole fungicides have been reported to cause synergistic effects when co-occurring with pyrethroids (Bjergager et al., 2011, 2012; Kretschmann et al., 2015; Nørgaard and Cedergreen, 2010; Pilling and Jepson, 1993). Neonicotinoids (Iwasa et al., 2004), organophosphates (Sejerøe, 2011), strobilurin fungicides (Cedergreen et al., 2006; Rösch et al., 2017) and bipyridylium herbicides (Cedergreen et al., 2008). These pesticides are frequently detected in agricultural streams (Inostroza et al., 2016; Knillmann et al., 2018; Munz et al., 2017; Münze et al., 2017; Shahid et al., 2018a). However, most studies on synergistic effects of pesticide mixtures only report interactions at higher concentrations than those commonly detected in the aquatic environment (Cedergreen et al., 2006; Nørgaard and Cedergreen, 2010). Additionally, studies on synergistic mixture effects are generally based on experiments without additional stress (Bjergager et al., 2017; Kretschmann et al., 2015; Nørgaard and Cedergreen, 2010). Organisms in the field

experience sub-optimal conditions and occasionally have to cope with severe environmental stress (Holmstrup et al., 2010). A recent meta-analysis revealed that environmental stress severely enhances the toxicity of individual pesticides (Liess et al., 2016). Examples in the meta-analysis include food stress (Beketov and Liess, 2005; Pieters et al., 2005), competition (Knillmann et al., 2012) and UVB radiation (Liess et al., 2001) that can increase the sensitivity of organisms to toxicants up to a factor of 100 depending on the strength of environmental stress.

Despite numerous studies on the influence of environmental stress on the effect of single toxicants, only little attention was paid to the combined effect of environmental stress and pesticide mixtures. For example, Bjergager et al. (2012) investigated mixtures of esfenvalerate and prochloraz on *Daphnia magna* under semi-field conditions and detected similar and even higher synergism in the outdoor microcosms compared to those in laboratory studies. Also Delnat et al. (2019) reported that the daily temperature variation can increase the toxicity of a pesticide mixture of an organophosphate chlorpyrifos and a biopesticide Bacillus thuringiensis var. To our knowledge, apart from these studies, there is no information on pesticide mixtures under relevant field conditions, including environmental stressors.

To determine protective concentration levels of individual pesticides for regulatory purposes, we need to understand and quantify to what extent pesticide toxicity is increased by synergistic interactions and additional environmental stressors. Until now, approaches are lacking to predict the effects of mixtures that act synergistically. Traditional approaches such as concentration addition (CA) for similar acting compounds and effect addition (EA, also known as "independent action") for dissimilar acting compounds assume additive effects. Among these two approaches, CA is usually considered the most conservative approach (Belden et al., 2007; Bjergager et al., 2017; Hassold and Backhaus, 2014). In comparison, Liess et al. (2016) recently developed a new model, the 'stress addition model' (SAM), to specifically predict the synergy between environmental stressors and individual toxicants. However, SAM has not been tested yet for pesticide mixtures alone or in combination with environmental stress.

The aim of the present study is to identify the synergistic interactions of a frequently applied pesticide mixture, esfenvalerate and prochloraz (UBA, 2019) in combination with a common stressor, food limitation (Beketov and Liess, 2005; Pieters et al., 2005; Rose et al., 2002). For this, we performed experiments with *D. magna* for 28 days that included mixtures of environmentally realistic concentrations of both pesticides and the additional environmental stress. Furthermore, we analysed the prediction of the combined effects using traditional approaches for toxicant mixtures (i.e., CA, Loewe and Muischnek (Loewe and Muischnek, 1926) and EA, Bliss (Bliss, 1939)). We further tested the SAM to predict combined effects of environmental and toxicant stressors.

4.3 Materials and Methods

We studied the combined effect of the insecticide esfenvalerate and the fungicide prochloraz under high and low food conditions. For pesticide exposure, we set up a fully crossed factorial design with eight esfenvalerate treatments (0, 0.0001, 0.001, 0.01, 0.1, 0.316, 1, 3.16 µg/L) × four prochloraz concentrations (0, 1, 32, 100 µg/L) × two food levels (high, low) (Table C1). The experiment was repeated three × for all treatments of prochloraz apart from 32 µg/L. In addition, very low concentrations of esfenvalerate (0.0001, 0.001 and 0.01 µg/L of esfenvalerate) were included at a later stage (i.e. in second or third repetition) to complement the concentration response curve in the low effect range of esfenvalerate especially under low food conditions (Table C1). Before pesticide exposure, organisms were acclimatized to the corresponding food conditions for 7 days. Organisms were exposed to pesticides for 24 h, and survival was monitored for 3 weeks. For each treatment, we tested 15 daphnids with one individual per vessel containing 80 mL of the test solution (see also Table C1). The mortality of the daphnids was checked daily and dead individuals were removed from the experiment. Neonates from each vessel were removed daily. The total duration of the experiment was 4 weeks including the period of 1 week for acclimation to the respective food levels.

4.3.1 Test organisms

In all experiments, we used *D. magna* individuals obtained from a clone "Aachen V" cultured at the Department System-Ecotoxicology, Helmholtz Centre for Environmental Research – UFZ, Leipzig, Germany. Daphnids were cultured in beakers (20 individuals/beaker) with 1800 mL of artificial Daphnia medium (ADaM) (Klüttgen et al., 1994). The temperature of the culture medium was maintained at 20.0 $\pm$ 1.0°C under a photoperiod of a 16/8 h light/dark cycle that facilitated continuous amictic reproduction (Sebens, 1982). Individuals were fed with a suspension of green algae *Desmodesmus subspicatus* at 0.5 $\times 109$ cells $\mathrm{ind}^{-1}\ \mathrm{day}^{-1}$ in the first week and 0.75 $\times 109$ cells $\mathrm{ind}^{-1}\ \mathrm{day}^{-1}$ in the second week. On weekends daphnids were additionally fed with yeast (0.6 mg/L). In the culture and during the experiments, the medium was changed every second day, and neonates were removed within 24 h. The microalgae *D. subspicatus* was cultured in a mixture of distilled water and algae medium (ratio 9:1) (Grimme and Boardman, 1972) at 20.0 $\pm$ 1.0°C under continuous light and shaken through a mixture of CO_2 and compressed air (air: 300 bar, CO_2: 3 bar). The algae were harvested in the exponential growth phase and centrifuged, and the pellets were re-suspended in ADaM to obtain the required dilutions. During the test, the organisms used in the high food treatment were fed with 0.5 $\times 109$ cells $\mathrm{ind}^{-1}\ \mathrm{day}^{-1}$

the first week, 1.15 $\times 109$ cells $\mathrm{ind}^{-1}\ \mathrm{day}^{-1}$ the second week, and 1.35 $\times 109$ cells $\mathrm{ind}^{-1}\ \mathrm{day}^{-1}$ the third and fourth weeks. In contrast, organisms in the low food treatment were fed with 0.5 $\times 107$ cells $\mathrm{ind}^{-1}\ \mathrm{day}^{-1}$ the first week, 1.15 $\times 107$ cells $\mathrm{ind}^{-1}\ \mathrm{day}^{-1}$ the second week, and 1.35 $\times 107$ cells $\mathrm{ind}^{-1}\ \mathrm{day}^{-1}$ in the third and fourth weeks. The food dosage for low food conditions was established according to preliminary range finding tests that showed a minor effect on the survival of individuals (around 15% as compared to high food conditions) until the end of experiment (i.e., 4 weeks). Fecundity rates at the low food condition were decreased (number of eggs per female over 21 days = 0.18) as compared to high food conditions, but comparable to temporary conditions in the field. In the field, cladoceran populations have been studied to experience severe food limitation that causes a reduction in egg production close to zero (Tessier, 1986) and a crash of the population under observation (Müller-Navarra and Lampert, 1996).

4.3.2 Exposure to contaminants

We selected the pyrethroid esfenvalerate (Chemical Abstracts Service (CAS) 66230-04-4, purity: 99.8%) and the azole fungicide prochloraz (CAS 67747-09-5, purity: 98.6%) for the pesticide mixtures. We selected these pesticides because (i) azole fungicides and pyrethroid insecticides are known to cause synergistic effects and (ii) are frequently applied in agriculture in the form of mixtures (UBA, 2019). We tested concentrations of esfenvalerate, except the highest concentrations (1 and 3.16 µg/L esfenvalerate), that are in the range of those detected frequently in the field ranging from trace concentrations to 0.166 µg/L (Bacey et al., 2005; Münze et al., 2017) or even 0.76 µg/L (Cooper et al., 2003). The lowest tested concentration was even below the regulatory acceptable concentration (RAC) of esfenvalerate (EU RAC, 0.0005 µg/L; European Food Safety Authority (EFSA (EFSA, 2014)). In comparison, prochloraz concentrations are in the range of low to environmentally unrealistic concentrations of 100 µg/L. Frequently detected concentrations of prochloraz in European surface waters range from trace concentrations to 2.9 µg/L (Kreuger, 1998; Kreuger et al., 2010; Münze et al., 2017). We applied prochloraz and esfenvalerate at analytical grades (Sigma-Aldrich, Germany). We used dimethyl sulfoxide (DMSO) as a solvent for the preparation of the stock solution of esfenvalerate and prochloraz. The DMSO concentration was always kept below 0.02% [vol/vol] that is two orders of magnitude lower than the LOEC (Lowest observed-effect concentration; 2%) (Bowman et al., 1981) and under the solvent limit suggested by Organisation of Economic Cooperation and Development (OECD) guidelines (OECD, 2000).

4.3.3 Chemical analysis of the test media

Exposure concentrations of esfenvalerate and prochloraz were analysed for all treatments per experimental repetition. Samples were analysed 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, Hennigsdorf, Germany). The software Trace Finder 3.2 (Thermo Fisher Scientific) was applied for data processing. The measured concentrations of esfenvalerate and prochloraz in the experimental repetitions are given in the Supporting Information (Table C2). The median measured concentration of each nominal concentration ranged in acceptable boundaries (± 20%). The concentrations below the detection limit (i.e., 0.0001 and 0.001 µg/L) were confirmed by higher concentrations serving as stock solutions for serial dilutions. Results in subsequent sections are displayed and analysed using nominal concentrations.

4.3.4 Statistics and comparison of predictive models

To compare the LC_{50} concentrations of esfenvalerate between the different levels of food stress and prochloraz, we calculated LC_{50} and the 95% confidence intervals using a five-parameter log-logistic model for concentration-response relationships (Ritz and Streibig, 2005). The LC_{50} values of esfenvalerate were derived by fitting a five-parameter log-logistic model to the survival per treatment. The survival per treatment was averaged over the three repetitions before fitting. Single LC_{50} for each repetition were also determined to calculate the confidence intervals. As the survival of *D. magna* did not significantly differ from 7 days to 21 days after exposure (paired sample t-test; *p*-value > 0.05), we used the data for day 7 for further analysis.

In the present study, we first investigated the toxicity of the pesticide mixture under high and low food conditions. For this purpose, we compared the LC_{50} of esfenvalerate for different prochloraz treatments under high and low food conditions in relation to the respective control groups (i.e., high and low food conditions at 0 µg/L prochloraz). Secondly, we investigated the combined effect of pesticide and environmental stressors. For this, we compared different prochloraz treatments under low food conditions in relation to the high food control at 0 µg/L prochloraz as the optimal laboratory condition.

We evaluated the predicted combined effects for the first and second analysis by applying different additive approaches (CA and EA) and one approach designed for synergistic interactions (SAM). Both the EA (Bliss, 1939) and CA (Loewe and

Muischnek, 1926) models are commonly applied to predict mixture effects and assume the additivity of effects. For the EA approach, the effect was predicted using the following equation (Eq. 4.1):

$$E(C_{mix}) = 1 - \prod_{i=1}^{n}(1 - E(Ci)) \tag{4.1}$$

where E(C_{mix}) is the total effect of all stressors E(C_i). For the CA approach, the prediction was based on the following equation (Eq. 4.2):

$$ECx_{mix} = \sum_{i=1}^{n} \frac{p_i}{ECx_i} \tag{4.2}$$

where ECx_{mix} is the total concentration of the mixture including environmental stress, pi indicates the proportion of component *i* in the mixture, and ECx_i is the concentration of component *i* producing a $\times$% effect. Environmental stress was converted into a concentration level via mortality based on the concentration-response relationship of the toxicant (for details see Liess et al. (2016)).

In comparison to the additive approaches CA and EA, the SAM was developed to predict synergistic effects of independent stressors, such as a toxicant and an environmental stressor (Liess et al., 2016). According to Liess et al. (2016) the prediction of the SAM model are based on three principal assumptions: (i) each individual has a certain stress capacity to tolerate all types of stress without showing an effect; (ii) every stressor can be transferred into a general stress level that ranges from 0 to 1 using stress-level related mortality as the common link (0 = no mortality, 1 = 100 % mortality); and (iii) the joint effect can be estimated by adding up general stress levels exerted by independent stressors. The details and formulas are given in Liess et al. (2016) and the software INDICATE.

We applied CA, EA and the SAM to predict LC_{50} using the software INDICATE (Version 1.0.0; http://www.systemecology.eu/indicate/). To quantify the predictive accuracy of the models, a model deviation ratio (MDR) was calculated for the CA, EA and SAM models by dividing the predicted LC_{50} values by the observed LC_{50} values. Belden et al. (2007) suggested the model deviation ratio as a simple measure of model accuracy. The authors further suggested the range of 0.5 < MDR < 2 as an arbitrary 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 the term "high synergism" or "strong synergism" when the MDR values were > 10 using concentration addition (CA) as the null model. Additionally, we calculated the mean deviation factor of all MDRs for different treatments of prochloraz and food using the three prediction models. In cases with MDR values < 1, we determined the deviation factor by dividing the predicted LC_{50} and the observed LC_{50}. Combined effects were considered to be significantly synergistic if the MDR values were > 2 and, if the 95%

confidence intervals of observed and predicted LC_{50} values of the three single repetitions did not overlap (Belden and Lydy, 2006; Coors and De Meester, 2008). Except the determination of observed and predicted LC_{50} values, we generated all figures and statistical analyses using the software R studio (version 1.0.44) and R (version 3.0.3).

4.4 Results

4.4.1 Synergistic potential of azole fungicide prochloraz

To reveal general differences between the toxicity of the pesticide mixture under different food levels, we compared the toxicity of esfenvalerate at different concentrations of prochloraz under high and low food conditions in relation to respective control groups (i.e., high and low food controls). Under high food conditions, prochloraz alone did not show any significant effect on the survival, even at the highest concentration. However, under low food conditions, the survival was significantly affected by higher concentrations of prochloraz ($\geq$32 µg/L prochloraz, Wilcoxon's rank sum test, *p*-value < 0.05; Figure 4.1B). Further, we observed that under both food conditions, the strength of synergism between esfenvalerate and prochloraz increased with increasing concentration of prochloraz. Under high food conditions, synergistic effects between both pesticides could only be observed at higher concentrations of prochloraz ($\geq$32 µg/L prochloraz; Figure 4.1A, Table 4.1). However, these synergistic effects in relation to CA were only moderate under high food conditions, as shown by an MDR of 0.82 to 2.18 but not significant (Table 4.1). In comparison, the threshold for the synergistic effects of prochloraz under low food conditions was lower than that under high food conditions ($\geq$ 1 µg/L prochloraz; Figure 4.1B, Table 4.1) using CA as the reference model. With increasing concentrations of prochloraz, the MDR for LC_{50} increased to 2.6, 13 and 1925 for 1 µg/L, 32 µg/L and 100 µg/L prochloraz, respectively.

Regarding the prediction of the mixture effects of esfenvalerate and prochloraz, we observed that under high food conditions, the mean deviation of the predicted combined effect from the observed effect was similar for all three approaches (Figure C1, Table 4.1). However, under low food conditions, EA and to a lesser extent CA provided the most accurate predictions at lower concentrations of prochloraz (1 and 32 µg/L prochloraz), while the SAM highly overestimated the combined effect. In contrast, at the highest concentration of prochloraz (100 µg/L), the SAM predictions were the most precise (Figure C2, Table 4.1). Additionally, when we took the average of all treatments (i.e., 1, 32 and 100 µg/L of prochloraz), the SAM predictions deviated two and six times less from the observed effect compared to the predictions of EA and CA, respectively (Figure C2, Table 4.1). The

results indicate that the SAM provides the best predictions of mixture toxicity if strong synergistic interactions are expected.

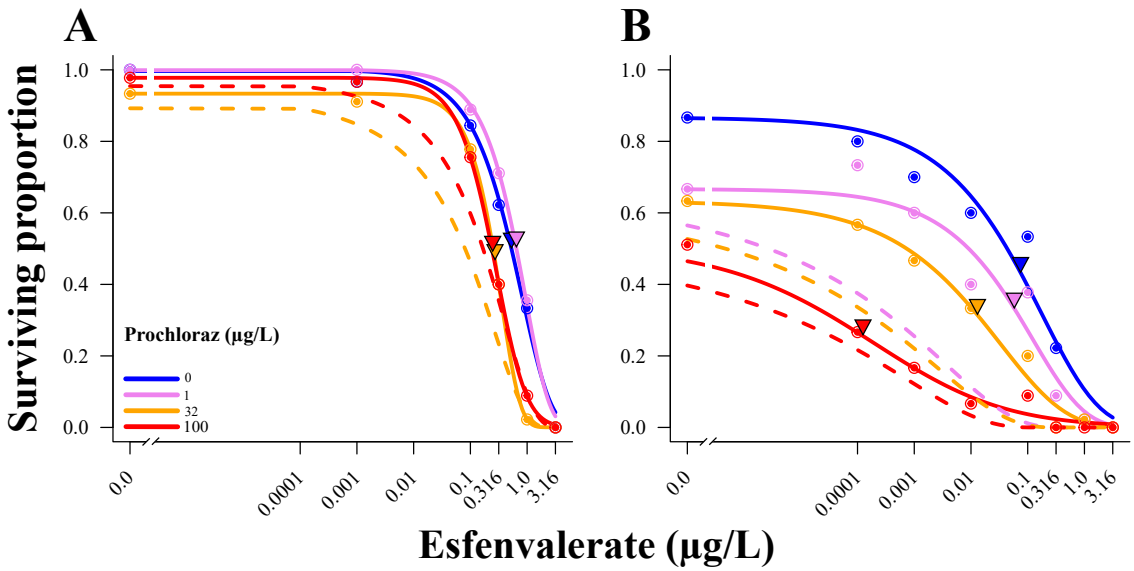

Figure 4.1: Survival of *Daphnia magna* at day 7 after an exposure of 24 h to the mixture of esfenvalerate and prochloraz under (A) high food and (B) low food conditions. Data points represent an average survival based on all experimental repetitions that was calculated relative to the initial number of individuals. The solid lines show the fitted observed concentration-response relationships, and the dashed lines represent the modelled concentration-response relationship under additional stress using the SAM. Under high food conditions (A), the predicted concentration-response relationship at 1 µg/L of prochloraz is not shown; because SAM requires an effect > 0% at control conditions (0 µg/L esfenvalerate). At 1 µg/L prochloraz alone there was no measurable effect on the survival of *D. magna* under high or low food conditions. Triangles display LC_{50} values of different concentration-response curves.

4.4.2 Interaction of three stressors including both pesticides and food limitation

For the combined effect of both pesticides and food stress, we performed similar analysis as in the previous chapter Synergistic potential of azole fungicide prochloraz at high and low food conditions. In comparison, we here compare all treatments of low food and prochloraz to the control with high food and without prochloraz as the optimal laboratory condition (best case). Our results show that in comparison to prochloraz and esfenvalerate under high food conditions (Figure 4.1A, Table 4.1), the combination of food stress and prochloraz notably

increased the sensitivity of daphnids to esfenvalerate (Figure 4.2, Table 4.1). The MDR values determined for the LC_{50} of esfenvalerate using CA were 7.7, 10.9, 50.2 and 5312 for the low food conditions with 0, 1 µg/L, 32 µg/L and 100 µg/L prochloraz, respectively. Where applicable, we determined significance of the synergistic effect (i.e. three repetitions per treatment of prochloraz) and identified significant synergistic effects at 0, 1 and 100 µg/L of prochloraz (Table 4.1). When comparing the predictions of CA, EA and the SAM for the effect of all three

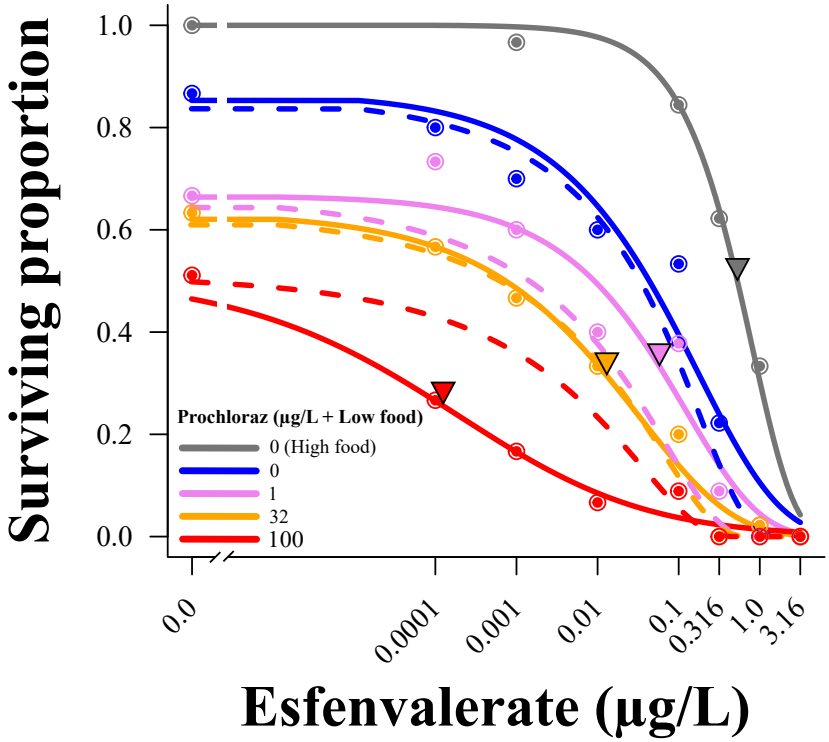

Figure 4.2: Survival and concentration-response curves of *Daphnia magna* exposed to a mixture of esfenvalerate and prochloraz and low food as an additional stress (interaction of three stressors). Data points represent an average survival based on all experimental repetitions that was calculated relative to the initial number of individuals. Organisms exposed to esfenvalerate alone under high food conditions were considered as control. The solid lines show the observed concentration-response relationships, whereas the dashed lines represent the modelled concentration-response relationships under the additional stress using the stress addition model (SAM). Triangles denote LC_{50} values for different concentration-response curves.

stressors, we found that the SAM performed best in terms of the modelled curve (Figure 4.2, Figure C3) and lowest MDRs (Table 4.1). The models of CA and EA substantially underestimated the combined effect of all three stressors by up to three orders of magnitude at the highest concentration of prochloraz (Table 4.1, Figure C3). On average, the underestimation by CA and EA of the observed effect was 1345 and 1072 times, respectively. In comparison, the SAM predicted

Table 4.1: Experimental observations and predictions of *Daphnia magna* exposed to esfenvalerate alone and in combination with prochloraz under high and low food conditions.

	Prochloraz (µg/L)	Observed LC_{50}[1] 95% CI[2]	Predicted LC_{50}[3] 95% CI	Significance of synergism	MDR CA	MDR EA	MDR SAM
High food	0 (high food)	0.529 (-0.023–1.367)	–	–	–	–	–
	1	0.647 (0.087–1.419)	0.529 (-0.023–1.367)	–	0.82	0.82	0.82
	32	0.272 (0.146–0.323)	0.556 (-0.065–1.363)	–	2.05	1.95	0.36
	100	0.247 (0.189–0.317)	0.54 (0.001–1.366)	–	2.18	2.14	0.71
Low food	0 (low food)	0.0746 (0.036–0.245)	–	–	–	–	–
	1	0.0576 (0.035–0.165)	0.15 (0.076–0.299)	–	2.6	1.3	0.0042
	32	0.0127 –	0.167 –	NA	13.2	5.9	0.0123
	100	0.000125 (-0.03–0.093)	0.241 (0.11–0.32)	*	1925	597	0.2742
Combination of three stressors	0 (high food)	0.529 (-0.023–1.367)	–	–	–	–	–
	0 (low food)	0.0746 (0.036–0.243)	0.577 (0.244–0.584)	*	7.7	7.1	0.743
	1	0.0576 (0.035–0.165)	0.628 (0.182–0.899)	*	10.9	9.2	0.287
	32	0.0127 –	0.636 –	NA	50.2	41.8	1.093
	100	0.000125 (-0.03–0.093)	0.664 (0.207–0.749)	*	5312	4229	58

[1]The observed LC_{50} was calculated using the mean survival of the all experimental repetitions. [2]The 95% CI is based on three LC_{50} values calculated for separate repetitions. [3]The predicted LC_{50} was calculated by CA model using the mean survival of all experimental repetitions. Whereas, the 95% CI is based on three values calculated for separate repetitions. [4]Organisms exposed to esfenvalerate alone under high food conditions were considered as overall control (optimal laboratory condition). Synergism was considered significant if the 95% confidence intervals of observed and predicted LC_{50} did not overlap. NA: Determination of CIs was not applicable, because under low food conditions prochloraz treatment with 32µg/L was repeated only two times.

best at 0, 1 µg/L, and 32 µg/L prochloraz (Figure C3; Table 4.1). Nevertheless, in the case of the highest concentration of prochloraz (100 µg/L), the SAM also underestimated the total effect by a factor of 58, which was still 92 and 73 times greater than those estimated by CA (i.e., 5312 times) and EA (i.e., 4229 times), respectively (Figure C3, Table 4.1).

In addition, the increase in toxicant sensitivity due to the combined effect of the three stressors compared to the survival under exposure to esfenvalerate alone,

was quantified as the shift in LC_{50} (LC_{50}/LC_{50}*). The LCx shifts modelled by the SAM and observed in different experiments were significantly correlated (LC_{50}: adjusted R^2 = 0.83, *p*-value = 0.006, n = 6; LC_{10}: adjusted R^2 = 0.64, *p*-value = 0.01, n = 7; Figure C4).

4.5 Discussion

In the present study, we revealed synergistic effects of the pesticide mixture of esfenvalerate and prochloraz under different food conditions. The results of our study show that synergistic effects between prochloraz and esfenvalerate were dramatically increasing under low food conditions. Based on CA, the threshold for synergy (MDR > 2) for both pesticides decreased from 32 µg/L prochloraz under high food conditions to 1 µg/L prochloraz under low food. This threshold concentration of 1 µg/L can be realistically expected in surface waters (Kreuger, 1998; Legrand et al., 1991; Weltje, 2013) and is lower than that reported in previous studies without additional stress. For example, Nørgaard and Cedergreen (2010) identified synergistic effects of alpha-cypermethrin and prochloraz on *D. magna* at higher concentrations of prochloraz ($\geq$ 99$\pm$8 µg/L). Bjergager and co-authors (2012) exposed *Daphnia magna* to different combinations of esfenvalerate with 90 µg/L prochloraz in microcosms and observed up to a 14 fold increase in mortality compared to the mortality in the CA predictions. In comparison, Bjergager et al. (2017) observed synergy of prochloraz and alpha-cypermethrin at 9.794 $\pm$ 4.897 µg/L prochloraz towards the immobilisation of *D. magna* under laboratory conditions. The authors also observed that the threshold of synergistic effects decreased to 5.651 $\pm$ 1.507 µg/L from 48 h to 14 days after contamination. This threshold concentration is still higher than that in our experiment, where we detected a synergistic effect at 1 µg/L prochloraz under low food conditions. In addition, Bjergager et al. (2017) exposed daphnids to fungicides during the whole experiment, while we applied a simultaneous peak exposure to both pesticides for only 24 h. The short exposure in our study might have led to a higher detected threshold concentration of synergistic effects than those in studies with longer or continuous exposure (Duquesne et al., 2006). Hence, this is the first study to reveal strong synergistic effects of pesticide mixtures at environmentally realistic concentrations under low food conditions.

In terms of the pyrethroid esfenvalerate, we recorded strong effects on the survival of *D. magna*. The LC_{50} of esfenvalerate at low food conditions decreased with increasing concentrations of prochloraz. At the nominal concentration of prochloraz ($\geq$1 µg/L), the LC_{50} of esfenvalerate was 0.058, which is more than one order of magnitude lower than the concentrations frequently detected in field (Cooper et al., 2003). Further, at higher concentrations of prochloraz (100 µg/L), the LC_{50} of esfenvalerate decreased up to 0.000125 µg/L that is two orders of

magnitude lower than the LC_{50} (0.012 µg/L) reported by Bjergager et al. (2012) for *D. magna* exposed to esfenvalerate and prochloraz. In the present study, this lower LC_{50} could be due to the additional environmental stress of low food.

MDR for the CA reference model underestimated the LC_{50} of esfenvalerate up to 5312 fold at 100 µg/L prochloraz and low food conditions compared to that of the control conditions without prochloraz and food stress (high food control). The identified MDRs were also much stronger than those detected for comparable concentrations of prochloraz (Bjergager et al., 2017; Nørgaard and Cedergreen, 2010). Until now, the highest synergism between two pesticides has been reported for *Ceriodaphnia dubia* exposed to cypermethrin in the presence of piperonyl butoxide with a 137 fold increase in toxicity by Wheelock et al. (2004). The high level of synergism of the pesticide mixture in the present study was due to the additional impact of food stress. The presence of food stress alone without prochloraz already increased the toxicity of esfenvalerate by a factor of seven. Starving organisms may have low energy reserves for physiological defence against stress and therefore show more sensitivity to contaminants (Sibly, 1999). As a possible consequence, some studies previously reported that the toxicity of metals and pesticides on invertebrates increased due to food limitation (Barry et al., 1995; Beketov and Liess, 2005; Koivisto et al., 1992; Pieters et al., 2005; Spadaro et al., 2008).

In the present study, we found that CA and EA generally underestimated the combined effects of the pesticide mixture under low food conditions as well as the interaction of all three stressors (Table 4.1, Figure C3). These results are not surprising for synergistic mixtures, because CA and EA assume additive effects. In contrast, the SAM, which is designed to predict synergism between toxicants and environmental stress, predicted the combined effects of both pesticides and food stress better than EA and CA (Figure 4.2, C4; Table 4.1). In general, SAM is able to predict a certain range of synergism with the most robust predictions for strong synergistic effects. However, even the SAM underestimated the combined effect of the pesticides and food stress at the highest concentration of prochloraz (100 µg/L). The underlying mechanisms for this high synergism should be the subject of future investigations.

The interactions of biotic– and abiotic stress factors are much more complex under field conditions, modifying the sensitivity of communities and populations to contaminants (Heugens et al., 2006; Jonker et al., 2004; Relyea and Hoverman, 2008). Recently, Delnat et al. (2019) investigated the effect of a common environmental stressor – daily temperature variation – on the combined toxicity of an organophosphate chlorpyrifos and a biopesticide Bacillus thuringiensis var towards vector mosquito *Culex pipiens*. A high variation in daily temperature changed the combined effect of both pesticides from additive to synergistic. Similarly, Gandar et al. (2017) reported higher toxic effect of a pesticide mixture to-

wards molecular response of a goldfish (*Carassius auratus*) at 32°C as compared to 22°C. Other investigations also have reported synergistic interactions among various environmental and toxicants (Holmstrup et al. (2010) and calculated by Liess et al. (2016)), however, only single toxicant exposure was considered.

4.6 Conclusions

As a conclusion, mixtures of pesticides and environmental stressors may act in a strong synergistic manner on non-target organisms. Environmental risk assessments should consider these combined effects in order to be protective for the environment. Additionally, approaches such as the SAM can improve the prediction of the combined effects of synergistic toxicant mixtures and environmental stress.

4.7 Author contributions

Study design: NS, SK; conducting experiments: NS; data analysis and interpretation of results: all; drafting of the manuscript: NS; revising manuscript: all.

4.8 Supporting information

Tables showing description of experimental setup, and concentrations of pesticides analysed during different experimental rounds. Figures showing the survival of *Daphnia magna* exposed to a common mixture of esfenvalerate and prochloraz under high and low food conditions, interaction of multiple stress (esfenvalerate, prochloraz and food limitation), and relationship between LCx—shifts modeled by SAM and observed in different experiments (Supporting Information for chapter 3).

5

Pesticide-induced metabolic changes and environmental stress

5.1 Abstract

In natural ecosystems, long-term detrimental effects of pesticides may occur at very low concentrations, below those considered safe by the governmental risk assessment. Mechanisms potentially responsible for this unexpected sensitivity include environmental stress-factors, such as food deficiency. To reveal the relevant mechanisms, we investigated how food stress – a common ecological parameter – interacts with insecticide induced biochemical fingerprints. Therefore we measured metabolomic perturbations in *Daphnia magna* following a 24 h exposure to the pyrethroid esfenvalerate under high and low food conditions. In total, 160 metabolites covering the groups of amino acids, fatty acids, lipids and sugars were analyzed. At 0.001 µg/L esfenvalerate – a factor of 50 below the NOEC (0.052 µg/L) provided by the regulatory authorities, and a factor of 200 below the acute LC_{50} – the endogenous metabolome was significantly affected. Further, the effect under low food conditions was considerably stronger compared to high food conditions. Individual metabolites showed up to 7-fold stronger effects under low food conditions. In general, the metabolomic changes were largely dose-specific and increased over seven days after contamination. We conclude that the metabolic profiles are altered for at least seven days after a pulse exposure, and therefore might be a key process to understanding population level changes at ultra-low pesticide concentrations in the field.

5.2 Introduction

Over the past decades, the large-scale use of agricultural pesticides has raised concerns about their presence in the environment (Liess et al., 1999) and their effects on ecosystem structure and functions (Landis et al., 2003; Liess and Schulz, 1999). In fact, several studies have shown a decline in aquatic and terrestrial biodiversity due to pesticides (Beketov et al., 2013; Benton et al., 2002; Butchart et al., 2010; Fox, 2013). Such pesticide effects have been reported far below the concentrations that are considered to be safe by regulatory authorities (Knillmann et al., 2018; Liess and Von der Ohe, 2005; Schäfer et al., 2012). Reasons for these low effect concentrations in the field include higher sensitivity of individuals to pesticides under suboptimal conditions (Liess et al., 2019, 2016).

Dynamic environmental factors, such as food availability, temperature and intraspecific competition may further enhance the potential effects of contaminants (Hines et al., 2007; Morrison et al., 2007; Viant, 2007). Lab investigations have also identified pesticide effects under ultra-low concentrations starting at around 100 times below the LC_{50} (Liess et al., 2019). For example Liess and Schulz (1996) observed delayed effects of a pyrethroid Fenvalerate on caddisflies at more than three orders of magnitude below the LC_{50}. Similarly, Siddique et al. (2020) and Cold and Forbes (2004) showed long term effects on key life traits in *Gammarus pulex* at very low pesticide concentrations. However, the underlying mechanisms of these unexpected effects at ultra-low pesticide concentrations are unclear. In such scenarios, it is important to link the molecular actions of pesticides to their possible interference with biological processes for a better understanding of pesticide toxicity, specifically at ultra-low concentrations. Ultimately, this knowledge can contribute to the extrapolation of the laboratory data to more realistic scenarios in the field.

Numerous promising techniques such as proteomics, transcriptomics and metabolomics have already been developed for characterizing the biological responses to environmental stressors (Escher et al., 2017; Jemec et al., 2010). Since food stress is supposed to directly affect the metabolism, the analysis of endogenous metabolites might provide information on the molecular adaptation under stress. Metabolomics is a well-established –omics technique, widely applied to analyze endogenous metabolites within a cell, tissue or biofluid (Bundy et al., 2004; Jones et al., 2008; Viant et al., 2006; Wu et al., 2011). Metabolomics can provide a comprehensive evaluation of a biological response of living organisms under stressed conditions (Bundy et al., 2008; Garreta-Lara et al., 2016), and adds to the base of knowledge on the potential effects of contaminants that are of great environmental concern (Bundy et al., 2008; Van Aggelen et al., 2009; Viant et al., 2003). This technique has been employed for a wide range of organisms to characterize the effects of toxicants (Jongeneelen, 2001; Kovacevic et al., 2016; Lanz

et al., 2009; Lucas et al., 1993) and environmental stressors (Garreta-Lara et al., 2018; Kullgren et al., 2013; Lin et al., 2006).

The drawback of metabolomics is that it detects the consequence of molecular adaptation or adverse effects rather than the regulation which is controlled on the transcriptional level. But on the other hand, the direct link to the phenotype results in the high sensitivity of metabolic changes as a response to external stressors. In spite of high relevance for the risk assessment of pesticides, only a few studies have investigated the metabolomic changes in freshwater invertebrates following an exposure to various toxicants (Jones et al., 2012; Maity et al., 2012; Nagato et al., 2013). For example, the crustacean *Daphnia magna* – a model test species – is extensively used in eco-toxicology, but metabolomics studies of this species are very rare (Taylor et al., 2008). Further, we are aware of only two metabolomics studies that investigated food stress induced changes to metabolite composition in *Daphnia magna* (Smolders et al., 2005; Wagner et al., 2015). However, none of the studies employed metabolomics to investigate the interaction between chemical and environmental stress. Therefore, more information is needed on metabolomic changes under conditions prevalent in the field (including chemical- and environmental stressors).

In the present investigation, we aim to explore the association between exposure to very low concentrations of the pyrethroid esfenvalerate and metabolic changes in *Daphnia magna*, under high and low food conditions. These key metabolic profiles can reveal the combined effects of chemical and environmental factors in the laboratory, which potentially lead to delayed/synergistic effects in the field.

5.3 Materials and methods

We studied lethal effects as well as metabolic changes in *Daphnia magna* related to the exposure of a pyrethroid insecticide esfenvalerate under high and low food conditions. In all experiments, we exposed *Daphnia magna* to low concentrations of esfenvalerate: 0.001, 0.01 and 0.1 µg/L for 24h and quantified metabolite contents at different time points (after 24h and on 4th and 7th day after exposure) using LC-MS-MS.

5.3.1 Culture of test organisms

Test organisms were obtained from a clone "Aachen V" cultured at the Department System Eco-toxicology, UFZ Leipzig. The culture was maintained at a constant temperature of 20.0 $\pm$1°C and a controlled photoperiod of 16h light/8h dark

cycle (Sebens, 1982). The daphnids were cultured in beakers (20 animals/beaker) with 1800 mL of ADaM (Artificial Daphnia Medium) (Klüttgen et al., 1994). The culture medium was renewed three times per week and daphnids were fed with green algae *Desmodesmus subspicatus* expressed in cells $\mathrm{ind}^{-1}\ \mathrm{day}^{-1}$. The quantity of food was 0.5 $\times$ 109 cells $\mathrm{ind}^{-1}\ \mathrm{day}^{-1}$ in the first week, which was then increased to 0.75 $\times$ 109 cells $\mathrm{ind}^{-1}\ \mathrm{day}^{-1}$. Additionally, organisms were also fed with yeast (0.6 mg/L) once per week. Algae were cultured in a mixture of distilled water and algae medium (ratio 9:1) (Grimme and Boardman, 1972) at 20.0 ± 1°C under continuous light and shaken through a mixture of CO_2 and compressed air (Air: 300 bar, CO_2: 3 bar).

5.3.2 Exposure to esfenvalerate and food stress

We selected a pyrethroid esfenvalerate (CAS 66230-04-4, purity: 99.8%) for pesticide exposure that was purchased from Sigma–Aldrich, Germany. We prepared the stock solution by diluting 5mg of esfenvalerate in 10mL of dimethyl sulfoxide (DMSO) solvent. The DMSO concentration was always kept below the solvent limit suggested by OECD guidelines (OECD, 2000). The stock solution was further diluted in ADaM to the required test concentrations. Briefly, we applied four esfenvalerate concentrations: control group, 0.001 µg/L (1/500 of LC_{50}), 0.01 µg/L (1/50 of LC_{50}) and 0.1 µg/L (1/5 of LC_{50}) and two food levels (i.e. high and low). After a 7 days period of acclimatization to the corresponding food conditions (low and high food), test organisms were exposed to esfenvalerate for 24 h following the OECD Guidelines for Testing of Chemicals (OECD, 2004). The applied esfenvalerate concentrations were somewhat lower than those concentrations frequently recorded in the field (Bacey et al., 2005; Cooper et al., 2003).

For both the control and pesticide concentrations, 120 daphnids were tested with 30 individual per beaker containing 1800 mL of the test solution. Thus, four glass beakers per concentration were prepared. The temperature of all experiments was maintained at 20.0 ± 1°C under a photoperiod of 16/8 h Sebens (1982). After a pulse exposure of 24 h, 30 surviving organisms from each concentration and control treatments were collected in 1.5 mL Eppendorf tubes and stored at –80°C for metabolite analysis. The remaining alive daphnids were transferred into beakers with uncontaminated medium, and fed with a respective amount of green algae. On day 4 and 7, again 30 surviving organisms from each treatment were collected for metabolite analysis. The experiment was performed in 11 replicates over a period of nine months. To verify exposure concentrations, we analyzed the test medium for all treatments throughout the experiment. The average measured concentrations of esfenvalerate showed an acceptable deviation ($\pm$20%).

5.3.3 Sample preparation

Daphnia magna individuals were stored in Eppendorf tubes at -80°C. For sample preparation, 1mL of acetonitrile/water (1:1) and 3 metal beads were added to each sample, and homogenized for 10 minutes using TissueLyser (30/s) model). Then, samples were centrifuged for 15 minutes (15,000 rpm at 5°C), dried in a vacuum centrifuge (V-AQ at 30°C for 4 h) and diluted in 1 mL of methanol/acetonitrile (1:1). Subsequently, these samples were treated with ultrasound under cooling with ice for 1 h and centrifuged again for 15 min (15,000 rpm at 5°C). Afterwards, supernatant was transferred into new Eppendorf tubes and dried again in a vacuum centrifuge (V-AQ at 30°C for 2 h).

5.3.4 Targeted Metabolomics

The metabolomic analyses were carried out with the AbsoluteIDQ® p150 Kit (Biocrates Life Science AG, Austria). The Kit identifies and quantifies 163 metabolites from 5 compound classes (acyl carnitines, amino acids, biogenic amines, glycerophosphol- and sphingolipids, and hexoses. The kit preparation was carried out following the manufacturer's instructions. Recently, Russo et al. (2018) successfully employed this metabolomics kit for analysing pesticide-induced metabolic changes in aquatic invertebrates.

The LC-MS/MS analysis was carried out by MRM acquisition using a Waters Acquity UPLC System (Waters, Eschborn, Germany) coupled with QTRAP 5500 (AB Sciex, Darmstadt, Germany). The following MS parameters were used: Ion Source: Turbo Spray, Curtian Gas: 20psi, CAD Gas: Medium, Ion Spray Voltage: 5500 V, Temperature: 500°C, Ion Source Gas 1: 40psi, Ion Source Gas 2: 50 psi. The analyses were performed by an isocratic FIA-MS-MS method with two runs (positive and negative ionisation mode) respectively as three min runs. Running solvent was a mixture of water and 5mM ammonium acetate in methanol (v/v) was used. Mass spectra were analyzed with Analyst Software version 1.6.2 and validated by the MetVal tool from the MetIDQ Software tool delivered by Biocrates Life Science AG. An automatic quality assessment was conducted by comparing the obtained values for blanks, internal standards and quality controls. The Kit has been validated according to the FDA Guidance for Industry.

5.3.5 Materials

Water: Milipore, PITC: Fluka (for proteine sequence analysis), Pyridine: Fluka (p.a.), Methanol (Merck KGaA, Darmstadt, Germany, hypergrade for LC-MS),

Acetonitril: Merck, Lichrosolv for LC/MS, Ammonium Acetate (Honeywell - Fluka, Seelze, Germany)

5.3.6 Data analysis

The data analyses were conducted using the statistical software R studio for windows (version 1.0.44) and R (version 3.0.3). To increase the reliability of analyses, the experiment was performed in 11 replicates. We exposed organisms to four different concentrations of esfenvalerate at two food levels, and analyzed 160 metabolites at four different time points. Thus, a data set of about 21,120 observations was analyzed (Tables D1-D4). The LC_{50} and 95% confidence intervals were calculated using the log-logistic model (Ritz and Streibig, 2005) with the five-parameter log-logistic function LL.5. We applied linear mixed-effects (LME) models to investigate the effect of esfenvalerate on (i) the overall metabolite content of the exposed daphnids and (ii) the content of each metabolite class separately. For the overall metabolite content, different pesticide concentrations were compared using the post hoc analysis with specific custom contrasts. Paired sample t-tests were used to compare treatments with control groups. A comparison of high and low food treatments was conducted using two sample t-tests. To improve the normal distribution of residuals, the data were ln(x) transformed prior to the analyses.

5.4 Results

5.4.1 Pesticide effect on overall metabolite content

We observed a significant effect of esfenvalerate on the overall metabolite contents of exposed organisms (Figure 5.1). The metabolic alterations were significantly stronger under low food conditions (Figure 5.1 b, d, Table D2). Under high food conditions, 24 h exposure to 0.01 µg/L and 0.1 µg/L of esfenvalerate caused a 10% and 6 % reduction of the overall metabolite content respectively (Figure 5.1a; $p < 0.01$). Under low food conditions, a dose-dependent down-regulation of metabolite content was observed (Figure 5.1b). The lowest concentration reduced 5% of the overall metabolite content ($p < 0.01$) followed by the medium (8%; $p < 0.001$) and the highest concentration (13%; $p < 0.001$). Further, the effect at lowest and highest concentration was significantly stronger than the effect observed under high food conditions (Table D2, two sample t-test; $p < 0.01$).

After a recovery time of 48 h (day 4), both an up- and down-regulation of metabo-

lites was observed (Figure 5.1c, d). Under high food conditions, the lowest and medium concentrations increased the metabolite content by 7 and 6% respectively (Figure 5.1c; $p < 0.01$). While the highest pesticide concentration resulted in a significant reduction (11%; $p < 0.001$). Under low food conditions, we observed up-regulation at 0.001 µg/L (10%; $p < 0.001$) and down-regulations at 0.1 µg/L (35%; $p < 0.001$). Remarkably, the effect at 0.1 µg/L was three times higher than the effect observed under high food conditions (two sample t-test; $p < 0.001$). Furthermore, on day 7, the reduction in metabolite content was notably increased

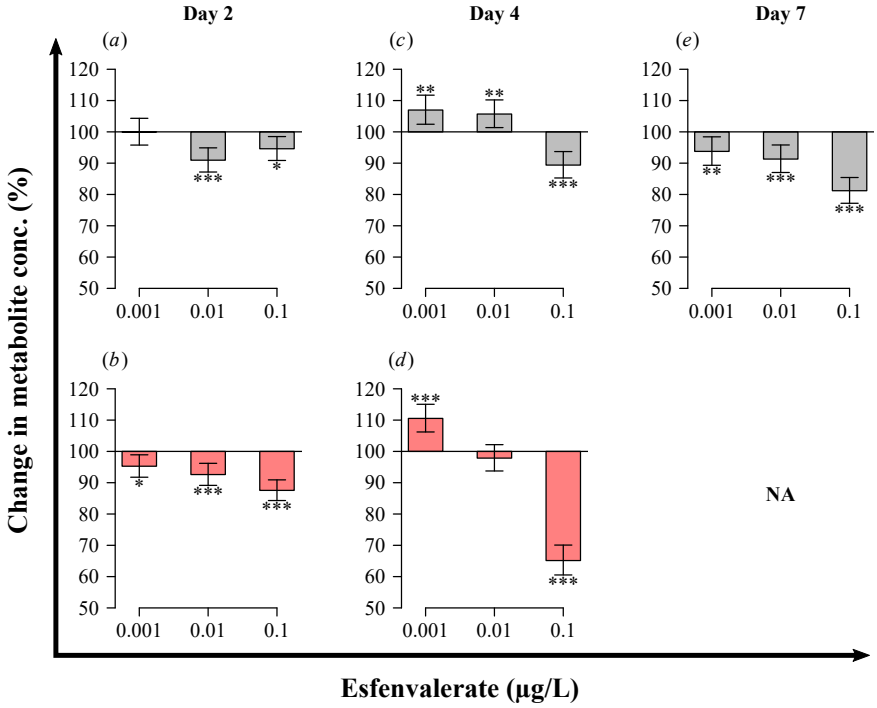

Figure 5.1: Percent change in the overall metabolite content of *D. magna* exposed to esfenvalerate in relation to the respective control groups. Daphnids were exposed to 0.001 µg/L, 0.01 µg/L and 0.1 µg/L of esfenvalerate for 24 h under (a, c, e; grey) high and (b, d; red) low food conditions. Metabolites were measured (a, b) directly after an exposure of 24h, (c, d) on day 4 (after a recovery time of 48h) and (e) on day 7. Changes are presented with 95 % confidence intervals and statistical significance is indicated with asterisks: "." $p < 0.1$ "*" $p < 0.05$ "**" $p < 0.01$ and "***" $p < 0.001$.

with an increasing concentration of esfenvalerate (Figure 5.1e). Exposure to 0.1 µg/L resulted in a 19% reduction in metabolic content ($p < 0.001$) followed by 0.01 µg/L (9%; $p < 0.001$) and 0.001µg/L (7%; $p < 0.01$).

5.4.2 Effect of pesticide exposure on metabolite classes

To understand the effect of pesticide exposure in respect to the underpinning mechanisms, we identified changes in different metabolite classes. On day 2 and day 4, we observed significant changes in two metabolite classes i.e., amino acids and glycerophospholipids under high food conditions (Figure 5.2a, 5.3a). Whereas under low food conditions, we even observed significant changes in five classes i.e., amino acids, glycerophospholipids, sphingolipids, acylcarnitines and sugars (Figure 5.2b, 5.3b). Furthermore, the effects under low food conditions were significantly stronger compared to those under high food conditions (Table D3).

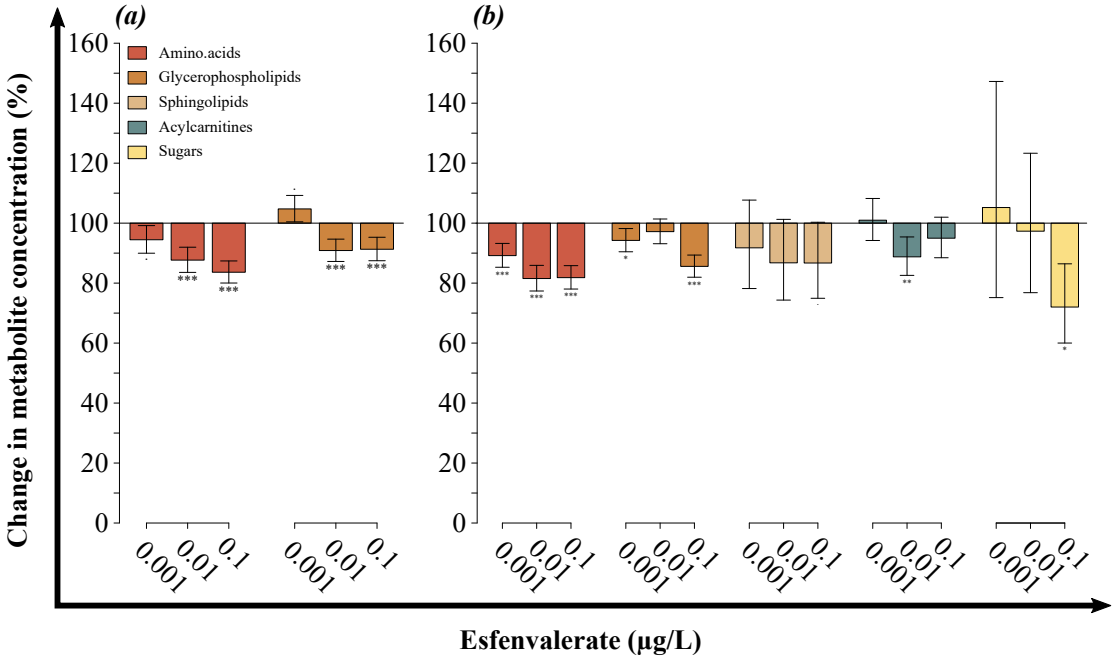

Figure 5.2: Percent change in the content of different metabolite classes of *D. magna* exposed to esfenvalerate in relation to the respective control groups. Daphnids were exposed to 0.001 µg/L, 0.01 µg/L and 0.1 µg/L of esfenvalerate for 24 h. Metabolites were measured directly after an exposure of 24h under (a) high and (b) low food conditions. Only metabolite classes with significant perturbations are reported. Changes are presented with 95 % confidence intervals and statistical significance is indicated with asterisks: "." $p < 0.1$ "*" $p < 0.05$ "**" $p < 0.01$ and "***" $p < 0.001$.

Under high food conditions, exposure to 0.1 and 0.01 µg/L significantly reduced the contents of amino acids by 17% and 13% respectively (Figure 5.2a, $p < 0.001$). Whereas, 0.001 µg/L marginally reduced the amino acids content (6%; p = 0.07). Similarly, exposure to 0.1 µg/L and 0.01 µg/L significantly reduced the contents of glycerophospholipid by 9% each ($p < 0.001$). Whereas, the lowest concentration

marginally increased the glycerophospholipid content (5%; p = 0.07). Under low food conditions, 0.1 and 0.01 µg/L each resulted in an 18% reduction in the amino acids content (Figure 5.2b, $p < 0.001$) followed by 0.001 µg/L (11%; $p < 0.001$). The decreased glycerophospholipid content was recorded at 0.001 µg/L (6%; $p < 0.05$) and 0.1 µg/L (15%; $p < 0.001$). However, reductions in sphingolipids and sugars were only observed at 0.1 µg/L (sphingolipids: 13%; $p < 0.1$, sugars: 28%; $p < 0.05$), and reduction in acylcarnitines was only recorded at 0.01 µg/L (11%; $p < 0.01$).

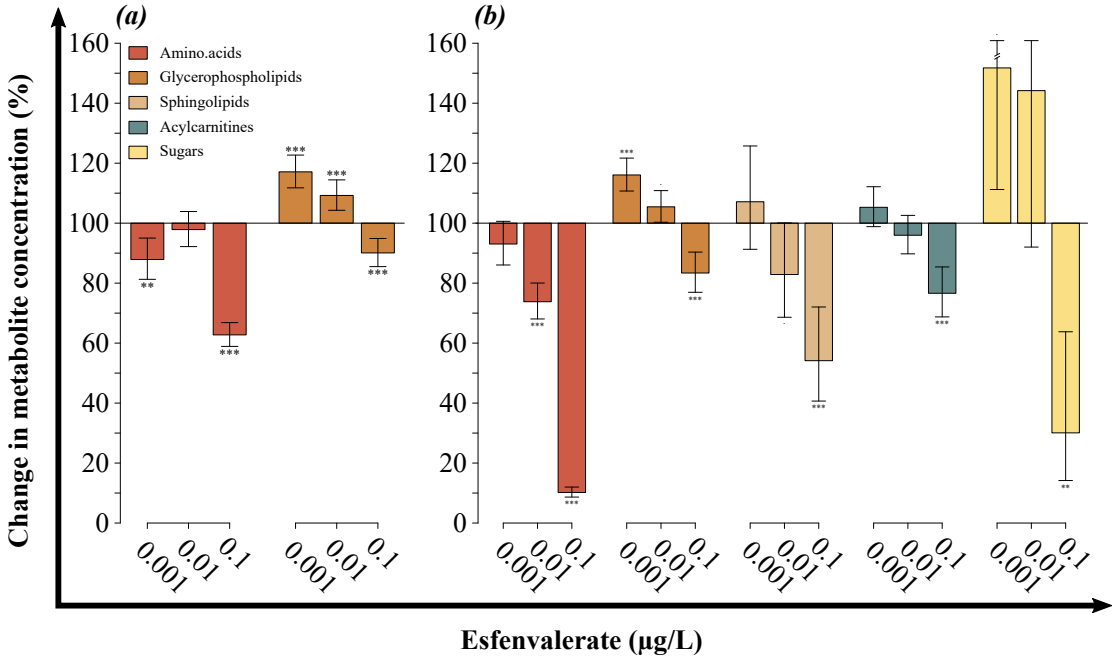

Figure 5.3: Percent change in the content of different metabolite classes of *D. magna* exposed to esfenvalerate in relation to the respective control groups. Daphnids were exposed to 0.001 µg/L, 0.01 µg/L and 0.1 µg/L of esfenvalerate for 24 h. Metabolites were measured on day 4 (after a recovery time of 48h) under (a) high and (b) low food conditions. Only metabolite classes with significant perturbations are reported. Changes are presented with 95 % confidence intervals and statistical significance is indicated with asterisks: "." $p < 0.1$ "*" $p < 0.05$ "**" $p < 0.01$ and "***" $p < 0.001$.

On day 4, there was an even greater reduction in the amino acids content of organisms exposed to 0.001 and 0.1µg/L ($p < 0.01$). Under high food conditions (Figure 5.3a), we observed down-regulation at 0.001 µg/L (13%; $p < 0.01$) and 0.1 µg/L (38%; $p < 0.001$). In contrast, exposure to 0.001 µg/L and 0.01 µg/L significantly increased the glycerophospholipid content by 17% and 19% respectively ($p < 0.001$). A reduction was only recorded at 0.1 µg/L (10%; $p < 0.001$).

Under low food conditions (Figure 5.3b), the metabolite content of amino acids down-regulated at medium concentration (13%; $p < 0.01$) and the highest con-

centration (13%; $p < 0.01$). Similar to high food conditions, glycerophospholipids showed up-regulation at low and medium concentrations (0.001µg/L: 16%; $p < 0.001$, 0.01µg/L: 5%; $p = 0.08$), and down-regulation at 0.1 µg/L (17%; $p < 0.001$). A significant reduction in sphingolipids, acylcarnitines and sugars was only observed at the highest concentration 0.1 µg/L (sphingolipids; 46%, acylcarnitines; 24%, sugars; 70%; $p < 0.01$).

On day 7, we only presented the results for high food conditions as the number of replicates under low conditions was not sufficient due to such high mortality. We observed a significant reduction in the contents of four metabolite classes including amino acids, glycerophospholipids, sphingolipids and acylcarnitines (Figure 5.4). In amino acids and glycerophospholipids, the reduction was recorded at all concentrations ($p < 0.01$). The reduction in amino acids content was increased considerably with an increasing concentration of esfenvalerate. The highest concentration 0.1 µg/L resulted in a 19% reduction in metabolic content ($p < 0.001$) followed by a medium concentration 0.01 µg/L (9%; $p < 0.001$) and the lowest concentration 0.001µg/L (7%; $p < 0.01$). However, reduction in sphingolipids and acylcarnitines was only observed at the highest concentration (0.1 µg/L).

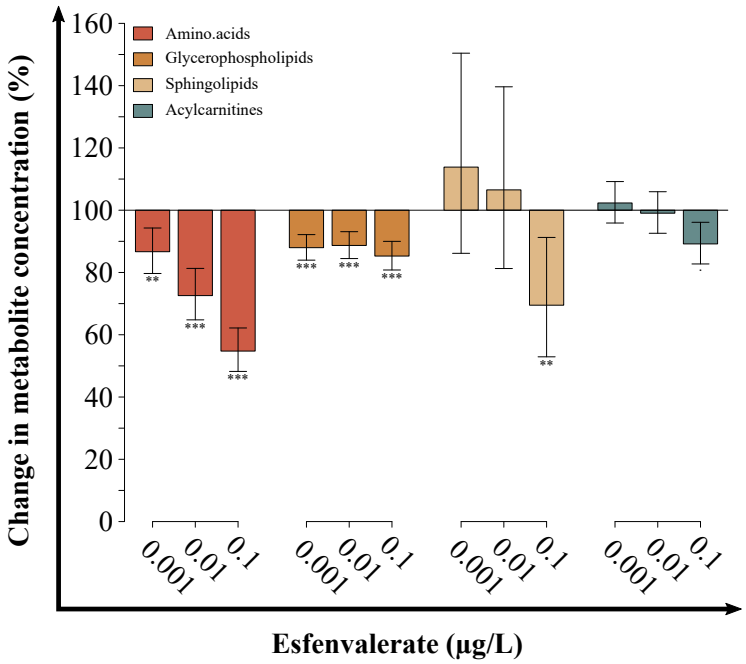

Figure 5.4: Percent change in the content of different metabolite classes of *D. magna* exposed to esfenvalerate in relation to the respective control group. Daphnids were exposed to 0.001 µg/L, 0.01 µg/L and 0.1 µg/L of esfenvalerate for 24 h. Metabolites were measured on day 7 only under high food conditions. Changes are presented with 95 % confidence intervals and statistical significance is indicated with asterisks: "." $p < 0.1$ "*" $p < 0.05$ "**" $p < 0.01$ and "***" $p < 0.001$.

5.4.3 The effect of pesticide exposure on individual metabolites

Out of 160 metabolites, we revealed significant changes in 14 metabolites under high food conditions and 15 metabolites under low food conditions. In total, 13 metabolites from amino acids (arginine, glutamine, glycine, methionine, phenylalanine, descriptionproline, serine, threonine, tyrosine, leucine, histidine, ornithine and tryptophan), one long-chain phospholipid phosphatidylcholine diacyl C42:4 (PC aa C42:4), one acylcarnitine (hydroxyvalerylcarnitine C5-OH (C3-DC-M)) and one sugar showed significant down-regulation (Figure D1) at the highest concentration (0.1 µg/L). However, under low food conditions, phosphatidylcholine diacyl C42:4 and sugar also showed a significant change at the lowest concentration (0.001 µg/L). In general, the effect on day 4 was significantly stronger compared to day 2 (p <0.05, Figure D2, D3). Furthermore, the effect under low food conditions regarding individual metabolites was up to 7-fold stronger than under high food conditions (Figure D3).

5.5 Discussion

Several investigations have reported significant effects of pesticides even at three to four orders of magnitude below the acute LC_{50} (Liess and Von der Ohe, 2005; Schäfer et al., 2012; Siddique et al., 2020). Reasons for these low effect concentrations and the unexplained variance of field effects on non-target invertebrates include an increased sensitivity of individuals to pesticides due to multiple stress conditions (Liess et al., 2019, 2016). Aquatic organisms are often impacted by multiple stressors including agrochemicals and environmental conditions. It is suggested that the presence of a large number of different toxicants in the field, coupled with environmental stress, could potentially result in synergistic interactions – contributing to unexpectedly high sensitivity of field populations. A meta-study showed that environmental stress severely enhances the toxicity of single pesticides. Examples include food stress, competition, pathogens, salinity, elevated temperature or UVB radiation. Accordingly, we investigated how the food supply interacts with insecticide induced biochemical fingerprints. In the present study, esfenvalerate exposure incurred significant effects on the overall metabolite contents of *Daphnia magna* at a concentration two orders of magnitude below the acute LC_{50}. Further, the effect was considerably stronger under low food conditions (Figure 5.1, Table D2). It is known that the detoxification mechanisms require a substantial investment of energy resources, therefore, starving organisms show a higher sensitivity to toxicants (Sibly, 1999). Although long-term sub-lethal effects of low pesticide concentrations have already been reported in the freshwater macroinvertebrate community structure (Beketov and Liess, 2008; Liess et al., 2013; Wieczorek et al., 2018), the metabolomic response to very low pesticide concentrations have rarely been studied (Russo et al., 2018).

5.5.1 Amino acids

Under both food conditions, we observed a significant decrease in amino acids content. Amino acids have been reported to play a significant role in the growth, reproduction and energy metabolism of *Daphnia magna* (Koch et al., 2011; Yebra and Hernández-León, 2004). During stress conditions, *Daphnia magna* may carry out gluconeogenesis to sustain the glucose levels required for survival. This process is usually characterized by the conversion of glucogenic amino acids (e.g. arginine, glutamine, glycine, methionine, proline, serine, tyrosine, histidine, tryptophan) into intermediates of pyruvate (Kokushi et al., 2012). Therefore, the down-regulation of glucogenic amino acids may be caused by their incorporation into energy production without regeneration through proteolysis. Moreover, general reduction can be attributed to increased synthesis of antioxidant proteins in response to toxicant stress (Knops et al., 2001; Smolders et al., 2005). Therefore, the decrease of amino acids in the present study might imply that the esfenvalerate exposure caused more severe oxidative stress in *D. magna* especially under low food conditions. With regard to oxidative stress, the changes in glutamine, cysteine and glycine are of specific interest, since they constitute the building blocks for glutathione which is the major antioxidant in insects that is rapidly consumed under oxidative stress. Consequently, in the first phase of oxidative stress an increase in glutathione synthesis is often observed. A down-regulation in amino acids content has already been reported in fish *Clarias batrachus* exposed to chlorpyrifos (Narra et al., 2011) and clam *Ruditapes philippinarum* exposed to arsenic (Wu et al., 2013). Lin et al. (2011) also reported down-regulation in amino acids of mice exposed to 2,3,7,8–tetrachlorodibenzo-p-dioxin (TCDD). Contrary to our findings, Kovacevic et al. (2016) and Martin-Park et al. (2017) reported an increase in amino acid content in organisms exposed to pesticides. They considered an increase in amino acids levels as a result of protein breakdown, releasing free amino acids for energy metabolism under stress conditions (Gillis and Ballantyne, 1996).

5.5.2 Glycerophospholipids

In the present study, glycerophospholipids showed significant up- and down-regulations under both food conditions. When we analyzed the individual metabolites, we identified a significant reduction of long-chain phospholipids phosphatidylcholine diacyl C42:4 (PC aa C42:4) under low food conditions. Both the up- and down- regulation of glycerophospholipids in rats have been associated with a high exposure to fipronil insecticide (Moser et al., 2015). Perturbations in glycerophospholipids have also been reported in *D. magna* exposed to different flame retardants (Scanlan et al., 2015; Stanley et al., 2013). Recently, Fuertes et al. (2018) reported the dynamics of glycerophospholipids in *Daphnia magna* exposed

to juvenoids and bisphenol A. Glycerophospholipids are an integral component of most of the cellular membranes (Ecker and Liebisch, 2014; Hishikawa et al., 2014), involved in many vital functions, such as survival, growth, development and reproduction (Fuertes et al., 2018; Hermansson et al., 2011; Kerr and Colucci, 2011). They also modulate the energy metabolism, neural activities and intracellular signaling pathways (Castro-Gómez et al., 2015; Farooqui et al., 2000). One of the two acyl chains in diacyl phospholipids can be arachidonic acid (20:4) that is involved in the synthesis of prostaglandin and the detoxification response. Therefore, a greater consumption of arachidonic acid – a building block of phospholipids – may cause a down-regulation of glycerophospholipids. Additionally, the observed change of glycerophospholipids in the present study might be resulted from esfenvalerate induced perturbation of neural cell membranes. Pyrethroid insecticides pose toxic effects by disrupting the voltage-dependent sodium channels in the nervous system, leading to a knockdown of the normal functioning of the nervous system and eventually to death (Davies et al., 2007). Here in the present study, we assume that different doses of esfenvalerate triggered different defense mechanisms, resulting in an up- and down-regulation of metabolites. For instance, recently Shi et al. (2018) reported dose-dependent responses of earthworms exposed to different concentrations of a flame retardant hexabromocyclododecane. Russo et al. (2018) also observed different levels of glycerophospholipids at different clothianidin concentrations and at different time points.

5.5.3 Sphingolipids

Sphingolipids exhibited up-regulation at the lowest concentration only under low food conditions, but down-regulated at the highest concentration under both food conditions. Like glycerophospholipids, sphingolipids are important constituents of neural membranes, and participate in a variety of indispensable metabolic, neurological, and intracellular signaling processes (Cole et al., 2012; Hermansson et al., 2011). Therefore, perturbation in sphingolipids might be attributed to the disturbance of neural cell membranes induced by the exposure to esfenvalerate. Moser et al. (2015) also reported changes in sphingolipids levels as the result of fipronil exposure.

5.5.4 Acylcarnitines

Acylcarnitines are widely known to be involved in energy production through the β-oxidation of fatty acids and are therefore considered as a widely used marker for metabolic disorders in mammals (Indiveri et al., 2011; Rodríguez-Sánchez et al., 2015). In the present study, acylcarnitines significantly decreased

under both food levels at the highest concentrations, suggesting a potential alteration in mitochondrial metabolism, energy production, and oxidative stress (Reuter and Evans, 2012). Recent investigations have suggested that the acylcarnitines also play a significant role in modulating neurotransmission (Jones et al., 2010). Therefore, down-regulation may be attributed to the potential neurotoxicity of esfenvalerate. Recently, Martin-Park et al. (2017) reported a significant decrease in acylcarnitine levels and associated it with the exposure to pyrethroid insecticide. However, organisms exposed to permethrin showed an up-regulation of acylcarnitines. By contrast, Russo et al. (2018) reported an up-regulation at low concentrations, and down-regulation at high concentration of clothianidin. In the present study, we also observed up-regulation of acylcarnitines at low concentrations, but it was not significant.

5.5.5 Sugars

A change in sugar was only recorded under low food conditions. Nutritional stress can induce significant changes in the metabolite composition of organisms (Wagner et al., 2015). In the present study, this appeared to be the case of extreme stress, where low food increased the toxic pressure by interacting with esfenvalerate. Under extreme stress conditions, all available energy and metabolic capacity is dedicated to maintain the survival of an organism until the return of favorable conditions (Sokolova et al., 2012). Recently, Zhang et al. (2018) and (2020) also reported a decrease in sugar levels of *Daphnia magna* exposed to multiple stress. In the present study, a significant decrease in sugar contents indicate the metabolic efforts of the organism for recovery after exposure to esfenvalerate under low food conditions. However, most of the organisms could not survive until day 7 especially at higher concentrations. Therefore, here we can assume that the perturbations of sugar in *Daphnia magna* were caused by the interaction of insecticide exposure and inadequate nutrition.

Taking together, we can suggest glucogenic amino acids, phosphatidylcholine diacyl C42:4 and hydroxyvalerylcarnitine C5-OH (C3-DC-M) as putative biomarkers of metabolic dysregulation, oxidative stress and inflammation caused by esfenvalerate exposure. However, further studies are necessary to explore the consistency of metabolomic responses across different toxicants, concentrations and species to identify the role of individual metabolites in the toxicity of esfenvalerate.

Exposure to esfenvalerate, even well below the regulatory acceptable concentrations, was observed to manifest significant metabolic effects. Further, the effect was considerably stronger under low food conditions. An interaction between food- and chemical stress was mainly responsible for extreme stress, and thereby

increased the energy demand for survival. Altogether, a strong depletion of energy reserves – due to food stress – can directly translate into lower fitness and may explain changes in the freshwater ecosystem structure in the field.

5.5.6 Author Contributions

Conceptualization: NS, ML and MvB; Study design: NS and URK; Investigation: NS and AS; Metabolomic analysis: NS and URK; Formal analysis: NS and ML; Writing Original Draft: NS; Review & Editing: All.

5.5.7 Acknowledgements

We thank the students, Miryam Nadkarni from Fern Universität in Hagen, Germany, and Anna Manukyan from Université Bourgogne Franche-Comté, France for their assistance in the experimental setup. We also acknowledge the German Academic Exchange Service (Deutscher Akademischer Austauschdienst, DAAD) for financially supporting NS through doctoral fellowship.

6

Discussion and outlook

In freshwater ecosystems, aquatic organisms are commonly exposed to a wide range of chemical and physiological stressors that may lead to adverse effects even at pesticide concentrations three to four orders of magnitude below the acute LC_{50} (Liess and Von der Ohe, 2005; Schäfer et al., 2012). At the same time, these low pesticide concentrations may exert a considerable pressure for adaptation. Effects at very low concentrations show that the current environmental risk assessments of pesticides fail to determine protective thresholds of risk. This scenario can be attributed to (i) an error prone estimation of pesticide exposure (Knabel et al., 2013, 2012) and (ii) because pesticides are commonly evaluated as single products without considering realistic environmental stress and exposure conditions (Liess et al., 1999). Therefore, in this dissertation, important factors for pesticide effects such as adaptation to pesticides, assessment of toxic pressure, interaction of mixtures, and the role of environmental stressors for the ecological effects were studied.

6.1 Development of pesticide tolerance

The development of pesticide tolerance in non-target species was determined by the local toxic pressure as well as the exchange rate with non-contaminated populations (Chapter 2). *G. pulex* from highly contaminated streams showed increased tolerance to an insecticide clothianidin as compared to individuals from less contaminated streams. Furthermore, distance to the nearby refuge areas was

an additional factor that drove pesticide tolerance. This increased tolerance in *G. pulex* may be due to physiological acclimation or the genetic adaptation. Irregular and rare pesticide exposure can induce detoxification enzymes that may result in physiological acclimation (Di Giulio et al., 1995). In contrast, the consistent pesticide exposure leads to acquire genetic adaptation (Lopes et al., 2008) and has been reported in several pest- (Bass et al., 2011; Jones et al., 2011) as well as non-target species (Vigneron et al., 2015; Weston et al., 2013). In the present study, the gammarid populations from agricultural streams had likely been experiencing a historical exposure to pesticides over several decades. Such continuous exposure imposes strong bouts of selection for resistance; hence, it is expected that the increased tolerance in *G. pulex* was might be due to evolutionary changes in genetics.

Presence of refuge areas is another aspect that influenced the resistance acquisition at contaminated sites. It is suggested that the undisturbed forested zones positively influence the quality of down-stream habitat and partly compensate the effects of pesticides (Liess and Ohe, 2005; Schäfer et al., 2007). However, migration of sensitive organisms to the downstream contaminated sites can slow down the evolution of pesticide tolerance. Therefore, it is suggested that the pesticide exposure and the distance from refuge areas should be considered in risk assessment when assessing the ecological effects of toxicants.

6.2 Use of pesticide body burden to assess the ecological effects in streams

In the second investigation (Chapter 3), pesticide body burden was applied to assess the pesticide exposure and potential effects in freshwater organisms. The toxic pressure derived from body burden and from water samples similarly explained the change in community structure. However, the increased tolerance of *G. pulex* to clothianidin was better explained by the toxicity derived from body burden (70%) as compared to the toxicity from water samples (53%). These findings are of great importance for improving risk assessments of pesticides.

The TU derived from body burden ($\mathrm{TU}_{\mathrm{max-Int}}$) was approximately one order of magnitude higher than the TU derived from water concentrations ($\mathrm{TU}_{\mathrm{max-Ext}}$). This difference could be due to uncertainty of calculated partition coefficients between *G. pulex* and the water phase or the disequilibrium at highly dynamic water concentrations during a peak event. However, the toxicity derived from both methods explained the changes in the community composition equally. Therefore, it is suggested that the internal concentrations are a useful and robust approach in the assessment of pesticide effects. In contrast, the $\mathrm{TU}_{\mathrm{max-Int}}$ explained

the increase in tolerance to the insecticide clothianidin in *G. pulex* considerably better. This difference resulted primarily from an underestimation of toxicity at one site using water samples. Whereas $\mathrm{TU}_{\mathrm{max-Int}}$, $\mathrm{SPEAR}_{\mathrm{pesticides}}$ and the insecticide tolerance in terms of EC_{50} indicated a high pesticide exposure. This observation suggests that the assessment based on body burdens was relatively more reliable as compared to water samples. Pesticide peaks in the stream water may be more subject to unpredictable short-term variation caused by the erratic combination of rainfall and pesticide applications than to the other exposure indicators that rely on longer-term processes. However, peak concentrations in the water generally provided an adequate measure of pesticide exposure as the difference from the other indicators became obvious only in one of the investigated streams.

6.3 Interaction between pesticide mixtures and environmental stress

Pesticide mixtures may produce synergistic effects especially under suboptimal conditions in the field. In this study (Chapter 4), food stress synergistically increased the effect of a mixture of esfenvalerate and an azole fungicide prochloraz up to 10-fold. Furthermore, the combined effects of pesticide stress and food limitation could be predicted best with the stress addition model (SAM).

About 20% of the total pesticide combinations used in Germany comes from two major pesticide classes i.e. pyrethroid insecticides and azole fungicides, and therefore co-occur in the aquatic environment (Riise et al., 2004; Werner et al., 2004). Azole fungicides have already been demonstrated to enhance the toxic effects of pyrethroid insecticides (Cedergreen et al., 2006). However, the threshold of 1 µg/L under low food conditions is lower than that reported in earlier studies without additional stress (Nørgaard and Cedergreen, 2010). The increased sensitivity could be due to limited availability of food. Starving organisms may have less energy budget for physiological defense against stress, and therefore, show more sensitivity to contaminants (Sibly, 1999). Under limited food supply, increased mortality of *Daphnia magna* has already been reported for exposure to metals and pesticides (Koivisto et al., 1992; Pieters et al., 2005).

Concerning the pyrethroid esfenvalerate, high synergism was recorded even below the ecologically acceptable concentrations (RAC of esfenvalerate: 0.08 (Weltje, 2013)) and nominal concentrations of prochloraz ($\geq$1 µg/L). Bjergager et al. (2017) also reported synergistic effects of a pyrethroid alpha-cypermethrin at very low concentration (0.01 µg/L) in the presence of prochloraz (500 µg/L); however the prochloraz concentration was quite far from the environmentally realistic scenario. The present study suggests that the environmental stressors

may interact with chemical stressors and can increase the potential toxic effects towards aquatic organisms even below their RACs, and therefore, realistic assessment of safe concentrations must consider the additional factors (Knillmann et al., 2013; Rasmussen et al., 2012).

Combined effects were better predicted by the SAM model because it assumes that the joint effect of independent stressors can be estimated by adding up individual stress to a universal stress capacity. Therefore, it is a good tool that quantitatively predicts the highly synergistic direct effects of independent stressor combinations (Liess et al., 2016). In contrast, effect addition (EA) and concentration addition (CA) assume additive effects and don't consider the interactions.

6.4 Metabolomic response to multiple stress

Exposure to pesticides may cause metabolomic changes even at very low concentrations, below those considered safe by the governmental risk assessment. The interaction between pesticides and environmental stress can further increase effect of pesticides (Chapter 5). Results presented in the chapter 5 showed that the pesticide exposure and food stress significantly affected different metabolite classes such as amino acids, glycerophospholipids, sphingolipids, acylcarnitines and sugars.

Under stress conditions, organisms need more energy for survival that can be usually achieved through gluconeogenesis. Therefore, reduction of glucogenic amino acids may be attributed to their incorporation in energy production without their renewal. Generally organisms respond to stress by allocating a larger portion of energy to maintain regular metabolism, or to activate the defense mechanisms (Spann et al., 2011), which consequently may lead to impaired growth and reproduction (Connon et al., 2008; Spann et al., 2011). Here it is assumed that the down-regulation could be either due to lesser production of amino acids, depletion in detoxification processes, or the exposed organisms suffered reduced growth.

Both glycerophospholipids and sphingolipids are involved in many vital functions, such as survival, growth, as well as neurological and intracellular signaling processes (Fuertes et al., 2018; Hermansson et al., 2011). Therefore, perturbation in glycerophospholipids and sphingolipids might be attributed to the disturbance of neural cell membranes induced by esfenvalerate exposure. Detoxification processes can be another for down-regulation of these metabolites.

Acylcarnitines and sugars are widely known to be involved in energy production. Down-regulation of these metabolites especially under low food conditions

suggesting potential alteration in mitochondrial metabolism, energy production, and oxidative stress (Reuter and Evans, 2012). Low food can increase the toxic pressure by interacting with esfenvalerate. Under extreme stress conditions, all available energy and metabolic capacity is dedicated to maintain the survival of an organism until the return of favorable conditions (Sokolova et al., 2012). Recently, Zhang et al. (2018) and Zhang et al. (2020) reported decrease in sugar levels of *Daphnia magna* exposed to zinc oxide nanoparticles (nZnO) at high temperature. Similarly, nutritional stress can induce significant changes in metabolite composition of organisms (Wagner et al., 2015). Altogether, strong depletion of energy reserves – due to interaction between pesticide and food stress – can directly translate into lower fitness and may explain changes in the freshwater ecosystem structure in the field.

6.5 Implications for the ecological risk assessment and outlook

In natural ecosystems, aquatic organisms often experience suboptimal conditions; whereas, lab investigations are generally conducted under optimal conditions. Thus, the obtained results from such lab experiments cannot be extrapolated to field-realistic exposure scenarios. In this dissertation, pesticide effects were studied under field relevant conditions; and therefore, the obtained results may have several implications for the ecological risk assessment of pesticides.

6.5.1 Adaptation to pesticides and prevailing factors

Evolution of pesticide tolerance in non-target species can have potential consequences for the local biodiversity. Especially, it is very important if it happens in non-target species that are being used for biomonitoring and policy making process. Guidelines based on the sensitivity of adapted populations may not be adequately protective of the environment and ecology. Present study reveals the extent to which a freshwater macroinvertebrate species (*G. pulex*) develops resistance under pesticide contamination, and the environmental parameters that govern the development of such resistance. Unraveling evolutionary processes in non-target populations has tremendous implications for the ecological risk assessment and management of biodiversity. Therefore, further field studies are required to understand underlying mechanisms. It is suggested that the adapted organisms may have less ability to compensate environmental stress such as climate change, a major concern in the near future. An interesting aspect would be to look at how non-target species respond to additional stressors when they

are adapted to pesticides. Future studies could explore the genetic diversity in adapted populations and associated fitness costs. Another dimension that should be investigated is the role of genetic adaptation to environmental stress.

6.5.2 Assessment of pesticide exposure

In regulatory practices, pesticide exposure in agricultural streams is commonly assessed based on the pesticide residues in water and sediment samples. However, measuring the short pulses of high peak concentrations after rainfall events is challenging. Realistic measurements of pesticide exposure in the water is necessary to explain long-term effects on the macroinvertebrate community. It is showed in Chapter 3 that the alternative quantification of pesticide exposure based on pesticide residues in macroinvertebrates is suitable to assess the overall pesticide exposure and effects in agricultural streams. However, the back-calculation from body burden to water concentrations is a subject of interest. To validate this method, further studies are needed. The comparison between body burden and synthetic passive samplers is an important area for future investigations.

6.5.3 Multiple stressors

The interactions of stress factors are much more complex in the field, modifying the sensitivity of communities and populations to contaminants (Heugens et al., 2006; Relyea and Hoverman, 2008). Negative effects of pesticides have frequently been reported in the field. It shows that the current environmental risk assessments of pesticides are not protective enough to determine safe thresholds of risk. The results reported in Chapter 4 showed that the pesticide mixtures with different mode of action may interact in a highly synergistic manner especially under suboptimal environmental conditions. To determine protective concentration levels of individual pesticides, we need to understand to what extent pesticide toxicity is increased by synergistic interactions and additional environmental stressors. Future studies that examine pesticide mixtures need to consider environmental stress conditions to fully understand the complexity of pesticide interactions in the real exposure scenarios. Moreover, it is interesting to study how genetically adapted organisms respond to pesticide mixtures under field relevant conditions.

Since it is not feasible to consider all possible mixtures experimentally, models are needed to predict the combined effects of mixtures from a single-substance toxicity data. Up until now, approaches are lacking to predict the effects of mix-

tures that act synergistically. Traditional approaches such as concentration addition (CA) and effect addition (EA) assume additive effects. However, (Liess et al., 2016) recently developed a new model, the 'stress addition model' (SAM), to specifically predict the synergy between environmental stressors and individual toxicants. Approaches such as the SAM can improve the prediction of the combined effects of synergistic toxicant mixtures and environmental stress. Assessment of the joint effects of multiple stress is still in developmental stages. Therefore, further investigations are required to develop predictive approaches and for their validation.

6.5.4 Metabolomics and toxicology

In chapter 5, results showed that the esfenvalerate exposure can cause significant metabolomic changes even far below the regulatory acceptable concentrations. This is one of the explanations that why pesticide effects in the field can be observed even at three to four orders of magnitude below the acute LC_{50}. Metabolomics is widely applied technique in toxicology, however, its application for regulatory purposes is limited to chemical grouping based on mode of action (MOA) and characterizing the adverse outcome pathways (AOP). For better understanding of pesticide toxicity, unraveling the molecular actions of pesticides to their possible interference with biological processes is imperative. Since the response of organisms to toxicant is regulated by a complex of genes, metabolites and proteins, integrated application of different -omics techniques is essential to understand biosystems. To better understand ecological effects of pesticides, future studies should consider broader range of non-target species. Knowledge of molecular pathways can ultimately contribute to the extrapolation of the laboratory data to more realistic scenarios in the field.

A

Supporting Information for chapter 2

Shahid, N., Becker, J. M., Krauss, M., Brack, W., and Liess, M. 2018. Adaptation of *Gammarus pulex* to agricultural insecticide contamination in streams. Science of the Total Environment, 621, 479-485. doi: https://doi.org//10.1016/j.scitotenv.2017.11.220

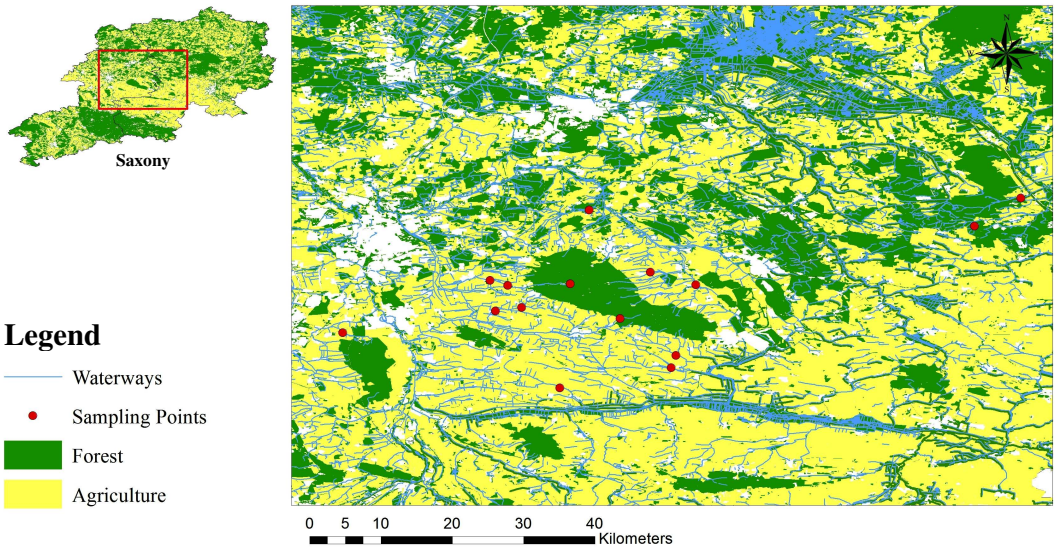

Figure A.1: Map of sampling sites selected within central Germany that cover a wide range from uncontaminated to highly contaminated streams.

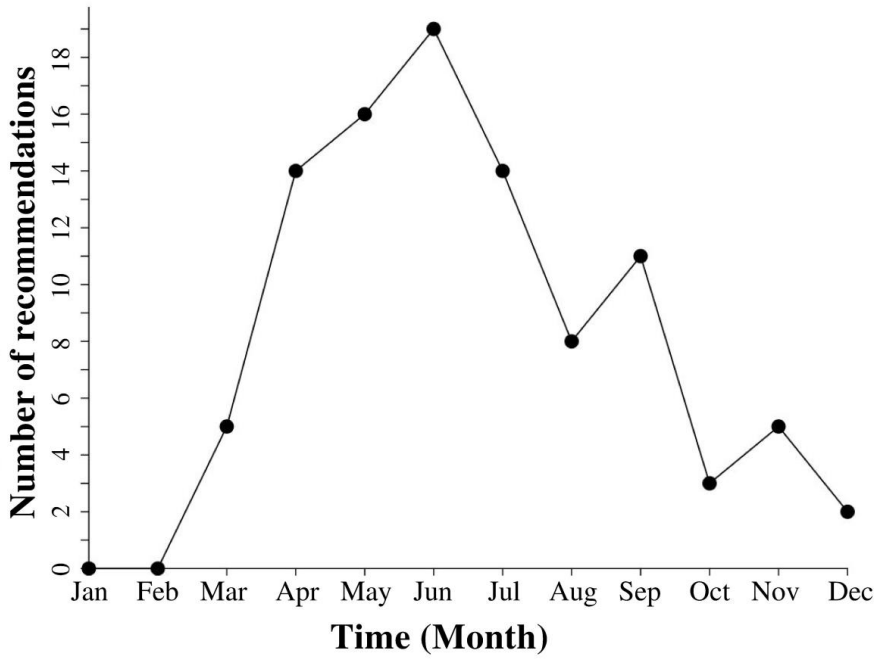

Figure A.2: Number of governmental recommendations for pesticide (herbicide excluded) application in croplands in the year 2015 provided by the Sachsen-Anhalt State Institute for Agriculture, Forestry and Horticulture (LLFG), Bernburg.

Table A.1: Chemicals considered for water analysis. Given data includes: CAS number, octanol-water partitioning coefficients (log K_{OW}*), molecular weights (MW, g/mol) of the target compounds; TP = transformation product.

Chemical name	CAS number	log K_{OW}*	MW	Compound group
Acetamiprid	160430-64-8	2.55	222.7	insecticide
Ametryn	834-12-8	2.98	227.33	herbicide
Atrazine	1912-24-9	2.82	215.7	herbicide
Azoxystrobin	131860-33-8	1.58	403.4	fungicide
Boscalid	188425-85-6	4.0	343.2	fungicide
Chlorotoluron	15545-48-9	2.58	212.7	herbicide
Chloroxuron	1982-47-4	4.08	290.7	herbicide
Clomazone	81777-89-1	2.86	239.7	herbicide
Clothianidin	210880-92-5	0.64	249.7	insecticide
Cyproconazole	94361-06-5	2.9	291.8	fungicide
Desethylatrazine	6190-65-4	1.78	187.6	TP of atrazine
Desethylterbutylazine	30125-63-4	2.23	201.7	TP of terbutylazine
Desisopropylatrazine	1007-28-9	1.36	173.6	TP of atrazine
Difenoconazole	119446-68-3	5.2	406.3	fungicide
Diflufenican	83164-33-4	3.53	394.3	herbicide
Dimethachlor	50563-36-5	2.59	255.7	herbicide
Dimethenamid	87674-68-8	2.92	275.8	herbicide
Dimethoate	60-51-5	0.72	229.3	insecticide
Epoxiconazole	133855-98-8	3.47	329.8	fungicide
Ethofumesate	26225-79-6	2.89	286.3	herbicide
Fenpropidin	67306-00-7	5.41	273.5	fungicide
Fenpropimorph	67564-91-4	5.5	303.5	fungicide
Fenuron	101-42-8	1.38	164.2	herbicide
Flufenacet	142459-58-3	2.39	363.3	herbicide
Fluoxastrobin	361377-29-9	5.22	458.8	fungicide
Flurtamone	96525-23-4	2.39	333.3	herbicide
Flusilazole	85509-19-9	4.89	315.4	fungicide
Imidacloprid	105827-78-9	-0.41	255.7	insecticide
Imidacloprid-guanidine	127202-53-3	0.69	210.7	TP of imidacloprid
Imidacloprid-urea	120868-66-8	3.7	211.6	TP of imidacloprid
Isoproturon	34123-59-6	2.84	206.1	herbicide
Lenacil	96639	3.09	234.3	herbicide
Metamitron	41394-05-2	1.44	202.2	herbicide
Metazachlor	67129-08-2	2.38	277.1	herbicide
Metolachlor	51218-45-2	3.24	283.8	herbicide
Metribuzin	21087-64-9	1.96	214.3	herbicide
Myclobutanil	88671-89-0	3.5	288.8	fungicide
Pethoxamid	106700-29-2	3.39	295.8	herbicide
Pirimicarb	23103-98-2	1.4	238.3	insecticide
Pirimiphos-methyl	29232-93-7	2.96	305.3	insecticide
Prochloraz	67747-09-5	4.13	376.7	fungicide

Continued on next page

Table A.1 – continued from previous page

Chemical name	CAS number	log K_{OW}*	MW	Compound group
Propiconazole	60207-90-1	4.13	342.2	fungicide
Propyzamide	23950-58-5	3.18	256.1	herbicide
Prosulfocarb	52888-80-9	4.23	251.4	herbicide
Prothioconazole-desthio	120983-64-4	3.05	312.2	TP of prothioconazole
Quinmerac	90717-03-6	2.87	221.6	herbicide
Quinoxyfen	124495-18-7	4.98	308.1	fungicide
Spiroxamine	118134-30-8	5.51	297.5	fungicide
Tebuconazole	107534-96-3	3.89	307.8	fungicide
Terbuthylazine	5915-41-3	3.27	229.7	herbicide
Terbuthylazine-2-hydroxy	66753-07-9	-1.29	211.3	TP of terbuthylazine
Thiacloprid	111988-49-9	2.33	252.7	insecticide
Thiacloprid amide	676228-91-4	1.06	270.7	TP of thiacloprid
Thiamethoxam	153719-23-4	0.8	291.7	insecticide
Triadimenol	55219-65-3	3.28	295.86	fungicide

Table A.2: Physicochemical properties of investigated streams (Summer).

Parameter	Unit	Min	Max	Mean	SD	t	df	*p*-value
Water temperature	°C	7.2	14.1	10.7	2.4	-3.8	12.9	0.002
Conductivity	µS	537	1367	898.3	243.4	-0.69	8.39	0.50
Water level	cm	5	38.5	15.5	10.1	-1.69	12.68	0.11
pH	-	7.9	9.0	8.5	0.3	0.29	10.91	0.77
D.O.	mg/L	9.0	11.5	10.5	0.73	-0.65	8.11	0.53

Table A.3: Seasonal variation in acute EC_{50} values of neonicotinoid insecticide clothianidin for *Gammarus pulex* collected from agricultural and non-agricultural streams. Given data: mean effective concentrations (EC_{50}) and standard error (SE) for winter and summer seasons.

Site ID	Winter		Summer	
	EC50	***SE***	***EC50***	***SE***
Agri-1	189.2	0.15	262.9	0.07
Agri-2	273.3	0.19	297.5	0.13
Agri-3	294.6	0.13	320.7	0.23
Agri-4	169.6	0.11	178.2	0.09
Agri-5	222.2	0.08	191.1	0.17
Agri-6	266.0	0.13	213.2	0.11
Agri-7	161.0	0.08	152.8	0.11
Agri-8	168.8	0.07	189.8	0.14
Agri-9	144.5	0.10	159.8	0.13
Control-1	122.7	0.12	82.9	0.14
Control-2	87.7	0.09	93.5	0.17
Control-3	59.4	0.12	67.1	0.29
Control-4	103.6	0.18	97.7	0.06
Control-5	90.2	0.18	92.6	0.17
Control-6	98.9	0.27	56.6	0.19

B

Supporting Information for chapter 3

Shahid, N., Becker, J. M., Krauss, M., Brack, W., Liess, M. 2018. Pesticide Body Burden of the Crustacean *Gammarus pulex* as a Measure of Toxic Pressure in Agricultural Streams. Environmental Science & Technology, 52(14), 7823-7832. doi: https://doi.org/10.1021/acs.est.8b01751

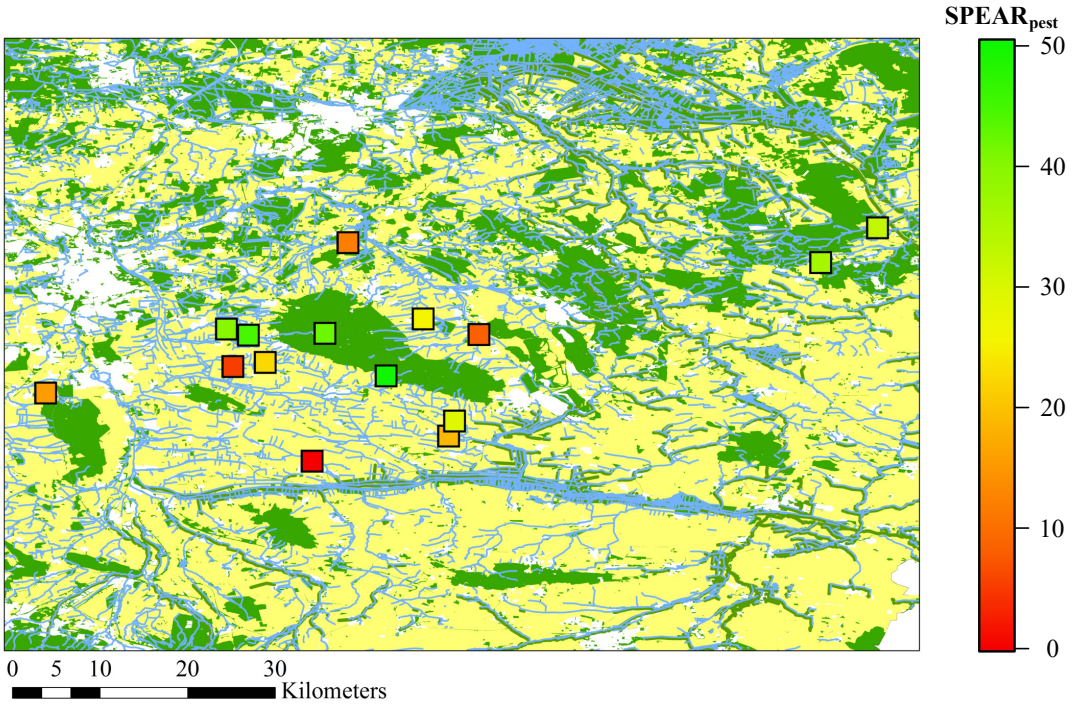

Figure B.1: Location of the sampling sites in central Germany that cover a wide range from non-contaminated to highly contaminated streams. Waterways are represented in blue, forests as green and agriculture in yellow colour. Square shapes are representing the sampling sites and are coloured according to the $\mathrm{SPEAR}_{\mathrm{pesticides}}$ values calculated during sampling in 2016, ranging from 50.3 (low pesticide pollution; green) to 7.0 (high pesticide pollution; red).

Figure B.2: Event-driven water sampler (EDS) used for collection of water samples from agricultural and non-agricultural streams. Two brown glass bottles are attached to a stainless rod. We used stainless steel tubes (4 mm diameter) for the water inlet and the air outlet. Opening of the water inlet of the both bottles are at heights of 5 and 15 cm from the regular level of the stream water. A silicon tube with a length of 15 cm on top of the air outlet was installed to provide sufficient distance between the openings of the water inlet and the air outlet. Additionally, we inserted a 100 µL pipette tip into the top end of the silicon tube, thus reducing the diameter of the air outlet and expanding the bottle's filling time.

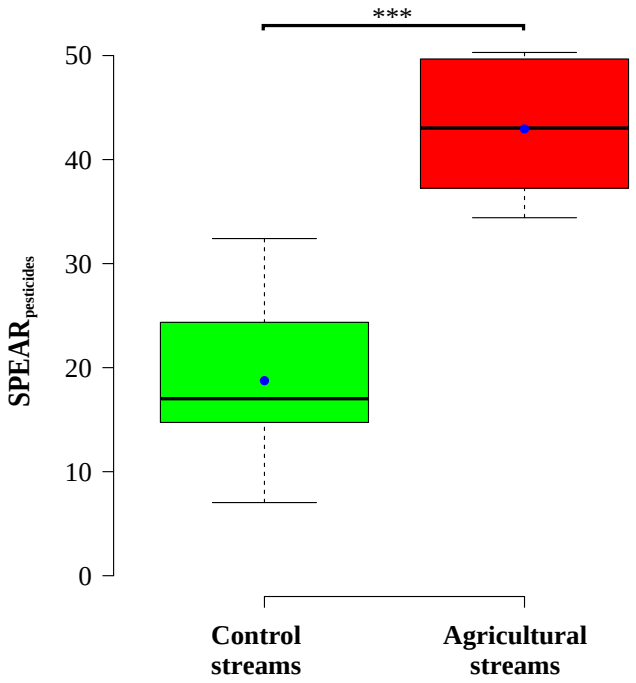

Figure B.3: Comparison of the ecological status of agricultural (red) and non-agricultural streams (green) in terms of $\mathrm{SPEAR}_{\mathrm{pesticides}}$ values. The boundaries of the central box are the 25th and 75th percentiles; the horizontal line is the median; the blue dot indicates the mean; and whiskers of the boxplot represent the minimum and maximum values. Non-agricultural streams showed significantly higher $\mathrm{SPEAR}_{\mathrm{pesticides}}$ values (Wilcoxon's rank sum test, W = 54, p-value < 0.001). The significance level for the observations is displayed as: *** = p-value < 0.001.

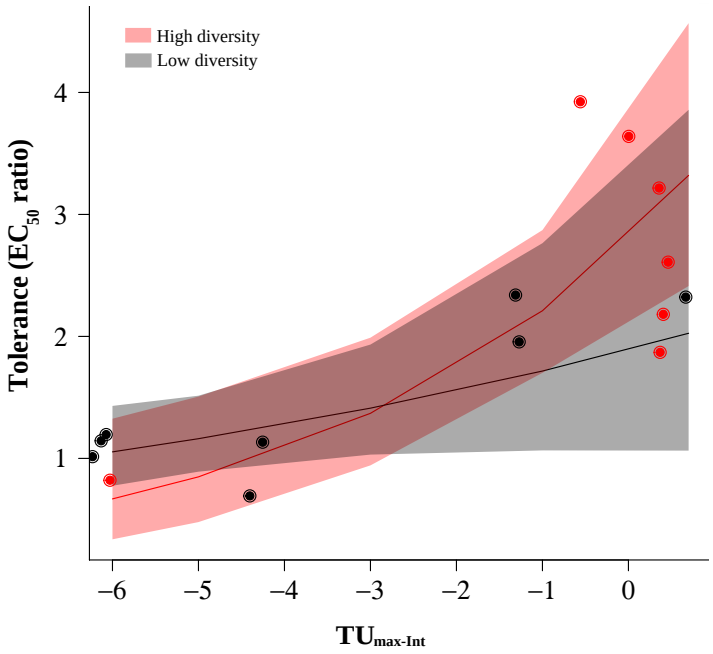

Figure B.4: Species diversity reduces the adaptation of macro-invertebrates to the pesticide contamination. The toxic units were calculated from equivalent pesticide concentrations in water derived from internal body burden of *G. pulex*. The tolerance was quantified as the ratio of the local EC_{50}/mean EC_{50} of all populations from non-contaminated streams ($\mathrm{TU}_{\mathrm{max-Int}}$ < –3). The light areas correspond to the 95% confidence interval. Adjusted R^2 = 0.72, F = 3.28, d.f. = 1, residual d.f. = 11, *p*-value = 0.097 for the interaction toxic unit:shannon.

Table B.1: Coordinates and physicochemical properties of investigated streams. Given data includes pH, water level (cm), conductivity (µS/cm), water temperature (°C) and levels of dissolved oxygen (D.O. (mg/L)) measured between 11:00 and 16:00 on the day of sampling.

Site ID	Coordinates	pH	Water level (cm)	Conductivity (µS/cm)	Water temp (°C)	D.O. (mg/L)
Agri 1	52°06′11.1"N 10°53′41.9"E	8.5	17.5	932.0	9.0	10.9
Agri 2	52°10′43.4"N 10°42′23.4"E	8.4	18.0	875.0	13.5	11.0
Agri 3	52°10′28.3"N 10°40′24.1"E	8.7	14.0	780.0	13.8	11.5
Agri 4	52°08′50.0"N 10°28′51.7"E	8.8	12.0	798.0	14.1	10.9
Agri 5	52°07′06.3"N 10°54′03.7"E	8.7	18.0	833.0	12.7	10.2
Agri 6	52°04′38.1"N 10°45′17.2"E	8.7	16.0	1005.0	12.1	11.1
Agri 7	52°12′26.6"N 10°55′34.4"E	7.9	7.0	885.0	9.3	10.3
Agri 8	52°13′24.4"N 10°52′08.0"E	8.3	38.5	1367.0	10.5	9.4
Agri 9	52°18′06.8"N 10°47′29.5"E	8.7	15.0	842.0	12.1	10.7
N-Agri 1	52°12′24.4"N 10°41′21.0"E	8.6	10.5	844.0	10.1	11.1
N-Agri 2	52°12′47.0"N 10°40′00.3"E	8.7	23.5	835.0	9.9	11.4
N-Agri 3	52°12′31.1"N 10°46′04.3"E	8.4	9.0	1296.0	8.6	10.4
N-Agri 4	52°18′24.1"N 11°17′40.3"E	8.6	9.0	537.0	7.2	10.7
N-Agri 5	52°16′53.2"N 11°16′38.6"E	8.1	7.0	723.0	7.9	9.6
N-Agri 6	52°09′53.6"N 10°49′50.6"E	9.0	5.0	815.0	9.5	9.0

Table B.2: Pesticides analysed in *G. pulex* samples. Given data includes: CAS number, acid dissociation constants (pK_a), neutral form acts as an acid (a) or as a base (b), octanol-water partitioning coefficients (log K_{OW}), organic carbon-water partitioning coefficients (log K_{OC}), molecular weights (MW, g/mol), method detection limits (MDL) of the target compounds determined according to U.S. EPA

Chemical name	CAS number	log pK_a	acid/base	log K_{OW}	log K_{OC}	MW	Compound group	MDL water
2-Hydroxyatrazine	2163-68-0	3.0	b	2.1	2.3	197.2	TP of atrazine	2.0
Acrinathrin	101007-06-1	-		6.7	5.9	541.5	insecticide	n.a
Ametryn	834-12-8	5.7	b	3.0	2.7	227.3	herbicide	1.5
Atrazine	1912-24-9	3.2	b	2.8	2.4	215.7	herbicide	1.0
Azoxystrobin	131860-33-8	-		1.6	3.4	403.4	fungicide	1.0
Bifenthrin	82657-04-3	-		8.2	6.4	422.9	insecticide	n.a
Boscalid	188425-85-6	-		4.0	2.9	343.2	fungicide	1.5
Chlorotoluron	15545-48-9	-		2.6	2.3	212.7	herbicide	1.0
Chloroxuron	1982-47-4	-		4.1	3.5	290.7	herbicide	1.5
Chlorpyrifos	2921-88-2	-		5.1	3.9	350.6	insecticide	n.a
Chlorpyrifos Methyl	5598-13-0	-		4.1	3.3	332.5	insecticide	n.a
Clomazone	81777-89-1	-		2.9	2.5	239.7	herbicide	1.2
Clothianidin	210880-92-5	-		0.6	2.1	249.7	insecticide	2.0
Cyfluthrin	68359-37-5	-		5.7	5.1	434.3	insecticide	n.a
Cyhalothrin	68085-85-8	-		6.9	5.5	449.9	insecticide	n.a
Cypermethrin	52315-07-8	-		6.4	4.9	416.3	insecticide	n.a
Cyproconazole	94361-06-5	2.3	b	2.9	3.1	291.8	fungicide	1.2
Deltamethrin	52918-63-5	-		6.2	4.9	505.2	insecticide	n.a
Desethylatrazine	6190-65-4	3.4	b	1.8	2.0	187.6	TP of atrazine	1.0
Desethylterbutylazine	30125-63-4	3.4	b	2.2	2.2	201.7	TP of terbutylazine	2.0
Desisopropylatrazine	1007-28-9	3.41	b	1.4	1.8	173.6	TP of atrazine	1.0
Diflufenican	83164-33-4	10.3	b	3.5	3.5	394.3	herbicide	20
Dimethachlor	50563-36-5	-		2.6	2.2	255.7	herbicide	1.0
Dimethenamid	87674-68-8	-		2.9	2.1	275.8	herbicide	1.0

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Table B.2 – continued from previous page

Chemical name	CAS number	log pK_a	acid/base	log K_{OW}	log K_{OC}	MW	Compound group	MDL water
Dimethoate	60-51-5	-		0.7	1.1	229.3	insecticide	1.0
Epoxiconazole	133855-98-8	2.3	b	3.5	3.0	329.8	fungicide	1.0
Esfenvalerate	66230-04-4	-		6.8	5.5	419.9	insecticide	n.a
Ethofumesate	26225-79-6	-		2.9	2.2	286.3	herbicide	4.0
Fenpropidin	67306-00-7	10.1	b	5.4	4.9	273.5	fungicide	2.0
Fenpropimorph	67564-91-4	8.5	b	5.5	3.5	303.5	fungicide	1.5
Fenuron	101-42-8	-		1.4	1.6	164.2	herbicide	2.0
Flufenacet	142459-58-3	-		2.4	2.6	363.3	herbicide	1.0
Fluoxastrobin	361377-29-9			5.2	5.9	458.8	fungicide	2.0
Flurtamone	96525-23-4	3.6	b	2.4	2.5	333.3	herbicide	1.0
Flusilazole	85509-19-9	2.3	b	4.9	3.2	315.4	fungicide	1.5
Fluvalinate	69409-94-5	-		6.8	5.9	502.9	insecticide	n.a
Imidacloprid	105827-78-9	-		-0.4	3.0	255.7	insecticide	2.0
Isoproturon	34123-59-6	-		2.8	2.1	206.1	herbicide	1.2
Lenacil	2164-08-1	6.6	b	3.1	2.2	234.3	herbicide	1.0
Metamitron	41394-05-2	2.8	b	1.4	1.9	202.2	herbicide	2.5
Metazachlor	67129-08-2	2.3	b	2.4	1.7	277.1	herbicide	1.5
Metolachlor	51218-45-2	-		3.2	2.1	283.8	herbicide	1.2
Metribuzin	21087-64-9	2.5	b	2.0	1.7	214.3	herbicide	2.0
Myclobutanil	88671-89-0	2.3	b	3.5	3.8	288.8	fungicide	4.0
Permethrin	52645-53-1	-		7.4	5.1	391.3	insecticide	n.a
Pethoxamid	106700-29-2	-		3.4	2.2	295.8	herbicide	1.0
Phthalimide	000085-41-6	8.4	a	1.3	1.0	147.1	TP of folpet	60
Pirimicarb	23103-98-2	5.0	b	1.4	1.7	238.3	insecticide	4.0
Propiconazole	60207-90-1	2.2	b	4.1	3.2	342.2	fungicide	2.0
Propyzamide	23950-58-5	6.9	a	3.2	2.6	256.1	herbicide	7.0
Prosulfocarb	52888-80-9	-		4.2	3.2	251.4	herbicide	1.5
Prothioconazole-desthio	120983-64-4	2.3		3.1	2.7	312.2	TP of prothioconazole	1.3

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Table B.2 – continued from previous page

Chemical name	CAS number	log pK_a	acid/base	log K_{OW}	log K_{OC}	MW	Compound group	MDL water
Quinmerac	90717-03-6	4.3	a	2.9	1.9	221.6	herbicide	4.0
Quinoxyfen	124495-18-7	3.9	b	5.0	4.9	308.1	fungicide	1.0
Spiroxamine	118134-30-8	9.3	b	5.5	3.4	297.5	fungicide	2.5
Tefluthrin	79538-32-2	-		7.2	5.7	418.7	insecticide	n.a
Terbuthylazine	5915-41-3	3.2	b	3.3	2.3	229.7	herbicide	1.0
Terbuthylazine-2-hydroxy	66753-07-9	-		-1.3	2.4	211.3	TP of terbuthylazine	3.0
Thiacloprid	111988-49-9	1.6	b	2.3	2.8	252.7	insecticide	1.5
Transfluthrin	118712-89-3	-		6.17	64880	371.16	insecticide	n.a
Triadimenol	55219-65-3	1.97	b	3.28	193	295.86	fungicide	1.5

Log K_{OW} and pK_a values were calculated using the Calculator Plugins, Instant JChem 2012, ChemAxon (www.chemaxon.com).
Log K_{OC} values were calculated using EPI Suite v4.11 based on the molecular connectivity index2. TP: transformation product, n.a: not analyzed.

Table B.3: Organic contaminants quantified in *Gammarus pulex* spp. tissues from agricultural and non-agricultural sites (concentrations in ng/g wet weight).

Chemical name	**LOQ**	**non-agricultural sites**						**agricultural sites**								
Fungicides		1	2	3	4	5	6	1	2	3	4	5	6	7	8	9
Flusilazole	0.33			+				0.57	+	+	+		3.77	+	+	0.78
Spiroxamine	0.23								12.74	2.32			5.01	0.73		
Epoxiconazole	0.04				+			+	0.58	1.7	+	+	1.57	0.38		+
Propiconazole	0.31							+	+	+	+	+	0.92	+		0.33
Fenpropidin	0.42								0.19	0.14						
Fluoxastrobin	0.02								+	+			0.99	0.52		
Prothioconazole-desthio	0.06	**0.17**	**0.22**	**0.28**	**0.25**	+	0.06	0.57	1.09	1.27	0.94	0.47	4.9	1.27	0.29	1.11
Herbicides																
Isoproturon	0.04								+	+		+	0.08		+	
Prosulfocarb	0.66				+	2.18	+			0.75		+	1.18	+	+	1.49
Ethofumesate	0.03						+	+	+	0.85		+		+		0.5
Lenacil	0.24						+						0.87			
Desisopropylatrazine	0.35		+										1.18			
Diflufenican	0.58					**2.58**		3.11	13.11	21.81		1.02	93.94	3.35	1.91	2.92
Insecticides																
Imidacloprid	0.22	+	+	+				**1.48**	+	+	**1.66**	+	**1.89**	**1.53**	**3.04**	+
Thiacloprid	0.47	+	+				+	0.94	**14.28**	**3.91**	+	**0.69**	+	+		**0.76**
Clothianidin	0.57							0.61			0.67	+		0.72		
Cyhalothrin*	0.18													0.604		
Cyfluthrin*	0.10						**0.238**						0.227		0.387	
Cypermethrin*	0.15						0.037								0.260	

+Compound detected, but below the method quantification limit; *Compounds were analyzed using GC-MS/MS. Other compounds were analyzed using LC-MS/MS. **Bold** values are responsible for maximum toxic units (TU_{max}).

Table B.4: Compound descriptors *A, B, L, S, V* calculated with ACD/Percepta and resulting partition coefficients protein/water K_{PW}, storage lipid/water K_{SLW} and membrane lipid/water K_{MLW} for the pesticides detected in *Gammarus pulex*.

Compound	***A***	***B***	***L***	***S***	***V***	**log** K_{PW}	**log** K_{SLW}	**log** K_{MLW}
Isoproturon	0.31	0.88	8.5	1.42	1.78	2.09	2.47	3.10
Diflufenican	0.47	1.15	11.7	2.3	2.43	3.19	2.77	3.93
Flusilazole	0	0.69	9.67	1.83	2.27	3.82	4.85	4.91
Imidacloprid	0.26	1.68	8.33	1.7	1.68	-0.89	-1.50	-0.41
Prosulfocarb	0	0.87	9.59	1.69	2.12	2.99	3.98	4.06
Ethofumesate	0	1.29	8.7	1.91	2.05	1.08	1.23	1.73
Spiroxamine	0	1.07	9.49	0.74	2.64	3.99	5.67	5.05
Epoxiconazole	0	0.91	11.37	2.19	2.22	3.36	4.24	4.49
Desethylterbutylazine	0.46	0.92	6.99	1.37	1.48	0.89	0.62	1.71
Propiconazole	0	0.98	11.16	2	2.34	3.44	4.37	4.51
Thiacloprid	0	1.33	8.75	1.7	1.73	0.41	0.79	1.24
Lenacil	0.31	1.15	8.94	1.47	1.80	1.40	1.57	2.29
Desthio prothioconazole	0.38	0.9	10.65	1.98	2.14	3.19	3.31	4.17
Fenpropidin	0	0.68	9.58	0.81	2.54	5.00	7.03	6.32
Fluoxastrobin	0	1.97	14.57	2.29	2.99	2.64	3.07	3.32
Clothianidin	0.4	1.55	7.75	1.67	1.58	-0.83	-1.72	-0.38
Cypermethrin	0	1.11	13.51	2.47	2.97	4.87	5.69	5.83
Cyhalothrin	0	1	12.7	2.17	3.04	5.28	6.30	6.24
Cyfluthrin	0	1.11	13.56	2.44	2.99	4.95	5.81	5.92

Table B.5: Median lethal concentrations (LC_{50}, µg/L) of different chemicals for given reference organisms after a 48 h exposure. LC_{50} values were obtained from the ECOTOX database US EPA and the Pesticide Properties Database PPDB. When data from several organisms were available for the same compound, the most sensitive organism was used. In few cases, the name of reference species was not given.

Chemical name	LC_{50} (µg/L)	**Reference Species**	**Reference**	**Compound group**
Acetamiprid	20.9	*Chironomus riparius*	US EPA	insecticide
Ametryn	28000	*Daphnia magna*	PPDB	herbicide
Atrazine	1000	*Chironomus riparius*	US EPA	herbicide
Azoxystrobin	187.5	*Daphnia magna*	US EPA	fungicide
Boscalid	5330	*Daphnia magna*	PPDB	fungicide
Chlorotoluron	67000	*Daphnia magna*	PPDB	herbicide
Chloroxuron	2950	*Daphnia magna*	US EPA	herbicide
Clomazone	12700	*Daphnia magna*	PPDB	herbicide
Clothianidin	22	*Chironomus riparius*	US EPA	insecticide
cyproconazole	26000	*Daphnia magna*	US EPA	fungicide
Desethylatrazine	5100	*Hyalella azteca*	US EPA	TP of atrazine
Desethylterbutylazine	-	-	-	TP of terbutylazine
Desisopropylatrazine	-	-	-	TP of atrazine
Difenoconazole	770	*Daphnia magna*	PPDB	fungicide
Diflufenican	240	*Daphnia magna*	PPDB	herbicide
Dimethachlor	24000	*Daphnia magna*	PPDB	herbicide
Dimethenamid	16000	*Daphnia magna*	PPDB	herbicide
Dimethoate	2900	Unknown species	US EPA	insecticide
Epoxiconazole	8690	*Daphnia magna*	PPDB	fungicide
Ethofumesate	13520	*Daphnia magna*	PPDB	herbicide
Fenpropidin	540	*Daphnia magna*	PPDB	fungicide
Fenpropimorph	2240	*Daphnia magna*	PPDB	fungicide
Fenuron	502000	Unknown species	PPDB	herbicide
Flufenacet	30900	*Daphnia magna*	PPDB	herbicide

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Table B.5 – continued from previous page

Chemical name	LC_{50} (µg/L)	Reference Species	Reference	Compound group
Fluoxastrobin	480	*Daphnia magna*	PPDB	fungicide
Flurtamone	13000	*Daphnia magna*	PPDB	herbicide
Flusilazole	3400	*Daphnia magna*	PPDB	fungicide
Imidacloprid	44.4	*Chironomus riparius*	US EPA	insecticide
Imidacloprid-guanidine	44.4	*Chironomus riparius*	US EPA	TP of imidacloprid
Imidacloprid-urea	44.4	*Chironomus riparius*	US EPA	TP of imidacloprid
Isoproturon	1000	Unknown species	US EPA	herbicide
Lenacil	8400	*Daphnia magna*	PPDB	herbicide
Metamitron	5700	*Daphnia magna*	PPDB	herbicide
Metazachlor	33000	*Daphnia magna*	PPDB	herbicide
Metolachlor	23500	*Daphnia magna*	PPDB	herbicide
Metribuzin	49000	*Daphnia magna*	PPDB	herbicide
Myclobutanil	17000	*Daphnia magna*	PPDB	fungicide
Pethoxamid	23000	*Daphnia magna*	PPDB	herbicide
Pirimicarb	17	*Daphnia magna*	PPDB	insecticide
Pirimiphos-methyl	0.19	Unknown species	US EPA	insecticide
Prochloraz	4300	*Daphnia magna*	PPDB	fungicide
Propiconazole	4900	Unknown species	US EPA	fungicide
Propyzamide	5600	*Daphnia magna*	PPDB	herbicide
Prosulfocarb	510	*Daphnia magna*	PPDB	herbicide
Prothioconazole-desthio	1300	*Daphnia magna*	PPDB	TP of prothioconazole
Quinmerac	100000	*Daphnia magna*	PPDB	herbicide
Quinoxyfen	80	*Daphnia magna*	PPDB	fungicide
Spiroxamine	6100	*Daphnia magna*	PPDB	fungicide
Tebuconazole	1770	*Daphnia magna*	PPDB	fungicide
Terbuthylazine	13100	Unknown species	US EPA	herbicide
Terbuthylazine-2-hydroxy	15000	*Daphnia magna*	PPDB	TP of terbuthylazine
Thiacloprid	37	*Hyalella azteca*	US EPA	insecticide

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Table B.5 – continued from previous page

Chemical name	LC_{50} (µg/L)	**Reference Species**	**Reference**	**Compound group**
Thiacloprid amide	37	*Hyalella azteca*	US EPA	TP of thiacloprid
Thiamethoxam	35	*Chironomus* sp.	US EPA	insecticide
Triadimenol	51000	*Daphnia magna*	PPDB	fungicide
Cyhalothrin	0.38	*Daphnia magna*	PPDB	insecticide
Cyfluthrin	0.335	*Daphnia magna*	US EPA	insecticide
Cypermethrin	0.21	*Daphnia magna*	PPDB	insecticide

TP: transformation product

Table B.6: Organic contaminants responsible for highest toxicity (expressed as $\mathrm{TU}_{\mathrm{max}}$) at each sampling site. Given data includes: Site ID, CAS number, octanol-water partitioning coefficients (log K_{OW}), molecular weights (MW, g/mol) and maximum concentration detected at that site (ng/L).

Site ID	Most toxic compound	CAS number	log K_{OW}	MW	Compound group	Max Conc.
Agri-1	Thiamethoxam	153719-23-4	0.8	291.7	insecticide	538.9
Agri-2	Diflufenican	83164-33-4	3.53	394.3	herbicide	156.3
Agri-3	Pirimiphos-methyl	29232-93-7	2.96	305.3	insecticide	2.3
Agri-4	Thiamethoxam	153719-23-4	0.8	291.7	insecticide	563.7
Agri-5	Clothianidin	210880-92-5	0.64	249.7	insecticide	278
Agri-6	Thiamethoxam	153719-23-4	0.8	291.7	insecticide	2827.8
Agri-7	Thiamethoxam	153719-23-4	0.8	291.7	insecticide	661.2
Agri-8	Thiamethoxam	153719-23-4	0.8	291.7	insecticide	741.1
Agri-9	Clothianidin	210880-92-5	0.64	249.7	insecticide	311.8
Non-Agri-1	Pirimicarb	23103-98-2	1.4	238.3	insecticide	4.1
Non-Agri-2	Prosulfocarb	52888-80-9	4.23	251.4	herbicide	7.3
Non-Agri-3	Clothianidin	210880-92-5	0.64	249.7	insecticide	12
Non-Agri-4	Clothianidin	210880-92-5	0.64	249.7	insecticide	8.4
Non-Agri-5	Clothianidin	210880-92-5	0.64	249.7	insecticide	3.4
Non-Agri-6	-					

Log K_{OW} values were calculated using the Calculator Plugins, Instant JChem 2012, ChemAxon (www.chemaxon.com).

C

Supporting Information for chapter 4

Shahid, N., Liess, M., Knillmann, S. 2019. Environmental stress increases synergistic effects of pesticide mixtures on *Daphnia magna*. Environmental Science & Technology. doi: https://doi.org/10.1021/acs.est.9b04293

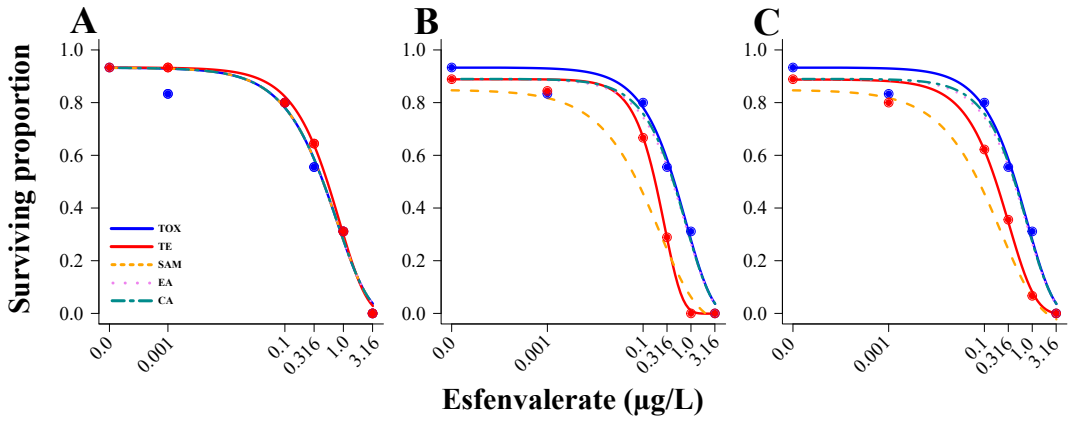

Figure C.1: Survival of *Daphnia magna* exposed to a common mixture of pyrethroid insecticide esfenvalerate and azole fungicide prochloraz under high food conditions. Dose-response relationships are displayed for day 21 – without additional stress (blue points, solid line) and in combination with different Prochloraz concentrations (Fig 3A; 1µg/L, B; 32µg/L and C; 100µg/L), as an additional stress (red points, solid line). Data points represent an average survival based on all experimental repetitions. The orange 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

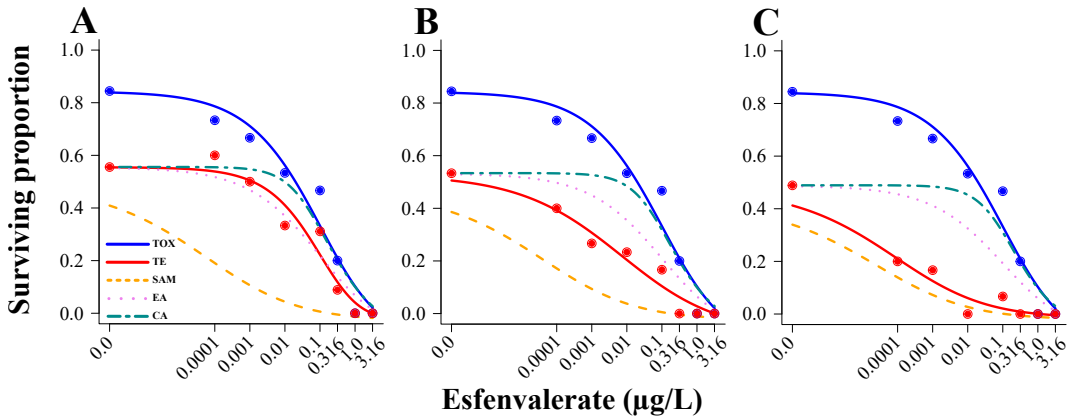

Figure C.2: Survival of *Daphnia magna* exposed to a common mixture of pyrethroid insecticide esfenvalerate and azole fungicide prochloraz under low food conditions. Dose-response relationships are displayed for day 21 – without additional stress (blue points, solid line) and in combination with different Prochloraz concentrations (Fig 3A; 1µg/L, B; 32µg/L and C; 100µg/L), as an additional stress (red points, solid line). Data points represent an average survival based on all experimental repetitions. The orange 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.

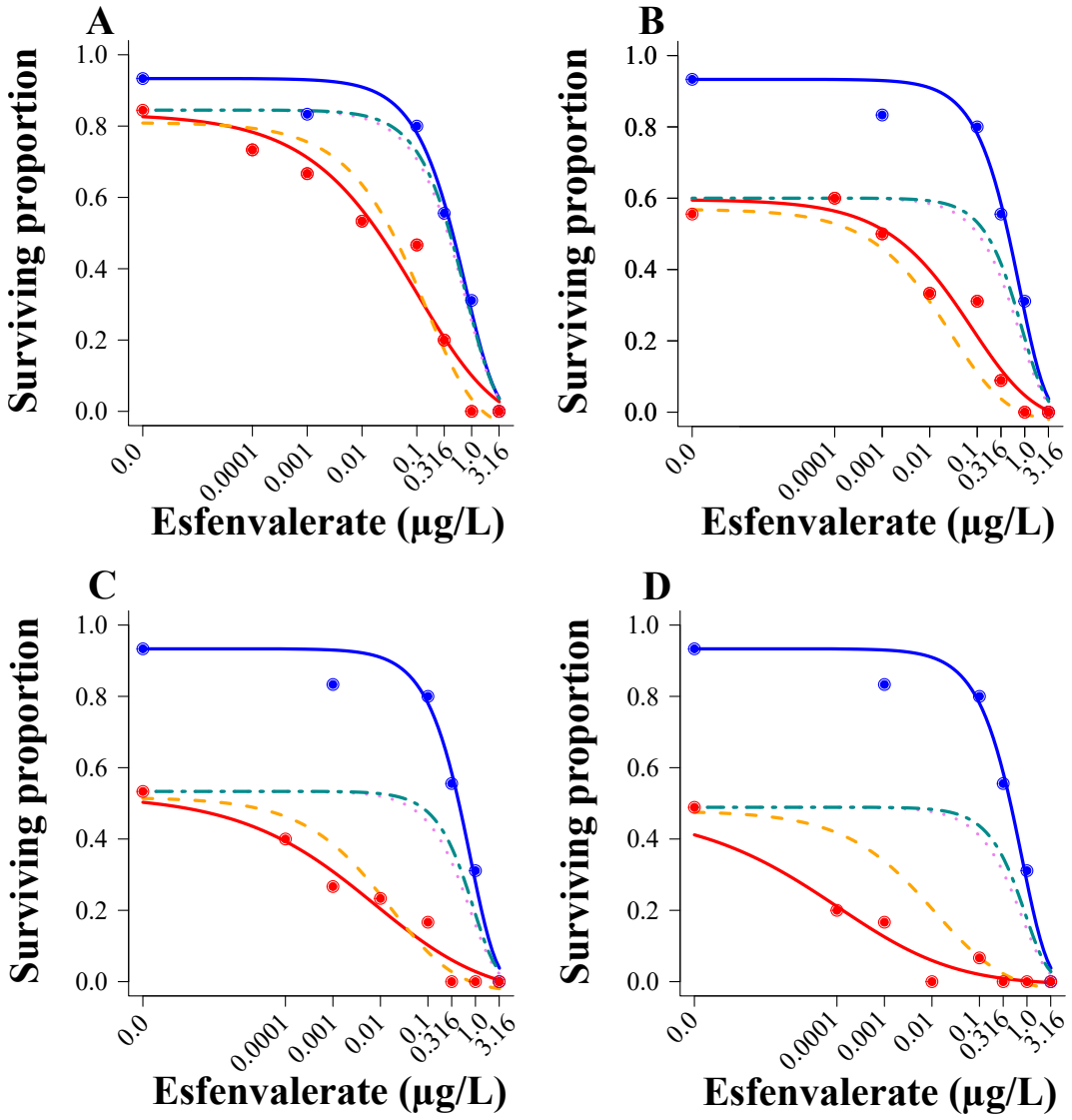

Figure C.3: Survival of *Daphnia magna* exposed to multiple stress (esfenvalerate, prochloraz and food limitation). Dose-response relationships are displayed for day 21 – without additional stress (blue points, solid line) and in combination with different Prochloraz concentrations (Fig 3A; 0µg/L, B; 1µg/L, C; 32µg/L and D; 100µg/L) and food limitation as additional stress (red points, solid line). Data points represent an average survival based on all experimental repetitions. The orange 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.

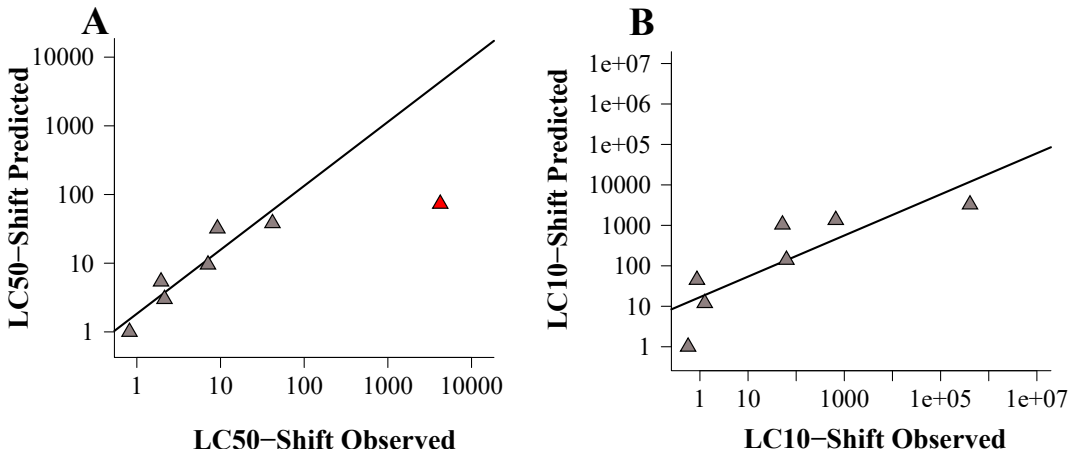

Figure C.4: Relationship between LC_x—shifts modeled by SAM and observed in different experiments. Shifts of LC_{50} (A) and LC_{10} (B) were calculated as the concentration of LC_x without environmental stress divided by the concentration of LC_x with environmental stress. In Fig A, red point was considered as an outlier and not included in the regression analysis. LC_{50}: adjusted R^2 = 83, *p*-value = 0.006, *n* = 6; LC_{10}: adjusted R^2 = 0.64, *p*-value = 0.01, *n* = 7.

Table C.1: Description of experimental setup including the number of repetitions for each concentration and individuals exposed to different combinations of esfenvalerate and prochloraz at high and low food conditions.

Concentration µg/L		**Rounds**	**Replicates**	**Rounds**	**Replicates**
Prochloraz	***Esfenvalerate***	***High food***		***Low food***	
0	0	3	45	3	45
	0.0001	-	-	1	15
	0.001	2	30	2	30
	0.01	-	-	1	15
	0.1	3	45	3	45
	0.316	3	45	3	45
	1	3	45	3	45
	3.16	3	45	3	45
1	0	3	45	3	45
	0.0001	-	-	1	15
	0.001	2	30	2	30
	0.01	-	-	1	15
	0.1	3	45	3	45
	0.316	3	45	3	45
	1	3	45	3	45
	3.16	3	45	3	45
32	0	3	45	2	30
	0.0001	3	45	2	30
	0.001	3	45	2	30
	0.01	3	45	2	30
	0.1	3	45	2	30
	0.316	3	45	2	30
	1	3	45	2	30
	3.16	3	45	2	30
100	0	3	45	3	45
	0.0001	-	-	1	15
	0.001	2	30	2	30
	0.01	-	-	1	15
	0.1	3	45	3	45
	0.316	3	45	3	45
	1	3	45	3	45
	3.16	3	45	3	45

Table C.2: Nominal and measured concentrations (µg/L) of esfenvalerate and prochloraz analyzed during different experimental rounds. The number of samples for each exposure concentration varied depending on the experimental setup (Table C1). Median and standard error are given for each exposure concentration.

Contaminant	Nominal Concentrations (µg/L)	Measured Concentrations Median (SE)
Esfenvalerate	0.0001*	-
	0.001*	-
	0.01	0.011 (0.003)
	0.1	0.1 (0.007)
	0.316	0.31 (0.06)
	1	1 (0.12)
	3.16	3.2
Prochloraz	1	1.4 (0.11)
	32	31
	100	99.5 (3.97)

*The lowest two concentrations (0.0001 and 0.001) were below the detection limit and were confirmed by higher concentrations used for serial dilutions.

D

Supporting Information for chapter 5

Shahid, N., Rolle-Kampczyk, U., Siddique, A., Von Bergen, M., Liess, M. 2020. Pesticide-induced metabolic changes are amplified by environmental stress. Submitted to the Journal "Journal of Hazardous Materials".

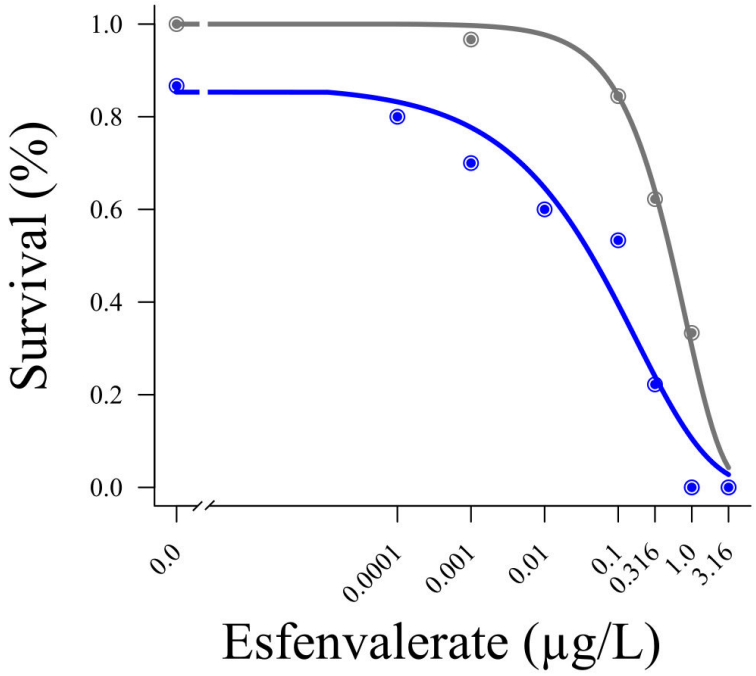

Figure D.1: Survival of *Daphnia magna* after an exposure of 24 h to different concentrations of esfenvalerate under (grey) high food and (blue) low food conditions. The given survival is for day 7 and calculated relative to the initial number of individuals. Forty-five replicates were used for each concentration level.

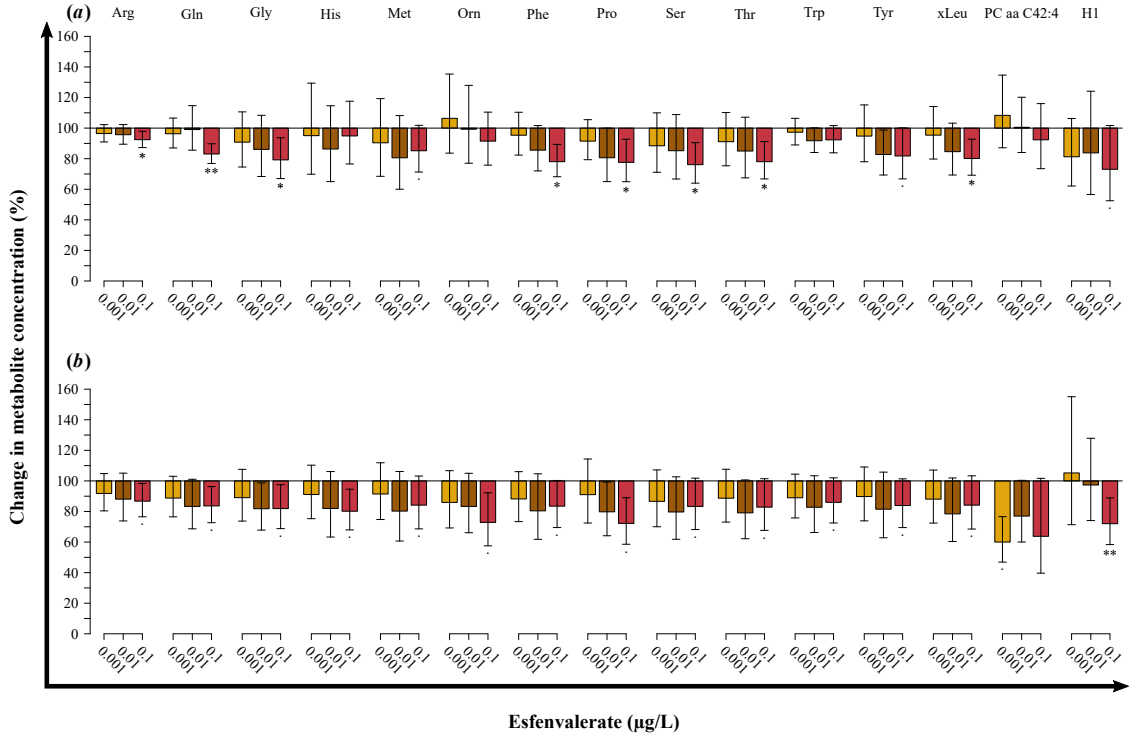

Figure D.2: Percent change in the content of individual metabolites of *D. magna* exposed to esfenvalerate in relation to the non-exposed control group. Daphnids were exposed to 0.001 µg/L, 0.01 µg/L and 0.1 µg/L of esfenvalerate for 24 h. Metabolites were measured directly after an exposure of 24h under (a) high and (b) low food conditions. The analysis is based on a linear mixed-effects model with overall 21,120 observations. Only classes with significant changes were reported. Changes are shown with their associated 95 % confidence intervals and statistical significance indicated with asterisks: "." $p < 0.1$ "*" $p < 0.05$ "**" $p < 0.01$ and "***" $p < 0.001$.

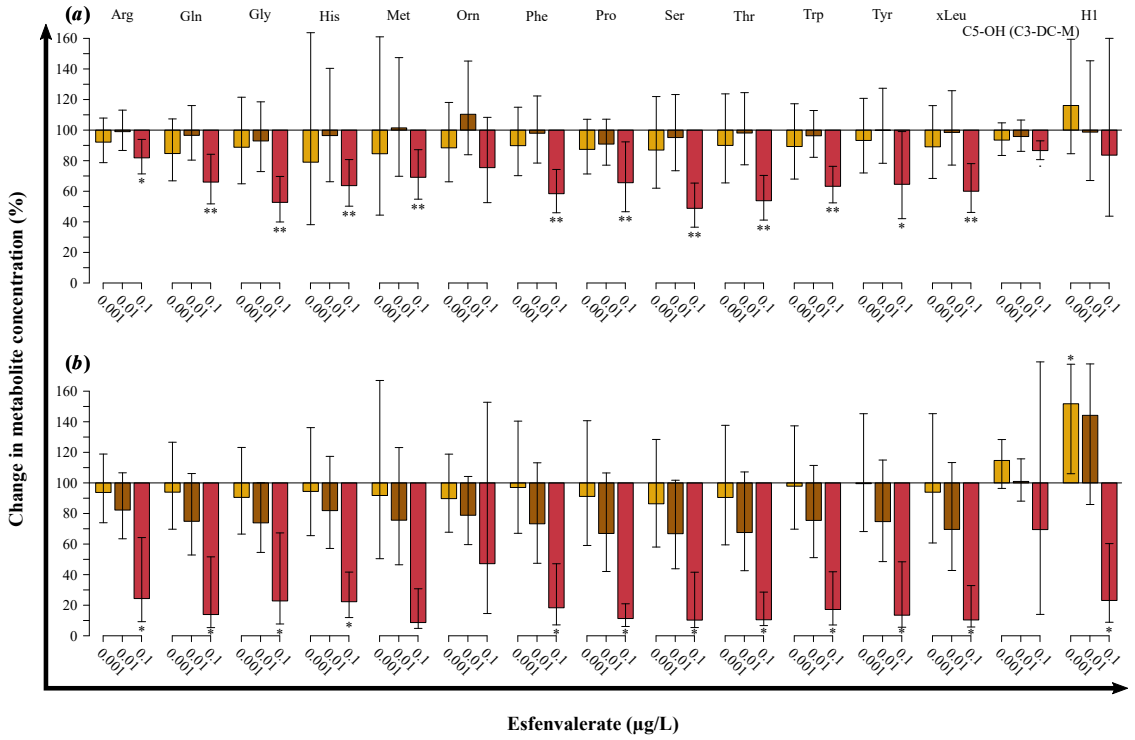

Figure D.3: Percent change in individual metabolites of *D. magna* exposed to esfenvalerate in relation to the non-exposed control group. Daphnids were exposed to 0.001 µg/L, 0.01 µg/L and 0.1 µg/L of esfenvalerate for 24 h. Metabolites were measured at day 4 (after a recovery time of 48h) under (a) high and (b) low food conditions. The analysis is based on a linear mixed-effects model with overall 21,120 observations. Only classes with significant changes were reported. Changes are shown with their associated 95 % confidence intervals and statistical significance indicated with asterisks: "." $p < 0.1$ "*" $p < 0.05$ "**" $p < 0.01$ and "***" $p < 0.001$.

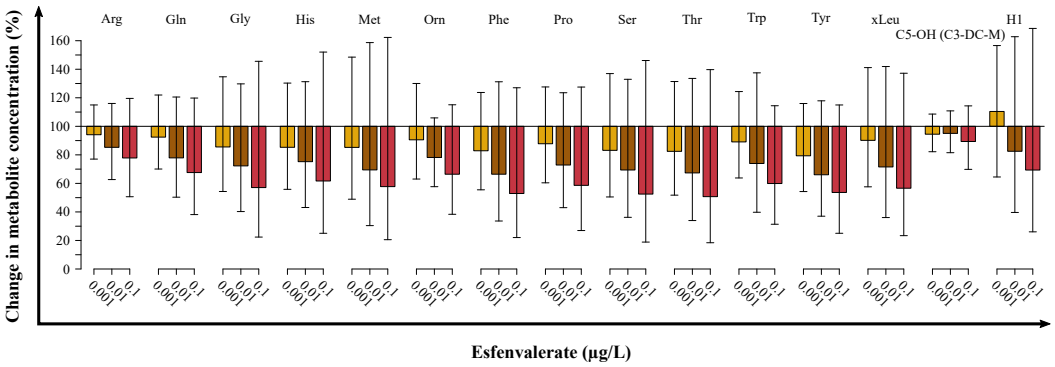

Figure D.4: Percent change in individual metabolites of *D. magna* exposed to esfenvalerate in relation to the non-exposed control group. Daphnids were exposed to 0.001 µg/L, 0.01 µg/L and 0.1 µg/L of esfenvalerate for 24 h. Metabolites were measured at day 7 only under high food conditions. The analysis is based on a linear mixed-effects model with overall 21,120 observations. Only classes with significant changes were reported. Changes are shown with their associated 95 % confidence intervals and statistical significance indicated with asterisks: "." $p < 0.1$ "*" $p < 0.05$ "**" $p < 0.01$ and "***" $p < 0.001$.

Table D.1: Nominal and measured concentrations (µg/L) of esfenvalerate analyzed during different experimental rounds.

Nominal Concentrations (µg/L)	Median measured Concentrations (SE)
0.001	0.0028 (0.0009)
0.01	0.01 (0.001)
0.1	0.98 (0.01)

Samples were analyzed by Wessling GmbH, Landsberg OT, Oppin, Germany (www.wessling.de) using a Thermo Fisher Scientific TSQ 8000 Evo Triple Quadrupole GCMS/MS. The lowest concentration (0.001) was further confirmed by higher concentrations used for serial dilutions.

Table D.2: Change in the overall metabolite content of *D. magna* exposed to esfenvalerate under high and low food conditions.

Time point	*Conc. (µg/L)*	**High Food**			**Low Food**			**Comparison (High vs. Low food)**			
		Factor of change	*p*	*Sig.*	*Factor of change*	*p*	*Sig.*	*t*	*df*	*p*	*Sig.*
Day 2	0.001	0.99			0.95	0.01	**	-3.248	5739	0.001	***
	0.01	0.91	< 0.001	***	0.93	< 0.001	***	1.147	5739		
	0.1	0.95	0.01	*	0.88	< 0.001	***	-5.012	5739	< 0.001	***
Day 4	0.001	1.07	0.003	**	1.11	< 0.001	***	2.369	5739	0.01	**
	0.01	1.06	< 0.01	**	0.98			-5.103	5739	< 0.001	***
	0.1	0.89	< 0.001	***	0.65	< 0.001	***	-11.406	4684	< 0.001	***
Day 7	0.001	0.94	< 0.001	***	–	–	–	–	–	–	–
	0.01	0.91	< 0.001	***	–	–	–	–	–	–	–
	0.1	0.81	< 0.001	***	–	–	–	–	–	–	–

We compared the overall metabolite content of *D. magna* exposed to different concentrations of esfenvalerate under high and low food conditions with respective non-exposed control groups. Metabolite contents were measured at day 2 (directly after the exposure), day 4 (48 h after the exposure) and day 7. The results reported under both food conditions refer to paired t-tests over ten experimental repetitions between the measured metabolite content in exposed organisms and respective control groups. *p* values (adjusted to account for multiple comparisons) are reported only for those classes that showed (even marginally) significant changes. Further, to identify the effect of food stress, different treatments under both food conditions were compared using t-tests. Comparison of both food levels was not applicable for day 7, because the number of alive daphnids under low food conditions were not sufficient for metabolomic analysis.

Table D.3: Change in the content of different metabolite classes in *D. magna* exposed to esfenvalerate under high and low food conditions.

Time point	*Metabolite class*	*Conc. (µg/L)*	**High Food**			**Low Food**			**Comparison (High vs. Low food)**			
			Factor of change	*p*	*Sig.*	*Factor of change*	*p*	*Sig.*	*t*	*df*	*p*	*Sig.*
Day 2	Amino Acids	0.001	0.94	0.07	.	0.89	<0.001	***	-3.16	447	0.002	**
		0.01	0.88	<0.001	***	0.82	<0.001	***	-3.38	447	0.001	***
		0.1	0.84	<0.001	***	0.82	<0.001	***	-1.09	447		
	Glycerophospholipids	0.001	1.05	0.08	.	0.94	0.011	*	-7.01	3219	<0.001	***
		0.01	0.91	<0.001	***	0.97			4.18	3219	<0.001	***
		0.1	0.91	<0.001	***	0.86	<0.001	***	-3.68	3219	<0.001	***
	Sphingolipids	0.001	0.83			0.92			1.15	555		
		0.01	0.84			0.87			0.42	555		
		0.1	1.10			0.87	0.07	.	-3.43	555	0.001	***
	Acylcarnitines	0.001	0.99			1.01			0.72	1419		
		0.01	0.96			0.89	0.003	**	-2.76	1419	0.006	**
		0.1	1.01			0.95			-2.51	1419	0.012	*
	Sugars	0.001	0.81			1.05			2.67	15	0.01	**
		0.01	0.84			0.97			1.57	15		
		0.1	0.73			0.72	0.011	*	-0.15	15		
Day 4	Amino Acids	0.001	0.88	0.003	**	0.93			1.84	447		
		0.01	0.98			0.74	<0.001	***	-9.24	447	<0.001	***
		0.1	0.63	<0.001	***	0.10	<0.001	***	-12.96	373	<0.001	***
	Glycerophospholipids	0.001	1.17	<0.001	***	1.16	<0.001	***	-0.61	3219		
		0.01	1.09	<0.001	***	1.05	0.08	.	-2.17	3219	0.030	*
		0.1	0.90	<0.001	***	0.83	<0.001	***	-2.86	2687	0.004	**
	Sphingolipids	0.001	0.93			1.07			1.83	555		
		0.01	0.99			0.83	0.08	.	-2.08	555	0.038	*
		0.1	0.88			0.54	<0.001	***	-3.06	433	0.002	**
	Acylcarnitines	0.001	0.98			1.05			3.25	1419	0.001	***
		0.01	1.03			0.96			-3.19	1419	0.002	**
		0.1	0.99			0.77	<0.001	***	-7.37	1187	<0.001	***
	Sugars	0.001	1.16			1.52	0.07	.	3.99	15	0.001	***
		0.01	0.99			1.44			3.71	15	0.002	**
		0.1	0.84			0.30	0.008	**	-2.37	15	0.029	*

Continued on next page

Table D.3 – continued from previous page

Time point	*Metabolite class*	*Conc.*	*Factor of change*	*p*	*Sig.*	*Factor of change*	*p*	*Sig.*	*t*	*df*	*p*	*Sig.*
Day 7	Amino Acids	0.001	0.87	0.0025	**							
		0.01	0.73	<0.001	***							
		0.1	0.55	<0.001	***							
	Glycerophospholipids	0.001	0.88	<0.001	***							
		0.01	0.89	<0.001	***							
		0.1	0.85	<0.001	***							
	Sphingolipids	0.001	1.14									
		0.01	1.07									
		0.1	0.69	0.011	*							
	Acylcarnitines	0.001	1.02									
		0.01	0.99									
		0.1	0.89	0.004	**							
	Sugars	0.001	1.10									
		0.01	0.83									
		0.1	0.67									

We compared the content of different metabolite classes in *D. magna* exposed to different concentrations of esfenvalerate under high and low food conditions with respective non-exposed control groups. Metabolite contents were measured at day 2 (directly after the exposure), day 4 (48 h after the exposure) and day 7. The results reported under both food conditions refer to paired t-tests over ten experimental repetitions between the measured metabolite content in exposed organisms and respective control groups. *p* values (adjusted to account for multiple comparisons) are reported only for those classes that showed (even marginally) significant changes. Further, to identify the effect of food stress, different treatments under both food conditions were compared using t-tests. Comparison of both food levels was not applicable for day 7, because the number of alive daphnids under low food conditions were not sufficient for metabolomic analysis..

Table D.4: Change in the content of different metabolite classes in *D. magna* exposed to esfenvalerate under high and low food conditions.

Time point	*Metabolite class*	*Conc. (µg/L)*	**High Food**			**Low Food**			**Comparison (High vs. Low food)**			
			Factor of change	*p*	*Sig.*	*Factor of change*	*p*	*Sig.*	*t*	*df*	*p*	*Sig.*
Day 2	Amino Acids	Arg	0.92	0.02	*	0.87	0.066	.	-1.61	15		
		Gln	0.83	0.007	**	0.84	0.09	.	0.28	15		
		Gly	0.79	0.02	*	0.82	0.09	.	0.59	15		
		His				0.80	0.09	.				
		Met	0.85	0.09	.	0.84	0.09	.	-0.17	15		
		Orn				0.73	0.06	.				
		Phe	0.78	0.02	*	0.83	0.09	.	1.00	15		
		Pro	0.78	0.02	*	0.72	0.06	.	-1.00	15		
		Ser	0.76	0.02	*	0.83	0.09	.	1.22	15		
		Thr	0.78	0.02	*	0.83	0.09	.	0.90	15		
		Trp				0.86	0.09	.				
		Tyr	0.82	0.07	.	0.84	0.09	.	0.34	15		
		xLeu	0.80	0.02	*	0.84	0.09	.	0.72	15		
	Glycerophospholipids	**PC aa C42:4**				0.60	0.09	.				
	Sugars	**H1**	0.73	0.06	.	0.72	0.006	**	-0.12	15		
Day 4	Amino Acids	Arg	0.82	0.01	*	0.24	0.02	*	-7.44	9	0.001	***
		Gln	0.66	0.007	**	0.14	0.02	*	-4.30	9	0.002	**
		Gly	0.53	0.002	**	0.23	0.02	*	-2.47	9	0.03	*
		His	0.64	0.004	**	0.22	0.02	*	-4.48	9	0.0015	**
		Met	0.69	0.009	**	0.09	0.02	*	-5.17	9	< 0.001	***
		Phe	0.58	0.002	**	0.18	0.02	*	-3.81	9	0.004	**
		Pro	0.66	0.02	*	0.11	0.02	*	-4.29	9	0.002	**
		Ser	0.49	0.002	**	0.10	0.02	*	-2.84	9	0.019	*
		Thr	0.54	0.002	**	0.10	0.01	*	-3.81	9	0.004	**
		Trp	0.63	0.002	**	0.17	0.01	*	-5.22	9	< 0.001	***
		Tyr	0.65	0.05	*	0.13	0.02	*	-3.00	9	0.01	*
		xLeu	0.60	0.004	**	0.10	0.02	*	-4.18	9	0.002	**
	Acylcarnitines	C5-OH (C3-DC-M)	0.87	0.06	.							
	Sugars	**H1**				1.52	0.02	*				
		H1				0.23	0.01	*				

We compared individual metabolite contents of *D. magna* exposed to different concentrations of esfenvalerate under high and low food conditions with respective non-exposed control groups. Metabolite contents were measured at day 2 (directly after the exposure), day 4 (48 h after the exposure) and day 7. Significant effect was only observed at highest concentration of esfenvalerate (0.1 µg/L) except sugars (**H1**) where the effect was also observed at 0.001 µg/L. The results reported under both food conditions refer to paired t-tests

over ten experimental repetitions between the measured metabolite content in exposed organisms and respective control groups. p values (adjusted to account for multiple comparisons) are reported only for those classes that showed (even marginally) significant changes. Further, to identify the effect of food stress, different treatments under both food conditions were compared using t-tests. Comparison of both food levels was not applicable for day 7, because the number of alive daphnids under low food conditions were not sufficient for metabolomic analysis.

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Contributions to the published articles and chapters

Chapter 1 This chapter is written by Naeem Shahid.

Chapter 2 This chapter is based on the manuscript "Adaptation of *Gammarus pulex* to agricultural insecticide contamination in streams" published in Science of the Total Environment. Naeem Shahid (>70%) designed the the study, collected water samples through event driven samplers, monitored macroinverebrates and did SPEAR calculation, conducted the sensitivity tests, analyzed the data and wrote the first draft of the manuscript. Jeremias Martin Becker (7%) contributed to the data analyses and interpretation of results. Martin Krauss (5%) and Werner Brack (5%) performed the chemical analyses. Matthias Liess (13%) conceived the concept of research, contributed to the interpretation of results and and supervised the study. All authors read, improved and approved the final manuscript.

Chapter 3 This chapter is based on the manuscript "Pesticide Body Burden of the Crustacean *Gammarus pulex* as a Measure of Toxic Pressure in Agricultural Streams" published in Environmental Science & Technology. Naeem Shahid (>70%) designed the study, collected water samples collected water samples through event driven samplers, collected invertebrates for the chemical analysis, monitored macroinverebrates and did SPEAR calculation, conducted the sensitivity tests, analyzed the data and wrote the the manuscript. Jeremias Martin Becker (7%) contributed to the data analyses and interpretation of results. Martin Krauss (5%) and Werner Brack (5%) performed the chemical analyses and contributed to the editing of the manuscript. Matthias Liess (13%) conceived the concept of research, contributed to the interpretation of results and and supervised the study. All authors read, improved and approved the final manuscript.

Chapter 4 This chapter is based on the manuscript "Environmental Stress Increases Synergistic Effects of Pesticide Mixtures on *Daphnia magna*" published in Environmental Science & Technology. Naeem Shahid (>70%) designed the the study, conducted all lab experiments, analyzed the data and wrote the the manuscript. Matthias Liess (15%) and Saskia Knillmann (15%) supervised the study, contributed to the interpretation of results and editing of the manuscript.

Chapter 5 This chapter is based on the manuscript "Pesticide-induced metabolic changes are amplified by environmental stress" submitted to the journal "Journal of Hazardous Materials". Naeem Shahid (>70%) was responsible for the concept of the study, performed lab experiments and metabolomic analysis, analyzed the data and wrote the the manuscript. Ulrike Rolle-Kampczyk (5%) contributed to metabolomic analysis, Ayesha Siddique (6%) contributed to sample preparation, and Martin von Bergen (4%) contributed to study idea and editing of the manuscript. Matthias Liess (15%) supervised the study, contributed to the study idea and interpretation of results. All authors read, improved and approved the final manuscript.

Chapter 6 This chapter is written by Naeem Shahid.

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Publications

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Journal Articles

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Professional Trainings

Workshops/courses offered by Helmholtz Interdisciplinary GRADuate School for Environmental Research (HIGRADE), Helmholtz Centre for Environmental Research (UFZ), Leipzig, Germany.

- Meta-analysis in Biology and Environmental Science
- Introduction to Solution-oriented Environmental Research

- Introduction to GIS
- Introduction Into Environmental Toxicology and Chemistry
- Toxicant Identification in Water, Sediment and Biota
- Risk Assessment of Chemicals in the Environment
- Introduction to Applied Statistics (incl Intro to R)
- Introduction to Multiple Regression and Model Selection
- Good Scientific Practice
- Time and Self-Management for PhDs