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Revealing the cascade of pesticide effects from gene to community

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HIGHLIGHTS

- Effects of pesticides cascade from the gene level to the community level
- Toxic pressure reduced genetic diversity in *Gammarus pulex* and altered allele distribution.
- This genetic adaptation was associated with a decrease in individual fecundity.
- Combined stress from pesticides and competition affects vulnerable invertebrates.
- Hence, pesticide-adapted *Gammarus pulex* dominate contaminated invertebrate communities.

GRAPHICAL ABSTRACT

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ABSTRACT

Global pesticide exposure in agriculture leads to biodiversity loss, even at ultra-low concentrations below the legal limits. The mechanisms by which the effects of toxicants act at such low concentrations are still unclear, particularly in relation to their propagation across the different biological levels. In this study, we demonstrate, for the first time, a cascade of effects from the gene to the community level. At the gene level, agricultural pesticide exposure resulted in reduced genetic diversity of field-collected *Gammarus pulex*, a dominant freshwater crustacean in Europe. Additionally, we identified alleles associated with adaptations to pesticide contamination. At the individual level, this genetic adaptation to pesticides was linked to a lower fecundity, indicating related fitness costs. At the community level, the combined effect of pesticides and competitors caused a decline in the overall number and abundance of pesticides susceptible macroinvertebrate competing with gammarids. The resulting reduction in interspecific competition provided an advantage for pesticide-adapted *G. pulex* to dominate macroinvertebrate communities in contaminated areas, despite their reduced fitness due to adaptation. These processes demonstrate the complex cascade of effects, and also illustrate the resilience and adaptability of biological systems across organisational levels to meet the challenges of a changing environment.

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1. Introduction

Despite elaborate regulation of agricultural pesticides, their occurrence in non-target areas has been linked since long to adverse ecological effects on insects in several field investigations, (Beketov et al., 2013; Liess et al., 2021b; Liess and Ohe, 2005; Rundlof et al., 2015) and microcosms studies (Liess and Groning, 2024; Rasmussen et al., 2017) even at ultra-low concentrations occurring in short pulses far below regulatory limits. This subtle but pervasive toxic pressure plays a pivotal role in shaping the evolutionary and ecological processes underlying the current biodiversity loss (Jaureguiberry et al., 2022; Marta et al., 2021) generally not considered in risk assessment (Agathokleous et al., 2022). Consequently, resulting in the emergence of adapted communities, quantified through the $SPEAR_{pesticides}$ index (quantifies proportion of pesticides vulnerable species - SPecies At Risk) (Liess and Ohe, 2005). These changes in community dynamics are influenced by a combination of factors. Firstly, they are determined by the direct effects of low pesticide concentrations on vulnerable species (Liess and Beketov, 2011). Secondly, they involve a comparative tolerance exhibited by insensitive species (Liess and Ohe, 2005). Thirdly, sensitivity (Liess and Beketov, 2011; Liess et al., 2013) and potential for recovery of vulnerable species is further impaired by competition with insensitive species (Knillmann et al., 2012). In addition, subpopulations with different sensitivities to pesticides develop (Becker and Liess, 2017) as identified also for algal communities (Wood et al., 2019) and terrestrial arthropods (Guedes et al., 2022).

Individual tolerance to toxicants is determined by a variety of mechanisms, including physiological acclimation, epigenetic modifications (Wolf and Wade, 2009), transgenerational effects (Tran et al., 2018) and genetic adaptation (Medina et al., 2007; Ribeiro and Lopes, 2013). While physiological acclimation provides a short-term response to stress (Ghalambor et al., 2007; Pigliucci, 2005), it is constrained by resource allocation (Auld et al., 2010). In contrast, long-term exposure requires evolutionary adaptation (Major et al., 2018). Genetic adaptation, among other mechanisms (Gaines et al., 2020), involves the evolution of tolerance at the population level through the selection of the most tolerant genotypes (Lopes et al., 2004; Orr et al., 2022). This adaptation process may lead to reduced genetic variability (Coors et al., 2009; Jansen et al., 2011) or changes in allele frequency when specific alleles are involved in resistance development (Gouin et al., 2019). Accordingly, populations may experience inbreeding due to increased homozygosity of deleterious alleles, possibly leading to local extinctions (Frankham, 2005; Keller and Waller, 2002). Therefore, understanding whether tolerance to contaminants is transient or has a genetic basis, is crucial since genetic adaptive responses may carry substantial fitness costs, impacting population performance under fluctuating conditions (Convey and Peck, 2019; Heim et al., 2018).

These adaptive responses may also be influenced by the nearby refuge sections. For instance, adaptation to pesticides was found to be reduced when the distance to nearby refuge sections was short, allowing for the migration of individuals (Becker et al., 2020; Shahid et al., 2018a). Contrary to this, a study reported increased pesticide tolerance in organisms from refuge sections as compared to those from agricultural areas (Schneeweiss et al., 2023b). Furthermore, the development of pesticide tolerance in *Gammarus pulex* was also reduced by the presence of competing species (Becker and Liess, 2017).

Although there are numerous studies on contamination-induced genetic adaptations, our understanding of their ecological consequences in natural populations is still in its infancy. This is because the cascading effects from individual trade-offs to community composition at the landscape level remain largely unknown, as also highlighted by Schneeweiss et al. (2023a). To investigate these processes, we conducted a comprehensive study, quantifying the ecologically relevant short-term peak exposure of agricultural pesticides in 38 small streams with a wide range of pesticide contamination in central Germany. Aiming to quantify the effect cascading through biological levels our biological endpoints covered the entire macroinvertebrate community in these streams. For the genetic analysis of *G. pulex*, we employed the AFLP (Amplified Fragment Length Polymorphism) technique developed by Vos et al. (1995) as the genome of *G. pulex* has not yet been sequenced. We also quantified the fecundity of field-collected *G. pulex* from streams with a range of pesticide contamination under non-contaminated laboratory conditions. Finally, we determined pesticide exposure and the invertebrate community within agricultural streams. Our hypotheses were:

(i) Increasing pesticide exposure in the field leads to increased tolerance in *G. pulex*.

(ii) Populations with increased insecticide tolerance exhibit lower genetic diversity.

(iii) The distribution of alleles within populations vary according to the level of field contamination.

(iv) Genetic adaptation to pesticides has a negative impact on general fitness of individuals quantified as fecundity (offspring/ind.) in the absence of pesticides.

(v) Tolerance development, genetic diversity, and allele frequency in *G. pulex* populations from contaminated sites are influenced by nearby refuge areas.

(vi) The dominant crustacean *G. pulex* may overcome the negative consequences of pesticide adaptation due to reduced interspecific competition with competing pesticide-vulnerable species.

2. Materials and methods

2.1. Study sites and sample collection

In the present study, we collected *G. pulex* from 38 streams in Germany during 2018–2019 (Liess et al., 2021a). Sites map and details in Fig. S1, Table S1. Briefly, 21 populations were sampled in 2018, 11 populations were sampled in 2019 spring (9 repeated from 2018 and 2 new populations added), and 6 populations were sampled in 2019 autumn (repeated from 2019 spring). Among these, 25 were contaminated with environmentally relevant levels of pesticides (log $TU_{max} \geq -3.0$) (Liess et al., 2021b), while 13 were contaminated with considerably low concentrations and thus considered as reference streams. The sites covered a variety of stream habitats such as numerous types of sediments, plants, organic matter and morphology (Liess et al., 2021a). Since the present study focused on effects of pesticide contamination, it was made sure there is no input from wastewater treatment plants or industrial facilities. Contrary to contaminated streams, the reference sites were located within or at the edge of forest (Liess et al., 2021a). The test organism, *G. pulex*, were sampled using kick–nets (0.5 mm) in, (i) Summer 2018 (April–June): during the peak pesticide application period conforming high pesticide exposure, (ii) Spring 2019 (March–April): before the beginning of main pesticide application, and (iii) Autumn 2019 (October–November): 3–4 months after main pesticide application (Huber et al., 2000; Liess et al., 1999) both conforming to "low pesticide exposure" in the field. To avoid the possibility of pseudoreplicates, we correlated the pesticide toxicity of the year 2018 and 2019, where the contamination between the years differed by 41 %. For acute sensitivity tests (Section 2.3, only performed in 2019 spring and autumn), approximately 100 individuals (7–9 mm size, both genders) were collected from each site and transported to the laboratory under constant aeration and cool boxes. Genetic analyses were conducted for all time points (2018, 2019 spring, autumn). For this purpose, 30 individuals (7–9 mm size, both genders) were collected from 38 sites and stored in absolute ethanol. Additionally, the physicochemical parameters such as temperature, pH, and dissolved oxygen (DO) were measured to make sure that these environmental parameters were in the benign range for gammarids (Table S2). The organisms for acute tests were kept overnight at 16 °C under continuous aeration before exposure to pesticide. For genetic analysis, samples were frozen at −20 °C in absolute ethanol until further analysis.

In addition to *G. pulex*, macroinvertebrates were sampled following standardized multi-habitat sampling (Meier et al., 2006) in June 2018 and 2019 (for details, refer to (Liess et al., 2021b), SI Invertebrate list) in order to analyze community changes potentially due to pesticide exposure. Sampled organisms were separated the from coarse organic debris using a column sieve set, preserved in 90 % ethanol, and later determined in the laboratory generally down to the lowest taxonomic level possible under the binocular. The invertebrate determination level, abundance and occurrence at sampling sites is provided in the (Liess et al., 2021b).

In addition, forested stream sections with a minimum 500 m area with no potential pesticide input either upstream or downstream were identified that may serve as potential refuge areas. The distance to the closest refuge sections was measured based on the waterway GPS positions using Google Maps. We considered refuge sections both upstream and downstream with a maximum distance of 7 km as *G. pulex* can migrate in both directions.

2.2. Quantification of pesticide contamination

The pesticide contamination in the streams was measured by collecting rain–event–triggered water samples (EDS) (Liess et al., 1999) under the Kleingewassermonitoring (KgM) project (https:// www.ufz.de/kgm/) (Liess et al., 2021b). EDS sampling for a duration of 3 h and 20 min was triggered by a 5 cm rise of water level, so that waves did not trigger the sampling and every runoff event could be captured, resulting in 200 mL unfiltered and cooled (4 °C) water samples.

The number of samples for each site varied from one to six, depending on the rainfall events, regardless of rainfall intensity. A total of 108 chemicals comprised of 75 pesticides (active substances) and 33 pesticide metabolites were analyzed in the water samples. Pyrethroid insecticides and the herbicide Glyphosate were not included due to analytical limitations. Targeted substances were analyzed using liquid chromatography–high–resolution mass spectrometry [LC–HRMS, Ultimate 3000 LC system coupled to a Q Exactive Plus MS equipped with a heated electrospray ionization (ESI) source, all from Thermo Scientific]. The maximum toxic unit (TU_{max}) was calculated as suggested by Liess and Ohe (Liess and von der Ohe, 2005) following eq. 1, since several field studies conducted in small streams showed that pesticide effects on macroinvertebrate communities are best explained by TU_{max} (Liess et al., 2021b; Liess and von der Ohe, 2005; Schäfer et al., 2012).

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

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

Effects of field pesticides exposure on macroinvertebrate community were quantified using the $SPEAR_{pesticides}$ (SPEcies At Risk) bioindicator, which quantifies the toxic pressure of pesticides based on the proportion of macroinvertebrates classified as highly vulnerable to pesticides ("SPEcies At Risk") to the total number of macroinvertebrates (Liess and von der Ohe, 2005). A low SPEAR value indicates high effects of pesticides and hence high pesticide exposure. A detailed description of the approach is provided in Liess et al. (Liess and von der Ohe, 2005).

In addition to SPEAR indicator, Shannon taxa diversity ((Shannon and Weaver, 1949), proportion of Ephemeroptera, Plecoptera and Trichopteran (% EPT (Lenat, 1988)) and an indicator that unspecifically responds to stressors- total number of insect taxa were also calculated in (Liess et al., 2021b) and were used for correlations under current study.

2.3. Selection of insecticide and acute toxicity tests

In order to analyze pesticide sensitivity of populations, we used clothianidin, a neonicotinoid insecticide commonly applied in agriculture and has often been detected in small streams of the study area (Knillmann et al., 2018; Liess et al., 2021b). The acute sensitivity of *G. pulex* to the neonicotinoid was tested following the OECD guidelines for chemical testing (OECD, 2004) and the rapid tests for community level risk assessment (Kefford, 2013). A 40 mg/L stock solution was prepared in distilled water using DANTOP (500 g/kg, Spiess–Urania Chemical GmbH, Germany) and stirred overnight on a magnetic stirrer. Individuals of *G. pulex* were acclimatized for 10 days in a climate chamber preset at 16 °C, a 16:8 light–dark cycle (to mimic day-night cycle) under constant aeration and fed with ~6 g of wet weight of preconditioned leaves (Siddique et al., 2020). After acclimation to lab conditions, organisms were exposed to a range of sublethal to lethal concentrations of clothianidin. Five clothianidin concentrations (i.e., 0, 8, 40, 200, and 1000 μg/L) were prepared by diluting the stock solution in artificial stream water ADaM (Klüttgen et al., 1994). Briefly, 16 individuals from each population (4 individuals per tea bag, ⌀ 6 cm) were exposed to 3 L test concentration medium of clothianidin for 96 h and kept in climate chambers at 16 °C with a 16:8 light–dark cycle and continuous aeration. Immobility was considered as endpoint and recorded after every 24 h. Individuals were considered as immobile when they did not show body movement (swimming) within 30 s after probing with a glass rod. Dead individuals were removed regularly. Acute toxicity experiments were only performed with populations collected in 2019 Spring and Autumn due to practical limitations.

To determine the actual concentrations of insecticides used for acute tests, we collected 1000 mL samples of stock and working solutions at the start of the exposure. Since the exposure lasted only for 48 h and degradation rate of clothianidin is low in water (DT50 14.4 days at pH 9, 50 °C), concentrations after 48 h were not measured. The test samples were analyzed by Wessling GmbH, Landsberg OT, Oppin, Germany, using a Thermo Fisher Scientific TSQ 8000 Evo Triple Quadrupole gas chromatography–mass spectrometry (GC–MS/MS). The detection limit of the instrument was 5.7 ng/L. The analytical column used was a TG–5HT guard column with a 0.53 mm ID and a 0.15 μm film thickness (Thermo Fisher Scientific, USA). The software TraceFinder 3.2 (Thermo Fisher Scientific) was applied for data processing. Actual concentrations recovered from the samples were in acceptable boundaries (±10 %) to the nominal concentrations.

2.4. Population genetic diversity assessment

Genetic structure and diversity parameters of *G. pulex* populations were analyzed using amplified fragment length polymorphisms (AFLP) in a total of 1035 individuals (size 7–9 mm, both genders) from 38 sites (25–30 individuals per site) during 2018 and 2019. AFLP is highly reproducible and larger numbers of amplified products can be generated in a single reaction (Villalobos-Barrantes et al., 2015), and have successfully been applied for the detection of genetic loci influenced by contamination showing potential for understanding the evolution deriving forces (Bach and Dahllof, 2012; Markert et al., 2010). Initially, we tested two primer combinations AAC/CTT and ACC/CTG (Restriction Endonucleases *Eco*RI/*Mse*I), followed by Bach and Dahllof (2012) and selected one primer combination AAC/CTT for final analysis (Table S7). A total of 451 polymorphic bands were obtained and used for further analysis.

2.5. DNA extraction and AFLP assay

Genomic DNA was extracted from each individual *G. pulex* using Dneasy Blood and tissue kit (Qiagen, Germany) following manufacturer's guidelines with minor modifications such as double elution and more tissue sample to increase the concentration of DNA required for

AFLP. DNA integrity was checked on 1.5 % agarose gel, and DNA concentration was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies Inc.).

AFLP assay used in the present study is a modified version of Vos et al. (1995) and Paun and Schönswetter (2012). We performed DNA restriction and adaptor ligation in a single step. Briefly, for each individual 400–500 ng of DNA was double–digested with 5 U *Eco*RI (Invitrogen) and 1 U *Mse*I (New England Biolabs) and ligated in 10 μl total volume including 0.6 U T4 DNA ligase (Invitrogen), 10 × T4 Ligase buffer (Invitrogen), 0.5 M NaCl, 1 mg/mL BSA (Bovine Serum Albumin), 5 μM MseI adaptor pair (Invitrogen), 5 μM EcoRI adaptor pair (Invitrogen) for 3 h at 37 °C followed by 3 h at 17 °C. MseI and EcoRI adapter pairs were heated at 95 °C for 5 min separately and cooled down at room temperature before adding to Restriction–Ligation (RL) reaction. The efficiency of the restriction reaction was checked on 1.5 % agarose gel in 1 × TBE buffer (Tris Borate EDTA). The Restriction–Ligation products were diluted 10–fold with 0.1 × TE buffer (Tris EDTA) and stored at −20 °C until further processing. Pre–selective amplifications were then performed on 5 μl of diluted RL products in 10 μl volumes containing 10 mM dNTP (deoxynucleotide triphosphate)–mix, 1 μM each of EcoRI and MseI preselective primers (premixed) (Invitrogen), 1 U Hotstart Taq polymerase (Invitrogen) all in 10 × PCR buffer (Invitrogen) and ddH_2O. The PCR (Polymerase Chain Reaction) conditions for the preselective amplification were: one hold of 72 °C for 2 min, 20 cycles of (94 °C for 1 s; 56 °C for 30 s, 72 °C for 2 min), and finish with a hold of 60 °C for 30 min. Preselective products were diluted 10–fold with 1 × 0.1 M TE buffer. Selective amplifications were performed on 5 μl of diluted preselective products in 10 μl volumes containing 10 mM dNTP–mix, 1 μM of EcoRI and 5 μM MseI selective primers (Invitrogen), 1 U Hotstart Taq polymerase (Invitrogen) all in 10 × PCR buffer (Invitrogen) and double distilledH_2O. The cycling conditions for selective PCR were as follows: one hold of 94 °C for 2 min, 9 cycles of (94 °C for 1 s, 65 °C; decreasing 1 °C every cycle for 30 s and 72 °C for 2 min), followed by 23 cycles of (94 °C for 1 s, 56 °C for 30 s and 72 °C for 2 min) and finish with a hold of 60 °C for 30 min. Table S2 provides information on choice of adaptor and primer constructions. Negative controls (TE buffer used as template) were processed in parallel. The final products were sequenced on the ABI Prism 3130XL Genetic Analyzer (Applied Biosystems) with Red 500 DNA size standard and analyzed in GeneMapper 5.0.

2.6. Genetic analysis

GeneMapper 5.0 (Applied Biosystems) was used to score AFLP alleles. DNA fragments <50 base pairs in length were excluded from the analysis to minimize the probability of including homoplastic AFLP bands. Peak data were converted to create a binary matrix by scoring for presence (1) or absence (0) of a DNA fragment. A data qualification selection was performed by which AFLP markers with <5 % abundance in total dataset (Bonin et al., 2005), and individuals with <140 AFLP markers were excluded. GenAlex v. 6.51 was used to calculate basic genetic statistics including No. of bands, percentage of polymorphic loci (*PLP*), average number of alleles per locus (*Na*, mean and standard error (SE)), effective alleles per locus (*Na*, mean and SE), expected heterozygosity (*He*, mean and SE), unbiased expected heterozygosity (*uHe*, mean and SE). The Nei's genetic distances and genetic differentiation (PhiPT) were also calculated in GenAlEx v. 6.501 (Peakall and Smouse, 2020; Peakall and Smouse, 2006). Population differentiation was further quantified by analysis of molecular variance (AMOVA). The program I4A, inbreeding for AFLPs (Chybicki et al., 2011) was used to estimate the inbreeding coefficient (F_{is}) for each population. Estimates were calculated at three different values (0.1, 1, and 5) for each parameter, alpha and beta (Oleksa et al., 2013), using 10,000 rejected steps and 20,000 sampling steps. By computing different values of alpha and beta, we assess how much the inbreeding coefficients change when the shape parameters change. The accuracy of this estimate is determined by comparing the similarities between the loglikelihood values among the different B–distribution parameters. High similarity implies high accuracy.

The program Past 3.25 (Hammer et al., 2001) was used to access genetic diversity by applying Bray–Curtis similarity index on individuals within and between populations at each sampling site, thus creating a similarity matrix between all individuals. A Bray–Curtis similarity matrix was used as input for ANalysis Of Similarities (ANOSIM). To test whether the geographic distance accounted for eventual differences observed in genetic diversity between populations, the geographic distances between each pair of sites were measured based on the waterway GPS positions using Google maps and correlated to the pairwise genetic dissimilarity based on ANOSIM computed by (SIMilarity Percentage) SIMPER. A maximum value of 600 km was allotted to pair of streams with no connection through water.

2.7. Populations' fecundity assessment

The long–term effects of pesticide exposure in field and adaptation were analyzed by culturing *G. pulex* under pesticide–free conditions in the laboratory (Siddique et al., 2020). Due to practical limitations and general difficulties regarding gammarids culture, only 6 populations from 2018 were cultured in lab. In general, maintenance of culture followed the descriptions given by (McCahon and Pascoe, 1988a, 1988b) with certain modifications by (Siddique et al., 2020) as follows. Briefly, 100 medium–sized individuals (7–9 mm, with approx. Similar sex ratio) from each population were cultured in a 5 L covered glass tank filled with 3 L aerated artificial stream water (ADaM) (Klüttgen et al., 1994). Organisms were fed ad libitum with alder leaves preconditioned in stream water for at least two to three weeks before use. Stones of different size pre-conditioned in stream water for at least two weeks were added to increase the water quality of the culture medium and to provide juveniles with the opportunity to hide from omnivorous adults. Continuous aeration was provided in order to avoid oxygen shortage during experiments and cultures. To maintain the quality of the culture medium, 500 mL of medium was replaced by fresh medium every 14 days and 1500 mL of old medium was replaced with fresh medium every 30 days, and dead organisms were removed. The culture was maintained at 16 °C, with 60 % humidity and artificial light (12 h light:12 h dark) in a climate chamber. Adults and offspring were counted every month until four months. For population growth, expressed as the fecundity rate, the total number of individuals was divided by the initial number of individuals. The study observed ARRIVE guidelines (Percie du Sert et al., 2020) and EU Directive 2010/63/EU for animal experiments.

2.8. Data analysis

All the statistical analyses were carried out using RStudio (V 2023.6.1) and R (V 4.1.3) (Team, R.C., 2020) for Windows, unless otherwise mentioned. For the acute sensitivity, the concentration that affected 50 % of the exposed population (EC_{50}) was calculated using a generalized linear model with a quasi–binomial error distribution and a logit link function (V 3.0–1) (Ritz et al., 2015). For the comparison of EC_{50} values (spring vs autumn), t–test was applied. The local contamination was based on TU_{max} values derived from water samples collected in summer 2018 and 2019. Similarly, for the quantification of ecological effects, the SPEAR was calculated based on invertebrate data collected in June for both years. Two sample t–tests (when the assumption of equal variances was fulfilled) and Welch's t–test (in the case of nonequal variances) were applied to compare genetic parameters among reference and agricultural populations. In case the data were not normally distributed, Wilcoxon's rank sum test was used. Populations were divided into three groups based on pesticide contamination-TU_{max} range from −6 to −4.1, −4 to −2.1 and − 2 to 0 and named as "Low Contamination", "Medium contamination" and "High contamination" respectively and compared using t–tests. The "low contamination alleles" were quantified based on the allele frequency significantly higher

in the low contamination populations as compared to contaminated populations, and average frequency of these alleles for each population was used for further analysis. Similarly, the "high contamination alleles" were quantified based on the allele frequency significantly higher in the high contamination populations as compared to reference populations, and average frequency of these alleles for each population was used for further analysis.

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

3. Results

3.1. Pesticide exposure and adaptation of G. pulex

The studied sites were characterized by a wide range of pesticide concentrations leading from no effects to severe effects on macroinvertebrate communities (maximum Toxic Unit - log TU_{max} – 0.13 to –6.0). Neonicotinoids and carbamate insecticides sampled during rainfall induced peak exposure exerted the highest toxic pressure. The pesticide tolerance of *G. pulex* increased with increasing toxic pressure as expected under hypothesis (i). The 48 h median effect concentration (EC_{50}) of *G. pulex* to insecticide clothianidin positively correlated with the TU_{max} (adj. $R^2 = 0.51$, $p < 0.005$, Fig. 1). Among other parameters, genetic diversity, area covered with forest (%), and $SPEAR_{pesticide}$ showed negative correlations while Nutrients (Total Phosphorus and Total Nitrogen) and pesticide contaminations showed positive correlations with acute EC50 of *G. pulex*. Fig. S2.

3.2. Genetic diversity and genetic structure of G. pulex

The primer combinations selected in the present study yielded 451 polymorphic loci across 1035 individuals from 38 populations. Non-metric Multi-Dimensional Scaling (NMDS) based on bray-curtis similarity index showed agricultural and reference populations as two slightly overlapping clusters indicating pesticide contamination based genetic differentiation between the two groups (Fig. S3). Furthermore, individuals from each population at each sampling time showed different population clusters differentiating on field pesticide exposure based on principal component analysis (PCA) in fig. S4. Percentage of within population genetic diversity based on Bray-Curtis index was on average significantly lower in contaminated populations as compared to reference populations ($p < 0.001$, average contaminated = 5.2 % vs average non-contaminated = 17.4 %, $p < 0.001$ Table S3). Similarly, percentage of between populations' genetic diversity was on average significantly lower in contaminated populations (average contaminated 34 % vs. average non-contaminated = 50 %, $p < 0.001$). Further details on no. of bands, percentage of polymorphic loci (*PLP*), average number of alleles per locus (*Na*, mean and standard error (SE)), effective alleles per locus (*Na*, mean and SE), expected heterozygosity (*He*, mean and SE), unbiased expected heterozygosity (*uHe*, mean and SE) are provided in supplementary information table S3. A significant, albeit weak relationship was observed between the pairwise genetic dissimilarity and geographic distance between populations (adj. $R^2 = 0.039$, $p < 0.001$, Fig. S5). Detailed pairwise genetic dissimilarity and geographic distance is provided in Table S4. Although in general, populations were significantly differentiated from each other (overall PhiPT = 0.45, $p < 0.001$), the contaminated populations were more differentiated from each other (average PhiPT = 0.51) as compared to reference populations (average PhiPT = 0.33, pairwise PhiPT, pairwise PhiPT values in Table S5). The AMOVA (analysis of molecular variance) analysis revealed that in reference streams, on overage 67 % of the variation was observed within population and 33 % among populations. Whereas, in contaminated streams, 49 % variation was observed within populations and 51 % among populations (Table S6).

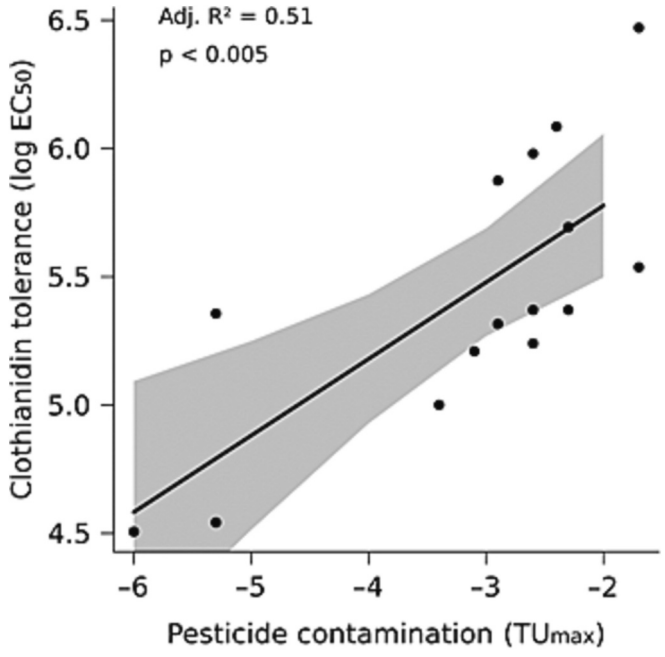

Fig. 1. Increased pesticide tolerance in *G. pulex*. Relationship between site specific pesticide contamination (TU_{max}) and pesticide tolerance of *G. pulex* quantified as EC_{50} of the insecticide clothianidin after 48 h constant exposure, log–transformed). Regression line represents least squared regression. Means ±95 % confidence intervals are shown.

3.3. Genetic diversity and adaptation of G. pulex

Overall, populations' genetic diversity decreased with increasing pesticide contamination and tolerance development as expected under hypothesis (ii). We observed a significant negative association between pesticide contamination in streams (TU_{max}) and population genetic diversity of *G. pulex* (adj. $R^2 = 0.63$, $p < 0.001$, Fig. 2a). Based on the range of toxic pressure exerted by pesticides (TU_{max}), we divided the populations into three groups, i.e., low (TU_{max} − 6 to −4.1), moderate (TU_{max} − 4 to −2.1), and high contamination (TU_{max}; −2.0 to 0). We revealed a higher genetic diversity in populations from low contamination sites (mean 17.57 %) followed by moderate (mean 9.45 %, $p < 0.005$) and high contamination sites (mean 5.18 %, $p = 0.015$, Fig. 2a). Furthermore, adapted populations were characterized by the reduced genetic variability (adj. $R^2 = 0.504$, $p < 0.005$, Fig. 2b), which also showed positive association with $SPEAR_{pesticides}$, Shannon index, total number of insect taxa, and % EPT species (Fig. S6). Furthermore, among other field parameters, pH, TU_{max}, area covered with agriculture (%), metal contamination and nutrients showed negative correlation while area covered with forest (%) showed positive correlation with genetic diversity in *G. pulex* (Fig. S7).

3.4. Contamination-specific alleles in G. pulex

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

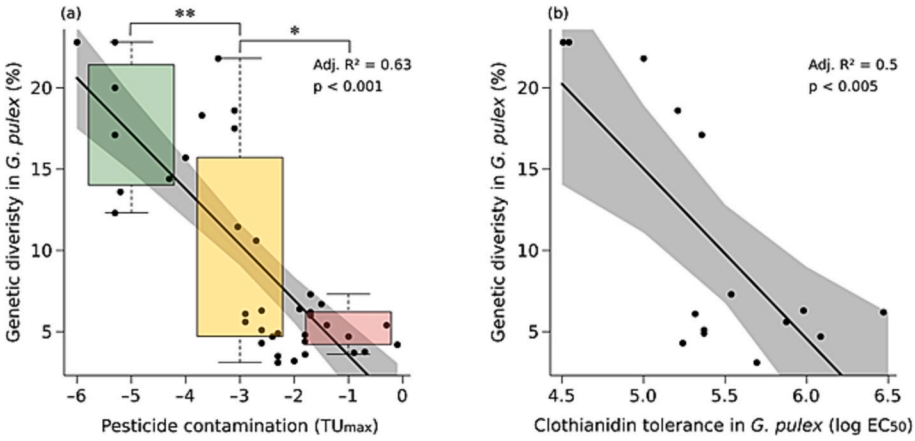

Fig. 2. Pesticide exposure, genetic diversity and adaptation of *G. pulex*. Relationship between site specific pesticide contamination (TU_{max}) and within population genetic diversity in *G. pulex* (a). Total pesticide contamination: The green box represents populations from low field exposure (TU_{max}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

contamination sites, however no significant difference was observed among populations from moderate and high contamination sites, revealing a toxic pressure dependent decline in allele frequency (Fig. 3a).

For populations occurring in highly pesticide contaminated sites we observed that the average frequency of 14 alleles termed "high contamination alleles" was significantly higher (0.54 vs. 0.34, $p < 0.05$), and positively correlated with site-specific pesticide contamination (adj. $R^2 = 0.55$, $p < 0.001$, Fig. 3b). Accordingly, the average frequency of these alleles was significantly different among populations from low, moderate and high contamination sites revealing toxic pressure dependent increase in allele frequency. Additionally, the ratio of high to low contamination alleles increased with increasing toxic pressure at sites (adjusted $R^2 = 0.55$, $p < 0.001$, Fig. S8).

3.5. Fitness trade–offs in genetically adapted populations

Pesticide adapted populations of *G. pulex* from a range of pesticide exposure in the field were characterized by a reduced fecundity when cultured under non–contaminated conditions in lab (adj. $R^2 = 0.92$, $p < 0.005$, Fig. 4a) as expected under hypothesis (iv). Furthermore, average fecundity was associated with within population genetic diversity (adj. $R^2 = 0.64$, $p < 0.05$, Fig. 4b).

3.6. Distance to refuge sections and genetic composition of G. pulex

As expected under hypothesis (v), *G. pulex* acquired a further increased tolerance when distance to nearby refuge area was high presumably decreasing the exchange rate between adapted and non-adapted populations (adj. $R^2 = 0.22$, $p = 0.06$, Fig. 5a). Additionally, increasing distance to refuge sections reduced the genetic diversity (adj. $R^2 = 0.36$, $p < 0.001$, Fig. 5b) and frequency of "low contamination alleles" (adj. $R^2 = 0.42$, $p < 0.001$, Fig. 5c).

3.7. Reduced competition and effects at community level

With increasing abundance of *G. pulex*, inbreeding coefficient of respective populations slightly decreased ($R^2 = 0.12$, $p < 0.05$, Fig. S9). The inbreeding coefficient was not associated with pesticide contamination or genetic diversity ($p > 0.1$, Fig. S10). The abundance of *G. pulex*

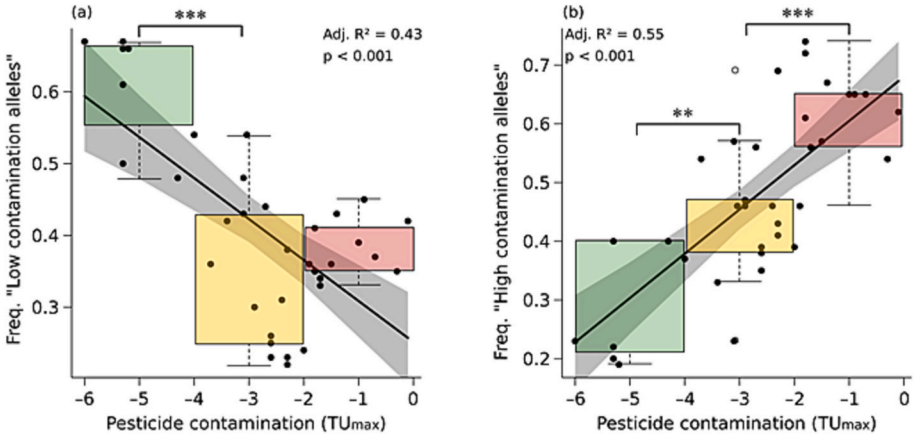

Fig. 3. Pesticide exposure and contamination-specific alleles of *G. pulex*. Relationship between site specific pesticide contamination (TU_{max}) and (a) average allele frequency of "low contamination alleles" in *G. pulex* populations based on alleles significantly higher in streams with low toxic pressure (TU_{max} range from −6 to −4), (b) average allele frequency of "high contamination alleles" in *G. pulex* populations based on alleles significantly higher in streams with high toxic pressure (TU_{max} range from −2 to −0). The green box represents populations from low field exposure (TU_{max} − 6 to −4.1), the yellow box represents populations from moderate field exposure (TU_{max} − 4 to −2.1), and the red box represents populations from high field exposure (TU_{max} − 2 to 0). Grey area represents ±95 % confidence interval and dots represent data points.

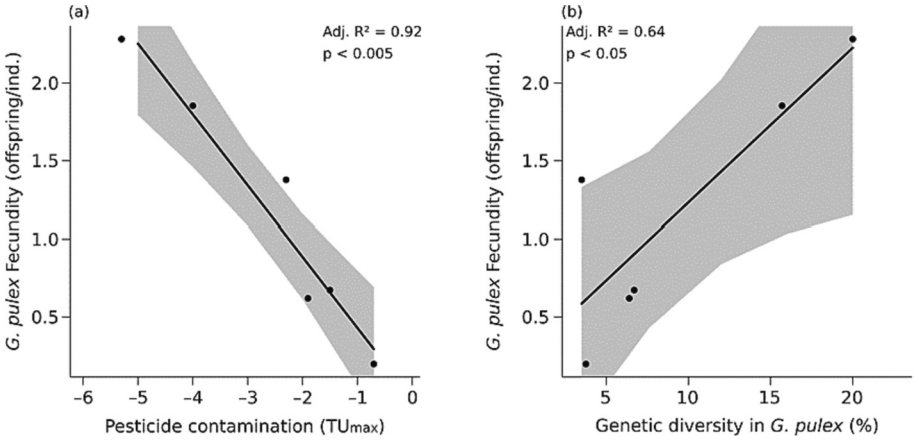

Fig. 4. Fitness costs of exposure to pesticides in *G. pulex*. (a) Relationship between site specific pesticide contamination (TU_{max}) and fecundity of gammarid populations cultured under non–contaminated conditions (average fecundity in cultures from month 2 to 4), (b) Linear regression between genetic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

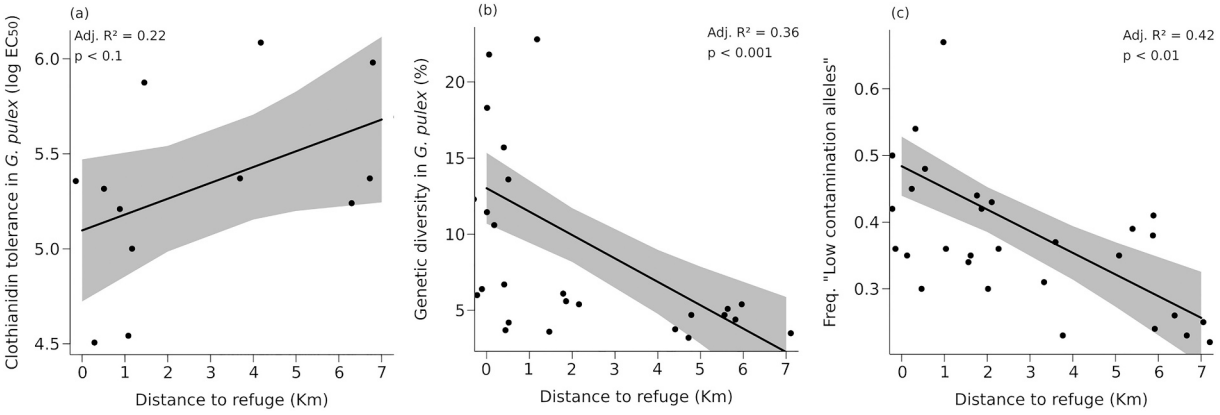

Fig. 5. The effect of nearby refuge areas. Relationship between distance to refuge and (a) Clothianidin tolerance (48 h EC_{50}) in *G. pulex*, (b) Within population genetic diversity and (c) Frequency of low contamination alleles in the studied populations of *G. pulex*. Grey area represents ±95 % confidence interval and dots represent data points.

populations was not affected by pesticide exposure ($p > 0.1$, Fig. S11), whereas the proportion of pesticide vulnerable species identified with $SPEAR_{pesticides}$ showed negative correlation between in-stream pesticide contamination (adj. $R^2 = 0.35$, $p < 0.001$; Fig. 6).

In terms of competition, *G. pulex* population density decreased with the increasing occurrence of vulnerable gammarids competitors. Such a relationship was established for the proportion of pesticide vulnerable species ($SPEAR_{pesticides}$; adj. $R^2 = 0.24$, $p < 0.001$, Fig. 7a) and also for Ephemeroptera, Plecoptera, and Trichoptera species competing with gammarids for food (% EPT; adj. $R^2 = 0.34$, $p < 0.001$, Fig. 7b) as expected under hypothesis (vi). In contrast, proportion of EPT species not competing with *G. pulex* for food did not show any association with its abundance (adj $R^2 = -0.054$, $p > 0.1$, Fig. S12). Correlation plot of abundance of macroinvertebrates groups is given in Fig. S13 indicating positive and negative co-occurrence of different taxa. Further details on macroinvertebrates and *G. pulex* abundance at each site are provided in table S7.

4. Discussion

Pesticide exposure triggers evolutionary processes in natural populations (Medina et al., 2007), and also alters the macroinvertebrate community composition as identified for agricultural streams in 2005 by Liess & Ohe and have since confirmed in several field studies (Liess et al., 2021b; Rumschlag et al., 2020; Stehle and Schulz, 2015). There are numerous investigations on contamination-induced genetic adaptations, however, understanding their ecological consequences in natural populations is still in its infancy. This knowledge gap primarily arises from the lack of a comprehensive understanding of the cascading effects of pesticides, extending from individual genetic composition to the broader community structure at the landscape level.

In detail, relevant biological effects include: (i) increased tolerance and a reduction in genetic diversity as an outcome of contamination (Bach and Dahllof, 2012), (ii) toxic pressure dependent distribution of alleles at field relevant exposure concentrations, (iii) fitness trade-offs associated with genetic adaptation (Heim et al., 2018), (iv) impact of geographical distance to the non-contaminated refuge populations (Becker et al., 2020; Shahid et al., 2018a) and (v) the interspecific competition with vulnerable species.

While some of these processes have undergone partial investigation, our study represents, to the best of our knowledge, the inaugural attempt to comprehensively integrate the complex cascade of pesticides effects and illustrates the resilience and adaptability of biological systems across organisational levels to meet the challenges of a changing environment.

At population level, we observed up to 4.5–fold (average 2.2–fold) increased pesticide tolerance in *G. pulex* collected from contaminated streams. Such a development of pesticide tolerance in different aquatic species including *G. pulex* (Becker and Liess, 2017; Shahid et al., 2018a, 2018b; Siddique et al., 2020; Siddique et al., 2021) and *Daphnia magna*

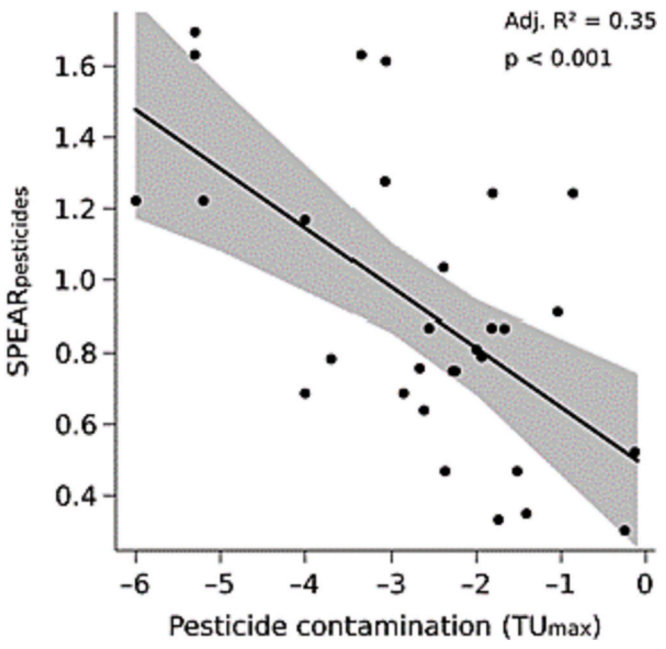

Fig. 6. Effect of pesticide exposure on macroinvertebrates. Relationship between site specific pesticide contamination (TU_{max}) and macroinvertebrate community structure quantified as $SPEAR_{pesticides}$ Means ±95 % confidence intervals are shown.

(Almeida et al., 2021) have been observed. Similarly, local adaptation to ore mine effluents have also been observed in *Dugesia gonocephala* (Weigand et al., 2018). The acquisition of increased tolerance in a natural population may occur through environmentally induced physiological acclimation (Maxwell et al., 2014; Siddique et al., 2021), epigenetic modifications (Wolf and Wade, 2009), loss of sensitive individuals, and selection of more tolerant genotypes which may reduce the genetic diversity (Forbes and Calow, 1996; van Straalen et al., 2011). The latter could be the reason for reduced genetic diversity in the current study (Fig. 2). Furthermore, reduced genetic diversity explained by toxic pressure reveal that even such low concentrations may significantly shape genetic metrics of populations and may describe the loss of sensitive species under long–term exposure that have not been studied so far.

In general, genetic erosion is widely accepted as a potential evolutionary outcome of long–term exposure to contaminants (Nowak et al., 2009; Ribeiro and Lopes, 2013; Ungherese et al., 2010). For example, low genetic diversity in >700 aquatic species from extensive cropland (Crossley et al., 2022), and reduction in allelic richness was observed in *G. pulex* from wastewater contaminated rivers (Švara et al., 2022). However, in these cases, investigations only focused on high exposure concentrations and did not investigate the effect of low exposure concentrations and potential local adaptation. Furthermore, tolerance acquired through genetic adaptation based on specific alleles may change allele frequency as a result of increased number of tolerant individuals in a population (Gouin et al., 2019). Likewise, in the present study, we observed a decrease in average alleles frequency of "low contamination alleles" with increasing toxic pressure (Fig. 3a). This change in frequency was significantly different among populations from low, moderate/high pesticide exposure indicating loss of alleles already at concentrations 3 to 4 orders of magnitude below the EC_{50}. Reduction in frequency of some alleles could be attributed to long-term consequence of contamination that selects for tolerant genotypes as mentioned earlier (Ribeiro and Lopes, 2013). On the other hand, an increasing average frequency of "high contamination alleles" with pesticide contamination observed in the present study (Fig. 3b) indicates effects on allelic distribution on such a low range of pesticide contamination. Further, this suggests that the "high contamination alleles" might be functionally associated with tolerance conferring mechanisms required to cope with pesticide stress. For instance, the allele frequency of two outlier SNP loci involved in insect cuticle resistance in natural populations of mayfly *Andesiops torrens* were significantly correlated with high pesticide exposure (Gouin et al., 2019).

Multiple stressors in the field shape the aquatic communities (Kuzmanovic et al., 2017). In the present study, pesticide exposure was mainly responsible for the observed effects on abundance of vulnerable invertebrates in the investigated streams (Liess et al., 2021b). However, several other environmental factors may affect the genetic diversity of populations. For example, geographic distance between sites partially explained the pairwise genetic dissimilarity (Fig. S5). Other studies also reported genetic structure of populations partially or completely explained by geographic distance (Švara et al., 2022; Whitehead et al., 2003). Furthermore, historical colonization events in crustacean *Gammarus fossarum* (Weiss and Leese, 2016) and barrier effects in gastropod *Ancylus fluviatilis* and flatworm *Dugesia gonocephala* (Weiss et al., 2022) have been associated with populations genetic structure and differentiation. In addition, abundance of sensitive species may also impact the evolutionary process as tolerance development can be affected by species diversity (Becker and Liess, 2017). Likewise, we observed a higher genetic diversity in *G. pulex* populations with higher macroinvertebrate diversity in terms of $SPEAR_{pesticide}$, Shannon Index, total number of insect taxa and percentage of EPT species (Fig. S6) indicating that the conditions that promotes species diversity may also preserve genetic diversity within species. Such a positive association between genetic–

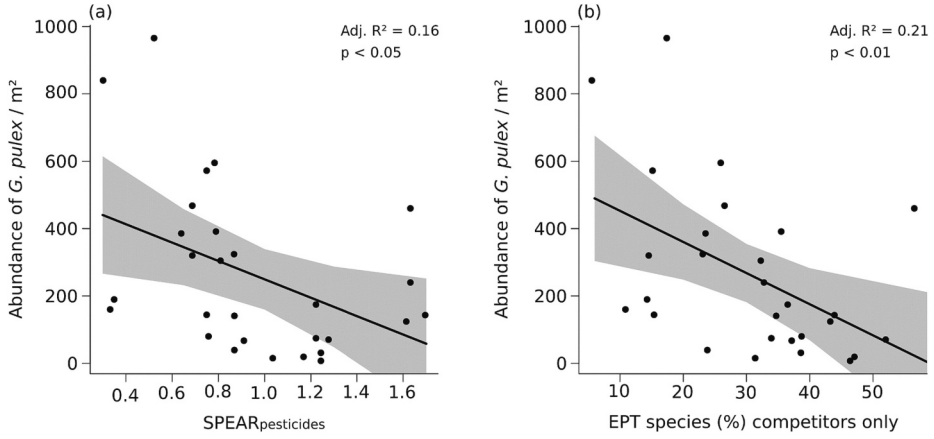

Fig. 7. Abundance of *G. pulex*. Relationship between Abundance of *G. pulex* and (a) $SPEAR_{Pesticides}$, and (b) Percentage of vulnerable species. Grey area represents ±95 % confidence interval and dots represent data points.

and species diversity has also been reported by Manel et al. (2020).

Fitness costs are often associated with increased tolerance due to energy trade–offs (Fournier-Level et al., 2019; Siddique et al., 2021). However, it has rarely been reported in natural populations (Jansen et al., 2011; Siddique et al., 2020). We observed that pesticide exposure and genetic adaptation to pesticides were associated with reduced fecundity in natural populations of *G. pulex* revealed under non-contaminated conditions (Fig. 4). Although, change in the environmental conditions from field to lab could also affect the growth rate, this finding suggests that survival in contaminated streams infers energetic costs reducing the energy available for vital functions and reduces plasticity for survival under slightly different conditions, also indicated elsewhere (Marchand et al., 2004). Yet, other studies revealed that pyrethroid resistant crustacean *Hyalella azteca* exhibited reduced thermal tolerance (Heim et al., 2018), and lower survival and lipid levels (Fulton et al., 2021) as compared to non-resistant individuals. However, these trade-offs were revealed in the presence of stressors, unlike our study where the fitness costs were observed under non-contaminated conditions.

Migration of organisms from nearby refuge areas often supports recovery for pesticide affected individuals (Becker et al., 2020; Shahid et al., 2018a) and populations (Liess and von der Ohe, 2005). Likewise, we observed that the clothianidin tolerance was reduced with increasing distance to the nearby refuge area (Fig. 5). However, genetic diversity and frequency of "low contamination alleles" increased with decreasing distance to refuge sections, suggesting that migration events from nearby refuge sections support populations to retain genetic diversity. In contrast, a significantly lower pesticide tolerance of *Gammarus* spp. at agricultural sites compared to edge and refuge sites has been reported (Schneeweiss et al., 2023b). The authors suggest that this could be due to higher environmental stress indicated by slightly lower lipid content per mg gammarid tissue at agricultural sites related to energy tradeoffs. Inbreeding coefficient of *G. pulex* populations decreased with increasing abundance (Fig. S9). However, neither local pesticide contamination nor reduced genetic diversity induced inbreeding depression (Fig. S10). Similarly, Švara et al. (2022) reported higher inbreeding coefficient in *G. pulex* from wastewater contaminated sites as compared to pristine sites, which could also be due to a smaller and thus genetically impoverished population.

On the community level, we observed that the abundance of *G. pulex* was independent of pesticide contamination (Fig. S11) despite their apparent fitness trade-offs and observed a decline in vulnerable species (Fig. 6) already below the regulatory compliant concentrations ($TU_{max} \geq -3$), mostly contributed by neonicotinoids and carbamate insecticides. The decline in pesticide vulnerable species is comparable with other investigations that identified strong impacts of nonpoint-source pesticide pollution on streams in Australia (Beketov et al., 2013), Europe (Beketov et al., 2013; Liess et al., 2021b; Liess and Ohe, 2005), North America (Chiu et al., 2016) and South America (Hunt et al., 2017). A situation that is in contrast to the aims of the regulatory authorization of pesticides that is supposed to prevent unacceptable effects in the environment (Australian Environment Agency (2009); EFSA (2013)). We hypothesize that this non-existent association of *G. pulex* abundance and pesticide exposure is due to the exposure-induced decline in competing pesticide-vulnerable SPEAR- and EPT-species (Fig. 7). Whereas, EPT species not competing with Gammarids for food do not show decline with increasing abundance of gammarids. This indicates that the combination of pesticide pressure and gammarids in field lead to the decline of pesticide vulnerable EPT species competing with gammarids in the field. In a two-species laboratory microcosm experiment, such combined effects of pesticides and pressure from competing species were also observed by Liess et al. (2013). Furthermore, species with low or even declining dominance may not be able to compensate individual trade-offs from pesticide effects at the community level (Liess and Beketov, 2011).

Thus, pesticide exposure at already low toxic pressure causes decline in vulnerable species, while on the other hand, selects tolerant genotypes and individuals with reduced fecundity in dominant species. Our working hypothesis suggests that these dominant species, however, benefits from reduced interspecific competition with declining pesticide vulnerable species. This represents a crucial process of species selection to site-specific environmental conditions. By unraveling these underlying mechanisms, we establish a vital link between genetic effects and ecosystem-level impacts.

In the present study, we considered the following Hill's criteria for ecological applications to show causality: (1) consistency and replication of results was observed in all the investigated streams, (2) dose-response relationships were always observed for acute toxicity tests conducted in the laboratory and SPEAR data from the field, (3) effects of confounding variables in the field were reduced by sampling sites with a wide range of various environmental parameter that were not interrelated and the application of the SPEAR approach that proved to be generally independent on confounding factors, lab experiments were conducted in controlled conditions in a climate chamber, and same methods were followed throughout the study, (4) biological plausibility was observed in terms of alignment with existing knowledge, mechanistic explanation, experimental evidence and links across scales of organization, and (5) the study design and analyses followed randomized controlled experiments to avoid potential bias.

CRediT authorship contribution statement

Ayesha Siddique: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Naeem Shahid:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Validation, Visualization, Writing – review & editing. **Matthias Liess:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ayesha Siddique reports financial support was provided by German Academic Exchange Service. Ayesha Siddique reports financial support was provided by Horizon Europe. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or S1.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.170472.

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