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Population structure and insecticide response of *Gammarus* spp. in agricultural and upstream forested sites of small streams

Anke Schneeweiss[1*], Verena C. Schreiner[1], Matthias Liess[2,3], Nina Röder[1], Klaus Schwenk[1,4] and Ralf B. Schäfer[1]

Abstract

Exposure to pesticides may cause adaptation not only in agricultural pests and pathogens, but also in non-target organisms. Previous studies mainly searched for adaptations in non-target organisms in pesticide-polluted sites. However, organisms may propagate heritable pesticide effects, such as increased tolerance, to non-exposed populations through gene flow. We examined the pesticide tolerance—as one of the pre-assumptions of local adaptation—of the freshwater crustacean *Gammarus* spp. (at genus level reflecting the gammarid community). The pesticide tolerance was quantified in acute toxicity tests using the insecticide imidacloprid. Gammarids were sampled at pesticide-polluted agricultural sites (termed agriculture), least impacted upstream sites (termed refuge) and transitional sites (termed edge) in six small streams of south-west Germany. Furthermore, we examined the population genetic structure of *Gammarus fossarum* and the energy reserves (here lipid content) of *G. fossarum* as well as of *Gammarus* spp. at the three site types (i.e. agriculture, edge and refuge). We found significantly lower imidacloprid tolerance of *Gammarus* spp. from agricultural sites compared to edge and refuge sites, potentially due to higher environmental stress at agricultural sites, as indicated by a slightly lower lipid content per mg gammarid tissue. We found no differences in pesticide tolerance between edge and refuge populations, indicating no propagation of pesticide effects to edges. The genetic structure among *G. fossarum* populations showed significant differentiation between streams, but not within a stream across the site types. We suggest that high gene flow within each stream hindered population differentiation and resulted in similar (pre)adaptations to local stress levels between site types, although they exhibited different pesticide pollution. Further studies on target genes (e.g., conferring pesticide tolerance), population structure and fitness of different phenotypes in particular among non-target organisms are required for adjacent pristine ecosystems to detect potential propagations of pesticide effects.

Keywords Pesticide tolerance, Imidacloprid, Edge effect, Invertebrate, Spatial, Lipid, Genetic

*Correspondence:
Anke Schneeweiss
schneeweiss@uni-landau.de
Full list of author information is available at the end of the article

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Introduction

Pesticides are applied over large areas in agricultural landscapes, where they can affect populations, communities and food webs in non-target ecosystems such as streams [1–5]. Exposure to pesticides can set off adaptation processes in agricultural pests and pathogens [6–9] and also in non-target organisms such as freshwater taxa [10–13]. A study reported an up to 3.6-fold average increase in insecticide tolerance across 17 freshwater invertebrate taxa [10]. Similarly, amphipods from pesticide-exposed streams exhibited a threefold higher insecticide tolerance compared to non-exposed populations [12]. In both studies, the authors suspected a predominant genetic basis underlying the tolerance development. First, the taxa were likely exposed regularly over decades [12], which is usually associated with genetic adaptation [14]. Second, the tolerance was similar during and after the pesticide spraying season, suggesting transgenerational effects that may be explained by genetic adaptation [10, 12]. Finally, genetic adaptation to pesticides has been detected or suspected frequently in pest species [15–18], and for non-target species [19] including freshwater invertebrates [11, 13, 20].

Overall, the development of increased tolerance in freshwater invertebrates may at least partly be based on genetic adaptations that occur through selection at gene loci affecting survival (e.g., target site encoding or metabolic resistance genes) [6, 21]. Directional selection can leave a long-term fingerprint on the gene pool (i.e. selective signatures in the genomes) [6], such as allele or genotype frequency changes in selected (e.g., pesticide-tolerant) populations [21–24], potentially translating into strong population differentiation [25, 26]. Strong small-scale population differentiation within the same catchment has been demonstrated for *Gammarus fossarum* [27], also in association with differences in pesticide susceptibility [28]. In addition, reduced genetic diversity has been found for strains carrying resistant alleles [29–31] and in taxa inhabiting polluted sites [13, 32–34]. A loss of genetic variation can affect numerous ecological processes at the population, community and ecosystem levels [35, 36]. Furthermore, increased tolerance may be associated with fitness costs, if the tolerance traits are genetically correlated with fitness traits (negative pleiotropy) or if the tolerance-related energy allocation to defence mechanisms leads to trade-offs in energy allocation [37–41].

To date, adaptations in non-target species have been mainly investigated in pesticide-polluted sites. However, the exchange of organisms may propagate heritable pesticide effects, such as increased tolerance, to non-exposed populations, for example, if related genes are transferred (i.e. gene flow) and if fitness costs are low. Historically theoretical and empirical research mainly focused on constraints or disruptive effects of gene flow on adaptation whereas to date an increasing number of studies suggests that local adaptation may develop or be maintained despite gene flow and that local adaptation can even be promoted by gene flow [42–49]. For example, already early (finite) population models suggested that gene flow can retard adaptation in treated fields, but can also increase the frequency of tolerance-promoting alleles in nearby untreated fields [50, 51] and may contribute to pre-adaptation of untreated populations [52]. For pest species, field studies suggest that insecticide resistance can spread through insect migration and related gene flow even between continents [53]. Similarly, fungicide resistance can spread over long distances [54, 55]. To which extent this applies to non-target species in freshwater ecosystems remains unknown. Such knowledge is required to reliably predict or explain non-target population and community dynamics in non-exposed habitats, which may inform pesticide management and contribute to biodiversity protection.

We examined pesticide tolerance as one of the pre-assumptions of local adaptation of the widely occurring freshwater crustacean *Gammarus* spp. We collected organisms from downstream pesticide-polluted agricultural sites (here termed agriculture), non-polluted upstream sections that are directly connected (here termed edges) and sites in forested sections further upstream (here termed refuges) in six small streams in south-west Germany. Based on between-sites distances and the maximal upstream dispersal distance of *Gammarus* spp. (for details see "Study area" Sect), we expected gene flow between site types. The highest levels of exchange (including genetic exchange) were expected for the edge areas following the edge concept [56]. Given the regular pesticide exposure at agricultural sections in our study region, we hypothesised local adaptation processes in terms of higher pesticide tolerance of *Gammarus* spp. and that these propagate via gene flow to the edge habitats (hypothesis 1). Pesticide tolerance was quantified in acute toxicity tests (similar to the studies of [10, 12, 28, 57]) using the insecticide imidacloprid. Further, the regular pesticide exposure in agricultural sites may leave a long-term fingerprint in the gene pool of populations despite considerable gene flow. This is hypothesised to result in a higher population differentiation between agricultural and refuge populations than between agricultural and edge populations (hypothesis 2a) and a decrease in genetic diversity from refuge over edge to agricultural sites (hypothesis 2b). Following the concept of resource allocation [58], we hypothesised that a higher tolerance is associated with a higher allocation of energy to defence mechanisms, resulting in lower

energy reserves in tolerant organisms compared to less tolerant organisms (hypothesis 3).

Materials and methods

Study area

We sampled at the three different site types "refuge", "edge" and "agricultural" of six streams (i.e. a total of 18 sampling sites) in summer 2019 in Rhineland-Palatinate, south-west Germany. Vineyards dominate land use near agricultural sites and forests characterise the catchment areas of upstream refuge and edge sites. The edge sites were located in the transition zone between agriculture and forest. The forested refuge sites were about 100 to 200 m higher than the agricultural sites (Additional file 1: Table S1). Refuge and edge sites were about 0.6 to 1.5 km apart, whereas edge and agricultural sites were about 1.4 to 4.8 km apart [59]. The distance between refuge and edge sites was selected based on estimates of the maximum gammarid upstream dispersal (i.e. approximately 1 km/lifespan see [59]; based on [60]), as we focus on gammarids in this study and assume that potentially acquired pesticide tolerance propagates via upstream dispersal. Analytical testing of stream water confirmed significant and repeated pesticide contamination that is likely to result in adverse effects on invertebrates at the agricultural sites (for details see: [59, 61]). Edge and refuge sites occasionally also had relevant pesticide toxicity, defined as log sum toxic units above − 3 [59, 62]. Accordingly, forested upstream sites correspond to "least impacted" sites relatively free of human influences, rather than completely "pristine" sites. However, the focus of this study was to examine site types with strong differences in pesticide stress. This was met by the site conditions because edge and refuge sites exhibited overall significantly lower pesticide exposure and toxicity than agricultural sites (average log sum toxic unit − 4.8, − 5.1 and − 2.1 in refuge, edge and agricultural sites, respectively [59]). The estimated toxicity to invertebrates was driven by insecticides in all site types [59]. Further details on the study area, site selection and environmental conditions at the site types can be found in [59].

Gammarid sampling

Sampling of gammarids for measurement of pesticide tolerance, population genetics and energy reserves was conducted from mid-June to mid-July 2019. This is a period when field populations likely have been exposed to high pesticide concentrations [63–65], which may lead to pesticide tolerance. For determining pesticide tolerance, we collected *Gammarus* spp. (Crustacea, Amphipoda) alive and transported them under cool and aerated conditions to the laboratory. All living organisms were cautiously handled and used in tests on the same day of

sampling without acclimation, because this reduced time in captivity and did not lead to relevant mortality [66]. We opted for random sampling to obtain a representative sample of the field communities within a genus (species level not identifiable by eye without harm) and therefore omitted prior identification and sorting (e.g., for size and sex) [66]. Most *Gammarus* spp. were *Gammarus fossarum,* but *Gammarus pulex* and *Gammarus roeselii* were also common, particularly at the downstream agricultural sites (Additional file 1: Table S2; Fig. 1C). We estimated the relative abundance of the tested *Gammarus* species based on the representative subsample taken for population genetics and considered the potential for interspecific differences by including the relative abundance of species as a random factor in the statistical modelling (see "Factors explaining pesticide tolerance" Sect). For population genetic analyses, we randomly sampled approximately 40 *Gammarus* spp. per sampling site and stored them in ≥ 96% ethanol (Roth, Karlsruhe, Germany) at − 20 °C. Within each sample, we identified and counted *G. roeselii* individuals microscopically. *G. pulex* and *G. fossarum* were distinguished via genetic analysis following the procedure described in "Population genetic analysis" Sect, given ambiguities in visual identification. The *G. fossarum* individuals ($n=20$, where abundances permitted; Additional file 1: Table S2) were further processed for population genetic analysis. For the analysis of energy reserves, we randomly collected approximately 20 *Gammarus* spp. per sampling site, which were stored in liquid nitrogen in the field and then at − 80 °C in the laboratory until lipid analysis ("Measuring energy reserves" Sect).

Experiments to determine pesticide tolerance

We conducted acute toxicity tests with *Gammarus* spp. principally based on the procedure described in [66]. Briefly, we exposed organisms for 96 h to the neonicotinoid insecticide imidacloprid (formulation Confidor® WG70—LOT: DE79858094D, Bayer CropScience AG, 40,789 Monheim, Germany). The formulation consisted of 700 g/kg active ingredient (AI) imidacloprid. In the following, concentrations refer to the AI concentrations. We selected this insecticide since it was of major relevance in terms of toxicity to the most sensitive freshwater invertebrate (highest toxic unit), high detection frequency, and widely occurring in the region under investigation in 2018 [61]. The analyses of 2019 stream water samples confirmed the major relevance of imidacloprid for the region and period under investigation [59, 61]. Specifically, imidacloprid was detected in 2019 in refuge, edge and agricultural sites at average concentrations of 1, 0 and 102 ng/L and in 20%, 0% and 73% of samples, respectively (edges only sampled
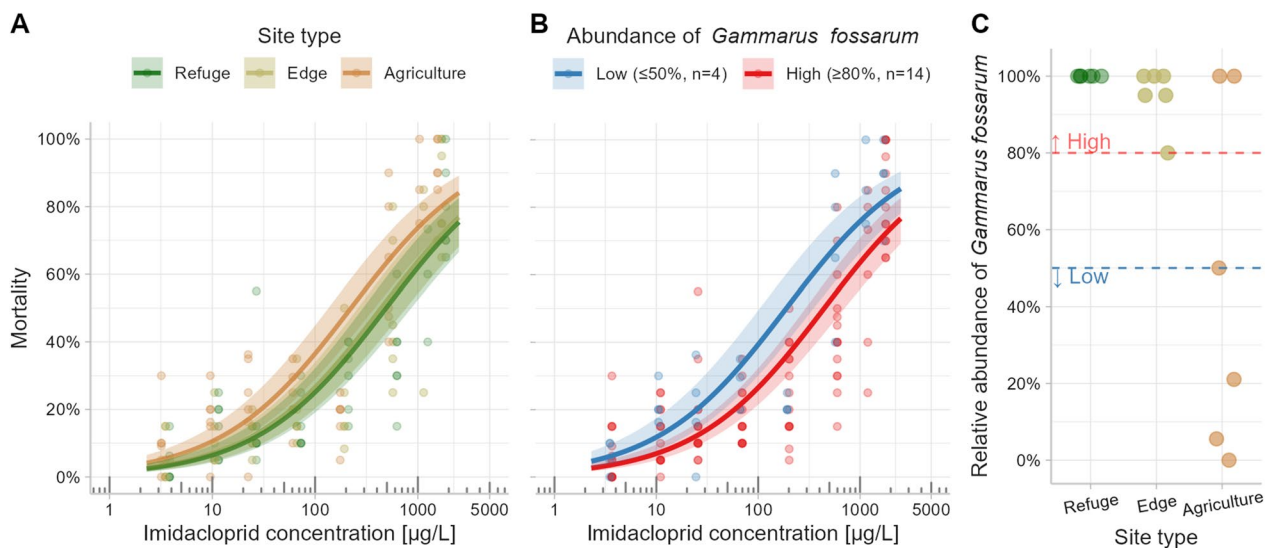
Fig. 1 Concentration–response relationship visualised for the mortality of *Gammarus* spp. individuals after 96 h of exposure to imidacloprid, visualised for the tested concentration range (**A**, **B**; test concentrations were log-transformed with half of the lowest non-zero test concentration added to avoid infinite numbers) and the relative abundance of *Gammarus fossarum* per site type (**C**). Each coloured dot represents the mean of replicates per concentration and sampling site (with different degrees of transparency for overlapping dots). The lines represent the average estimate of mortality per exposure concentration for a typical stream with the shadowed areas indicating the 95% confidence intervals (uncertainty of random effects not taken into account). Colours represent the site types (**A**, **C**) or the relative abundance of *G. fossarum* (**B**). Note that in **A** the line of the edge is slightly covered by the line of the refuge

in June, pesticide raw data from [59] at https://github.com/rbslandau/schneeweiss_refuge). For logistical reasons, we implemented test setups for each stream consecutively (for test start per stream see Additional file 1: Table S3). In each assay, we applied six increasing imidacloprid concentrations (7, 21, 63, 189, 567 and 1701 µg/L) together with pure medium controls (0 µg/L). A seventh concentration (1134 µg/L) was applied when testing the last three streams to increase the reliability of the concentration–response relationship (Additional file 1: Table S5). The concentration range was selected to capture the complete concentration–response curve based on toxicity values from the literature for *Gammarus* spp. The tests were run in medium Elendt M7 (Annex 2 of [67]), which we prepared on the day before the test setup in ultrapure water. Test vessels (volume of 150 mL) were aerated and kept at 16 °C, mimicking stream temperature (on average 13.3 °C, 13.7 °C and 15.7 °C at refuge, edge and agriculture, respectively [59]). We tested four vessel replicates per concentration each with five test organisms per vessel. We added 4*4 cm of stainless-steel mesh to each test vessel to provide a substrate for hiding and clinging to. Given that these taxa are photosensitive, the tests were run in complete darkness. We recorded mortality every 24 h until 96 h after test start. After 96 h exposure, we additionally recorded immobility (no swimming after disturbance with forceps).

At both the beginning and the end of the test, we measured water temperature, dissolved oxygen, electrical conductivity and pH in at least one replicate per concentration using a multi-parameter portable meter (WTW® Multi 3630 IDS Set G; Xylem Analytics, Rye Brook, USA; Additional file 1: Tables S3, S4). We took one 10 mL water sample per test concentration at the beginning of each test setup for the chemical analysis of imidacloprid. In addition, we took 10 mL triplicate water samples every 24 h during the test of the highest test concentrations (i.e. 1700 µg/L) to detect potential degradation of imidacloprid. For details on chemical analysis see Additional file 1: Text S1. Nominal and measured imidacloprid concentrations showed good agreement (Additional file 1: Table S5), we report nominal concentrations hereafter. Imidacloprid concentrations remained relatively stable throughout the test (Additional file 1: Figure S1).

Population genetic analysis

We analysed a fragment of the mitochondrial cytochrome c oxidase subunit one (CO1) gene in 20 *G. fossarum* individuals (where abundances permitted; Additional file 1: Table S2). Briefly, we cut off animals` heads with a sterile scalpel and extracted the total DNA from them using a slightly modified salt-extraction protocol after *Aljanabi* [68] (for modifications see Additional file 1: Text S2). We stored DNA extracts at − 20 °C until further examination. We used a nanodrop spectrophotometer (NanoDrop

1000, NanoDrop products, Wilmington, Delaware, USA) to measure the amount and quality of extracted DNA and, if necessary, diluted to final DNA extraction stocks of 10–20 ngDNA/μL. We amplified an approximately 650 base-pair (bp) long fragment of CO1 with the primers LCO1490-JJ and HCO2198-JJ [69]. These primers are based on the standard primers LCO1490 and HCO2198 [70] and have been successfully used for *Gammarus* spp. [71]. We used the following polymerase chain reaction (PCR) protocol per reaction: 1.5 μL PCR Buffer (1x), 1.2 μL dNTPs (0.2 mM), 0.75 μL each primer (0.5 μM), 0.125 μL Takara Ex Taq® (0.625 U/μL; TAKARA BIO INC., Shiga, Japan), 0.75 μL DNA template, filled to 15 μL with sterile water (PCR grade, autoclaved). We set the PCR setting for amplification as follows: initial denaturation at 94 °C for 60 s; 35 cycles of denaturation at 94 °C for 30 s; annealing at 51 °C for 45 s, and extension at 65 °C for 60 s; final extension at 65 °C for 5 min (after *Katouzian* et al. [71] with minor modifications). After a PCR product quality check using gel electrophoresis, PCR products were purified and sequenced by SEQ-IT GmbH & Co. KG (Kaiserslautern, Germany).

Measuring energy reserves

The energy reserve analysis was performed in two separate runs, one with a random sample of the field *Gammarus* spp. community and one with *G. fossarum* only (identified via microscopy and genetic analysis of two legs per individual following the procedure described in "Population genetic analysis" Sect). For each run and sampling site, five shock-frozen gammarids (each of the five individuals is treated as a replicate on the measurement subunit) were freeze-dried for 24 h and subsequently weighed to the nearest 0.001 mg. We then quantified the total lipid content of each gammarid after *Van Handel* [72] with minor modifications as described in Additional file 1: Text S3 and *Zubrod* et al. [73].

Data processing and statistical analysis

All statistical analyses and figures were produced in R (version 4.2.0 [74]). For visualisation, we used the ggplot2 package (version 3.3.6; [75]). We provide all raw data and the R script on GitHub at https://github.com/rbslandau/schneeweiss_tolerance.

Factors explaining pesticide tolerance

To compare the pesticide tolerance between site types (i.e. agricultural, edge, refuge), we modelled the mortality and immobility (which includes dead organisms) of *Gammarus* spp. after 96 h imidacloprid exposure as response variables explained by the imidacloprid concentration (nominal, log-transformed with half of the lowest non-zero test concentration added to avoid infinite

numbers, see [76]), site type and an interaction term. We chose a binomial distribution with a logit link function, given that the response was binary (dead/alive). We accounted for the nesting of site types within a stream by using stream as random factor (categorical, six levels) in a generalised linear mixed model (GLMM) [77]. Furthermore, vessel replicates related to each sampling site and concentration, representing a measurement subunit in our study design, were added as random factor (categorical, 120–135 levels). To examine potential interspecific differences in tolerance, we also added the relative abundance of *G. fossarum* (categorical, two levels: ≥ 80% = high, ≤ 50% = low; Additional file 1: Table S2) as a random factor to the models, but singular fits required changing to fixed factor (only two levels; e.g., [78]). Moderate collinearity between the covariates site type and the relative abundance of *G. fossarum* required removing the latter covariate from the full models (Pearson correlation coefficient = − 0.7; VIF = 2.5; [79]). We constructed a second, similar model, keeping the *G. fossarum* abundance instead of the site type as fixed predictor to illustrate the explanations given in the discussion section. For GLMM, we used the lme4 package (version 1.1–29; [80]). To test for the significance of single effects in GLMM, we applied a type II Wald Chi-squared (X^2) test available in the car package (version 3.1–0; [81]). We calculated lethal concentrations that killed 50% of the test organisms (LC_{50}) as well as concentration–response relationships per site type by applying the estimated parameters of each model to the logistic link function or its inverse, respectively.

Estimation of genetic differentiation

We edited and aligned sequence chromatograms in the software Geneious Prime (version 2022.0.1; http://www.geneious.com; [82]). Details on the sequence data preparation, quality control and taxonomy assignment are described in Additional file 1: Text S4. We computed and visualised population genetic metrics using the software R (version 4.2.0.; [74]) and following mainly a workflow provided by *Toparslan* et al. [83]. Briefly, we extracted haplotypes and plotted a minimum spanning (haplotype) network using the pegas package (version 1.1–2; [84]). A hamming distance matrix ("*N*") was used for the haplotype network. We calculated the genetic diversity as haplotype- and nucleotide diversity by applying the methods of [85] and [86], respectively, both of which are implemented in the pegas package (version 1.1–2; [84]). We modelled the haplotype diversity as a response explained by site type using a linear mixed model (LMM) with stream as random factor [77]. For LMM, we used the lme4 package (version 1.1–29; [80]) and fitted the models using restricted maximum likelihood (REML).

We applied a type III analysis of variance with Kenward–Roger's method available in the lmerTest package (version 3.1–3; [87]) to test for the significance of single effects in the LMM. This method has been shown to perform well for small sample sizes [88]. Additionally, we calculated the fixation index (F_{ST}) after *Nei* [86] between all populations as a measure of genetic population differentiation using the hierfstat package (version 0.5–11; [89]) for haploid genetic data. To determine potential drivers of genetic distance (i.e. site types, streams or sampling sites), we performed an analysis of molecular variance (AMOVA) as described in *Excoffier* et al*.* [90] using the pegas package (version 1.1–2; [84]). For the AMOVA, we used a hamming distance matrix as a measure of genetic distance.

Factors explaining energy reserves

For comparing the energy reserves between site types, we modelled the lipid content and dry weight per gammarid separately as response explained by site type using a LMM with the stream and replicate (related to each sampling site) as random factor [77]. To avoid growth effects, we previously normalised the lipid content to the gammarids' dry weight (µg/mg gammarid) [73]. For the *Gammarus* spp. experimental run, we constructed similar models using the *G. fossarum* abundance instead of the site type as fixed predictor (categorical, two levels: ≥ 80% = high, ≤ 50% = low; Additional file 1: Table S2).

Results

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

The mortality of *Gammarus* spp. was explained by "Concentration" (GLMM, $p < 0.001$; Additional file 1: Tables S6, S7) and by the factor "Site type" (GLMM, $p = 0.02$; Additional file 1: Tables S6, S7), where agricultural sites exhibited higher mortality than edge and refuge sites (Fig. 1A; pairwise differences: agriculture – refuge: $p = 0.03$, agriculture – edge: p = 0.07, edge – refuge: p = 0.93). The estimated LC_{50} was 492, 439 and 224 µg/L for refuge, edge and agricultural sites, respectively (Additional file 1: Table S8). The relative *G. fossarum* abundance was high (≥ 80%) at all refuge and edge sites, however, it was low (≤ 50%) at four of the six agricultural sites (Fig. 1C). The model including the relative abundance of *G. fossarum* as a covariate (Fig. 1B) indicated interspecific differences in tolerance (GLMM, $p = 0.005$; Additional file 1: Tables S6, S7). The immobilisation of *Gammarus* spp. was similar across all site types (GLMM, $p = 0.78$) and can be seen in the SI (Additional file 1: Figure S2; Table S6, S7).

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

We generated 397 CO1 sequences for *Gammarus* spp. from 18 sampling sites, 69 for *G. pulex* (seven sampling sites) and 328 for *G. fossarum* (17 sampling sites). Quality was sufficient for 309 sequences of *G. fossarum* (for threshold definition see Additional file 1: Text S4) for further population structural analysis. In the 511 bp alignment (293 sequences, 16 removed due to insufficient length) for *G. fossarum*, we detected 18 variable sites, all of which were synonymous substitutions (Additional file 1: Table S9). The individuals were clustered into 15 haplotypes, five of which had a frequency below 1% (Additional file 1: Table S9). The most common haplotype was found among 24.6% of individuals belonging to 6 sampling sites (Additional file 1: Tables S9, S10). At 12 of the 17 sampling sites, populations exhibited only one or two haplotypes (Table SI 10, Fig. 2), whereas the refuge and agricultural site of one stream (Triefenbach) exhibited seven haplotypes (Additional file 1: Table S10, Fig. 2). The distribution of haplotypes was comparable across refuge, edge and agricultural sites, as indicated by the haplotype network (Fig. 3). Note that H5 and H4 have been found at edge and refuge of Kropsbach where no samples could be taken at agricultural sites due to the absence of *G. fossarum*. The genetic diversity of *G. fossarum* populations measured as haplotype- and nucleotide diversity was similar across site types (Fig. 4; LMM, factor site type not significant at $p = 0.4$; Additional file 1: Tables S11, S12). F_{ST} values ranged from 0 to 0.2 among site types within the same stream and from 0.43 to 1 among sites of different streams (Additional file 1: Figure S3, Table S3). The AMOVA revealed that most of the total variance in the CO1 gene was found among streams and no variance among site types (Additional file 1: Table S14). For *G. pulex*, the 67 sequences belonged to five streams and two site types (agriculture and edge). For a 352 bp alignment (61 sequences, six removed due to insufficient length), we detected no variable sites and all individuals clustered into one haplotype (Additional file 1: Table S15). Accordingly, there was no genetic diversity or distance among populations, site types and streams for the *G. pulex* individuals. A brief discussion of the preliminary data for *G. pulex* can be found in Additional file 1: Text S5.

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

The energy reserves, in terms of average lipid content per mg gammarid tissue [µg/mg gammarid], decreased from refuge over edge to agriculture for *Gammarus* spp. (113, 101 and 88 µg/mg gammarid; Fig. 5A), although not
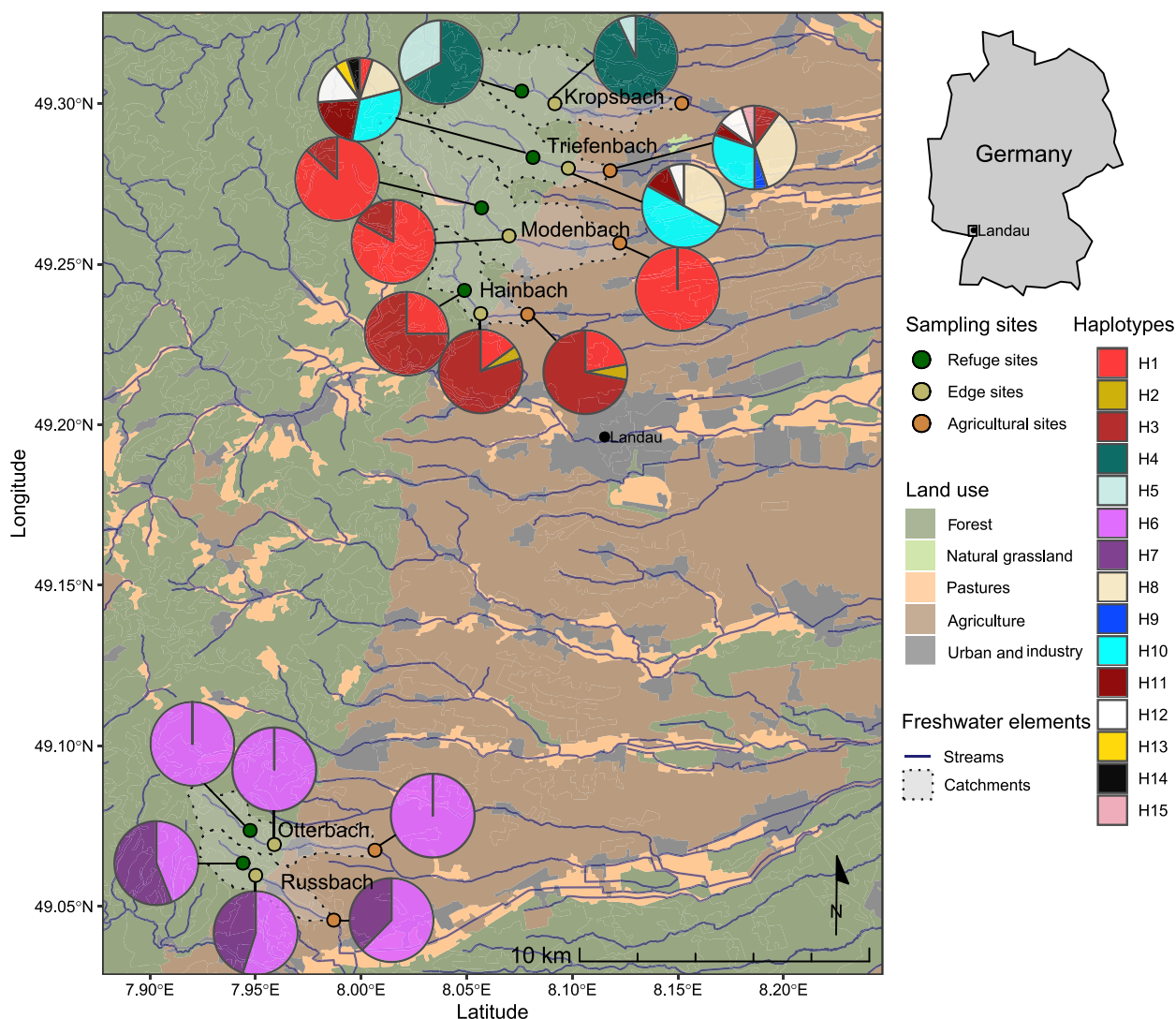
Fig. 2 Overview of the sampling sites, i.e. refuge, edge and agricultural sites at the six streams and their catchments in Rhineland-Palatinate, Germany, with different land use categories based on the CORINE land cover 2018 [91]. H1 to H15 refer to the haplotypes presented in Additional file 1: Table S10. The map was used from [59] and slightly formatted

significantly (LMM, $p = 0.296$; Additional file 1: Tables S16, S17). Similarly, for the model considering the relative abundance of *G. fossarum*, we observed a decrease in energy reserves from sites with high to sites with low *G. fossarum* abundance (106 and 82 µg/mg gammarid; Fig. 5B), albeit also statistically non-significant (LMM, $p = 0.143$; Additional file 1: Tables S16, S17). It should be noted that we have previously normalised the lipid content to the gammarids' dry weight (µg/mg gammarid) and that the dry weight itself was significantly related to the site type (LMM, $p = 0.029$; Additional file 1: Table S16, S17) and even stronger to the relative abundance of *G. fossarum* (LMM, $p = 0.003$; Additional file 1: Table S16, S17; separate models; Fig. 5C, D). Both the lipid content

and dry weight were similar across site types for the second experimental run involving *G. fossarum* individuals only (LMM, factor site type statistically not significant at $p = 0.302$ and 0.712, respectively; Additional file 1: Tables S16, S17; Additional file 1: Figure S4).

Discussion

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

We hypothesised that pesticide exposure sets off local adaptation processes in *Gammarus* spp. observable as increasing pesticide tolerance (hypothesis 1) and decreasing energy reserves (hypothesis 3) from refuge over edge to pesticide-polluted agricultural sites. By contrast,
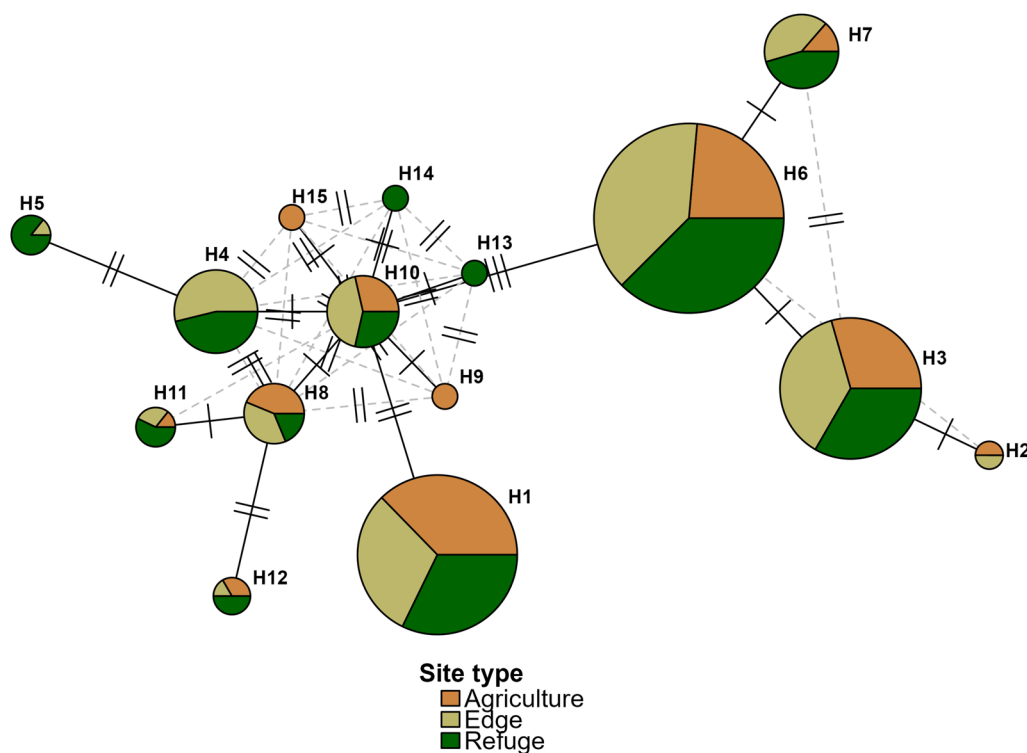
Fig. 3 Minimum spanning network created from *Gammarus fossarum* CO1 sequences using the pegas package 1.1–2 [84]. The circles represent sampled haplotypes (H1–H15) and their dimensions are scaled based on the number of sequences given in Additional file 1: Table S9 and 10. Short vertical lines on the connecting lines between haplotypes represent mutations. Colours represent site types

pesticide tolerance was significantly lower (around two-fold) at agricultural sites and similar in refuge and edge sites. This result may be influenced or partly explained by interspecific differences in physiological sensitivity and body size differences between site types.

Regarding interspecific differences, we found a mixture of three *Gammarus* species (*G. fossarum*, *G. pulex* and *G. roeselii)* for most of the downstream agricultural sites, whereas almost all edge and refuge sites were character-ised by individuals of *G. fossarum* (Fig. 1C), reflecting a turnover in the gammarid community from up- to down-stream consistent with the results of a previous study [59]. As explained in the method "Gammarid sampling" Sect, the examination of pesticide tolerance at the genus level of *Gammarus* spp. permitted rapid handling of the living organisms and avoided stress for organisms through deeper taxonomic identification. We suggest that the results (next paragraph) and main conclusions (Conclusion Sect) hold irrespective of the testing at genus level. This is because, previous laboratory studies indicate higher tolerance of *G. pulex* compared to *G. fos-sarum* for imidacloprid [92] and another insecticide [93], which would rather lead to a higher downstream toler-ance. Studies with *G. roeselii* suggest a higher pollution tolerance compared to *G. pulex* [94, 95] and *G. fossarum*

[96]. However, individuals from sites with high relative *G. pulex* and *G. roeselii* abundances exhibited lower imida-cloprid tolerance.

Regarding body size, individuals at agricultural sites were significantly heavier (Fig. 5C). This may be due to interspecific differences (*G. pulex* and *G. roeselii* are typically larger, consistent with our results see Fig. 5D), higher nutrient inputs and a higher temperature at agri-cultural sites [57, 59]. Again, tolerance rather increases with body size [97], whereas we observed a lower toler-ance. Thus, the interspecific differences in physiological sensitivity and differences in body size may rather have partly masked the lower tolerance in the downstream sites. In other words, at downstream sites where only *G. fossarum* occurs, the tolerance might even be lower. Hence, the same ecological explanations and conclusions given below would apply.

The hypothesis 1 of higher tolerance at the agri-cultural sites is based on previous studies that found generally higher insecticide tolerance in freshwater invertebrate populations in polluted stream sections compared to populations in non-polluted sections [10, 12, 76]. According to a recent meta-analysis, however, field populations of the freshwater amphipod *G. pulex* exhibited particularly high tolerance in tests (1) when
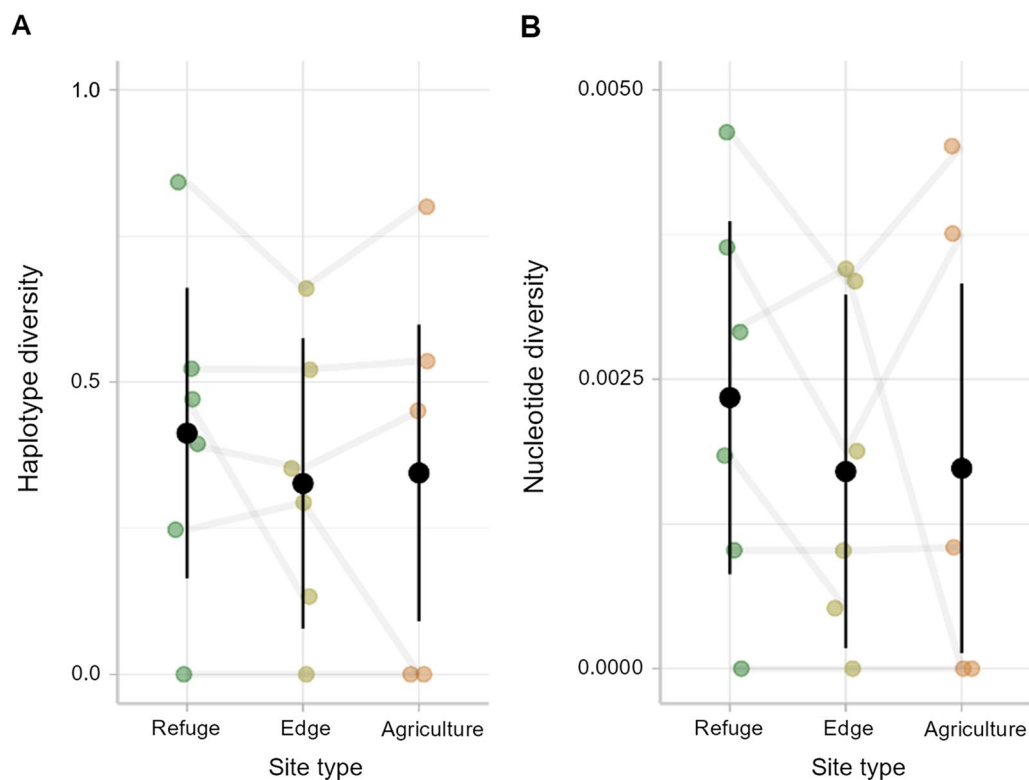
Fig. 4 Dot plots [75] visualising the haplotype—(**A**) and nucleotide diversity (**B**) of *Gammarus fossarum* populations. Each coloured dot represents a population sampled in each of the six streams, with the colours representing the site types. The black dots and ranges represent the predicted mean and 95% confidence intervals, respectively. The factor site type was not significant at $p = 0.4$ (Additional file 1: Tables S11, S12)

sampled populations were more than 3.3 km distant from the nearest refuge because of immigrating sensitive organisms; (2) when sampling occurred outside summer because of additional stress factors such as temperature during that time and (3) when the species diversity at the site of the sample population was low because of stronger inter- then intra-specific competition in more diverse sites [76]. In the present study, the distance to forest was on average 3.1 km, sampling took place in summer with higher temperatures observed in agricultural sites and multiple closely related *Gammarus* species were present in agricultural sites, potentially contributing to the lower tolerance (more details on environmental conditions and species diversity in [59]). The underlying explanation is that additional stress factors require energy trade-offs to the benefit of defence processes and detriments of other processes [98] that lower the capability of an organism to deal with other stressors such as subsequent pesticide exposure [99–101]. Also other studies found a lower tolerance of gammarids in pesticide-polluted compared to non-polluted sites [28, 57, 100] potentially related to energy trade-offs in multiple stressor environments. Indeed, concerning energy reserves, we found slightly lower lipid content per mg tissue in gammarids

at agricultural sites (Fig. 5A), albeit not statistically significant, possibly indicating decreased feeding activities of gammarids in response to pollution [102] or a higher allocation of energy to defence mechanisms including detoxification [58]. However, this decrease in energy reserves was not detected for *G. fossarum* alone (Additional file 1: Figure S4) and may therefore simply reflect a turnover in the gammarid community. Nevertheless, we highlight that *Gammarus* communities with *G. fossarum*, *G. pulex* and *G. roeselii* showed higher sensitivity in the acute toxicity tests than pure *G. fossarum* populations, which might partly be explained by multiple stressor effects in agricultural streams, especially in summer. However, further studies are required to confirm the role of gammarid community composition under multiple stressor effects.

Finally, given that we found no differences in the mortality response between edge and refuge populations which have been similar in the relative abundance of *Gammarus* species (*G. fossarum* ± 100%), we suggest that acute effects potentially related to a multiple stressor environment have not propagated to the edge of least impacted upstream sections. At agricultural sites, the reduced tolerance may, however, have long-term
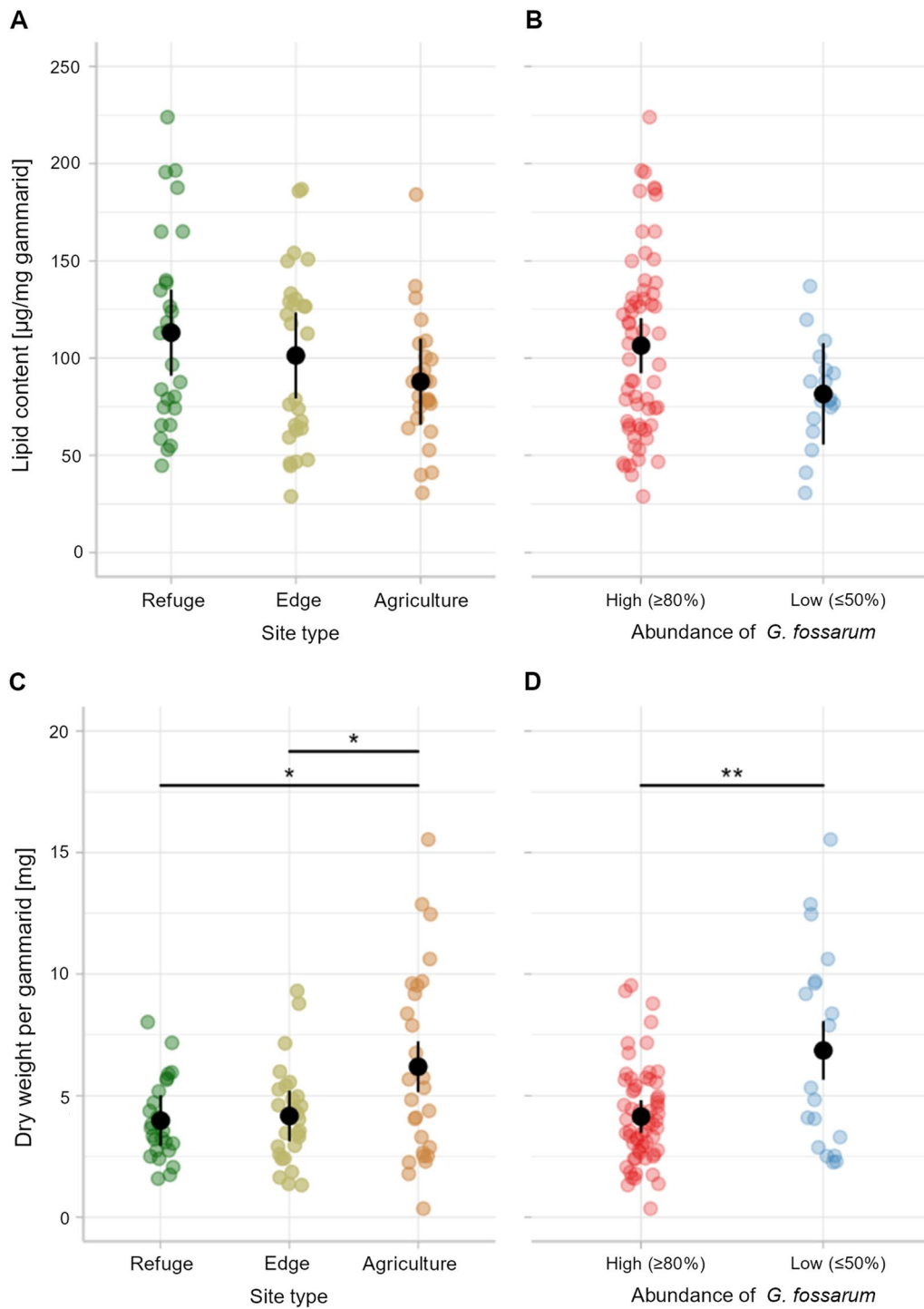
Fig. 5 Dot plots [75] visualising the total lipid content [µg] normalised by the dry weight per gammarid [mg] (**A**, **B**) and the dry weight per gammarid [mg] (**C**, **D**). Each coloured dot represents a replicate on the measurement subunit ($n = 3$–5 per sampling site) with the colours representing the site types ($n = 3$) and different degrees of transparency for overlapping dots. The black dots and ranges represent the predicted mean and 95% confidence intervals, respectively. The significance annotations " *" and " ** " refer to a *p*-value of < 0.05 and < 0.005, respectively

consequences for the gammarid populations with potential effects on the community and ecosystem functions [62, 103].

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

G. fossarum populations are genetically differentiated at the regional scale partitioned within the riverine network ($F_{st} > 0.5$, Additional file 1: Table S13), but differentiation is much lower at the local scale within the same stream ($F_{st} < 0.15$, Additional file 1: Table S13 and ANOVA, Additional file 1: Table S14), despite the strong gradient in pesticide pollution. This observation contrasts our second hypothesis (2a) that assumed small-scale population differentiation related to local pesticide pollution differences but is in line with the recent results for a single stream [57]. In the study by *Švara* et al*. [57], no genetic differentiation was found between *G. pulex* populations from sites of the same stream with different levels of pollution, while populations from polluted sites were also more sensitive to imidacloprid. The authors concluded that populations are well-connected and homogeneous. The F_{st} pattern observed in the present study suggests much higher levels of gene flow within the streams than between streams. However, geographically close streams (e.g., Otterbach and Russbach or Hainbach and Modenbach) share some haplotypes (Fig. 2), suggesting at least a low level of genetic exchange among neighbouring stream systems.

Eleven of the 15 haplotypes occur at two or even all three site types (Fig. 3), and haplotypes which occur only in one site types were rare ($n = 4$; Additional file 1: Table S9), supporting the view of hardly any local differentiation. In addition, the overall structure of the haplotype network, showing the most abundant and central haplotypes (H10 and H6) to occur in all site types, suggests that gene flow overrules local differentiation.

The pattern of strong population differentiation among rivers (versus low differentiation among river sections) likely reflects historical colonisation events by differentiated subpopulations originating from a diverse source population (river Rhine) or initial invasion of diverse populations but subsequent random genetic drift and bottleneck events resulting in loss of genetic variation over time. As such, 2018, the year preceding this study, was the warmest year on record in the study region and precipitation in the summer of 2018 was more than 50% below average [104], resulting in many streams falling sporadically dry (personal observation). Sporadic drought events that might convey population bottlenecks are frequently reported for streams along the palatine forest [105].

The application of molecular markers that provide a higher resolution, such as microsatellite DNA or single nucleotide polymorphism (SNP) analyses might have uncovered more information on small-scale local differentiation, however, they would not alter the overall pattern of larger differentiation between streams than among locations within a stream. Furthermore, similar patterns have been found in studies based on microsatellite DNA (e.g., [106, 107]), which unravelled population genetic structures reflecting the riverine network and colonisation history. On the other hand, some studies found genetic differentiation even at a local scale within the same stream [34, 106, 108, 109], and a weak correlation with anthropogenic organic pollutants [34]. A recent study investigating a pollution gradient in one large river catchment including adjacent catchments observed local differences in the genetic structure of *G. fossarum* populations matching differences in insecticide sensitivity [28]. However, these studies differed in terms of their geographic settings, e.g., larger distances and barriers such as weirs or dams between populations compared to *G. fossarum* populations in the present study.

Previous studies found no discrepancies between nuclear and mitochondrial markers [57, 106, 110], thus we expect no considerable different population structure if nuclear DNA information had been included. In addition, to the best of our knowledge, there are no known relevant differences in migratory behaviour among males and females of *G. fossarum*.

Given that stream systems are characterised by unidirectional water flow, downstream-biased gene flow in *Gammarus* is most likely [108, 111]. This may cause gene swamping of the downstream agricultural genepool with upstream (potentially maladapted) genotypes and hinder or mask local adaptation to pesticide pollution despite strong selection pressures [45, 57]. Notwithstanding, genotypes adapted to local pollution may occur at agricultural sites despite the observation of lower tolerance at these sites. For example, when adaptations are masked by acute pesticide effects or multiple stressors [76]. Evidence of evolutionary adaptation requires evidence at multiple levels of complexity [112] in addition to tolerance differences between populations. Testing the local occurrence of genotypes adapted to pesticide exposure is relevant, because gene flow may propagate adaptive potential (to pesticide pollution) from agricultural sites to upstream sites, given that our data on population structure suggest bidirectional gene flow (absence of many private haplotypes at either site type). Further, considerable upstream movements for gammarids have been documented [113, 114]. Thus, these "refuge" populations even if they never experienced significant

pollution levels might be genetically and evolutionarily impacted by pollution (e.g., already pre-adapted). If locally adapted genotypes convey fitness deficiencies, the exchange could result in an overall reduction of fitness of refuge populations. Such processes might have strong implications for nature conservation. For instance, refuge populations, in either natural habitats or managed nature protection areas, cannot be considered pristine or completely pollution naive if gene flow with impacted populations exists.

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

In contrast to our second hypothesis (2b) and a previous study [34], we found no gradient of genetic diversity associated with the pollution gradient (high at refuge and low at agricultural sites). This supports our findings based on the population structure ("Pollution gradient is not associated with Gammarus fossarum population structure" Sect), suggesting gene flow among site types, which in turn may mask the effects of natural selection [32, 45].

The alternative hypothesis compared to a "downstream decrease in genetic diversity" is a "downstream increase in intraspecific (neutral) genetic diversity (DIGD)" hypothesis. DIGD is a general spatial pattern of intraspecific diversity that has been shown for many taxa and was particularly pronounced for aquatic dispersers, due to processes such as downstream-biased dispersal or increases in habitat availability downstream [108, 115–117]. Our data, however, reveal no DIGD pattern. The lack of such a gradient may indicate pesticide effects at the downstream sites, but also other explanations have been suggested such as unstable conditions and associated higher allopatric diversification potential of headwaters (e.g., through drought events; [27, 28, 117, 118]). In addition, the lack of a typical DIGD pattern may be explained by the absence of gene flow from further downstream sites (e.g., confluences) for *G. fossarum*, which can be assumed (1) given that the genetic structure revealed only low exchange among streams and (2) for *G. fossarum* the 200 m isobar is typically the lowest distribution limit in our region [95], which matches our observations (Additional file 1: Table S1) and suggests that our agricultural sites are at the very top of the *G. fossarum* population distributional range. Furthermore, DIGD patterns have mainly been shown for larger spatial scales (e.g., 20–40 km [108]; > 50 km [115]) than those reported here (3–4 km). Thus, we consider it likely that gene flow between site types at very small scales potentially dilutes or masks the effects of pollution-related local genetic differentiation and adaptation.

Conclusion

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

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

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12302-023-00747-y.

Additional file 1: Table S1. Coordinates in decimal degrees and elevation in metre above sea level of the sampling sites. **Table S2.** Relative abundance [%] of *Gammarus fossarum*, *Gammarus pulex* and *Gammarus roeselii* at the sampling sites. **Table S3.** Physicochemical parameters of test medium at test start. **Table S4.** Physicochemical parameters of test medium at test end. **Table S5.** Nominal and measured concentrations applied in acute toxicity test setup at test start (0h). **Table S6.** Model outputs of separate full models for *Gammarus* spp. mortality or immobility after 96 h exposure to imidacloprid. **Table S7**. Analysis of deviance table for the generalised linear mixed-effects models (GLMM) listed in Table S6 using type II Wald Chi-squared tests. **Table S8**. Estimated lethal and effect concentrations in μg/L after 96 h exposure to imidacloprid where 50 % of test organisms (*Gammarus* spp.) have been affected for each site type. Table S9. Overview of the absolute and relative haplotype frequency as well as variable sites sampled per haplotypes in a fragment of the mitochondrial cytochrome c oxidase subunit one gene (CO1) of *Gammarus fossarum*. **Table S10**. Overview table of population genetic metrics

for *Gammarus fossarum* per sampling site. **Table S11.** Model outputs of separate full models for the haplotype- and nucleotide diversity in a fragment of the mitochondrial cytochrome c oxidase subunit one gene (CO1) of *Gammarus fossarum* individuals as response to site type. **Table S12.** Type III analysis of variance table with Kenward–Roger's method for linear mixed effect models listed in Table S11. **Table S13.** Pairwise CO1 fixation index (FST) values between all sampling sites. **Table S14.** Analysis of molecular variance (AMOVA) results for mtDNA CO1 data of *Gammarus fossarum* individuals from 17 sampling sites (=populations) modelled separately for two different groups (i.e. 6 streams*Gammarus fossarum* and 3 site types). **Table S15.** Overview table of population genetic metrics for *Gammarus pulex* per sampling site. **Table S16.** Model outputs of separate full models for the lipid content in µg/mg gammarid or the dry weight in mg as response to site type or the abundance of *Gammarus fossarum*. **Table S17.** Type III analysis of variance table with Kenward–Roger's method for linear mixed effect models listed in Table S16. **Text S1.** Chemical analyses in acute toxicity tests. **Text S2.** Slightly modified salt-extraction protocol for the extraction of genomic DNA. **Text S3.** Slightly modified lipid-quantification protocol. **Text S4.** CO1 sequence data preparation, quality control and taxonomy assignment. **Text S5.** Lower between stream population differentiation for *Gammarus pulex* compared to *Gammarus fossarum*—extended discussion. **Figure S1.** Stability of the highest toxicity test concentration from start to the end of the test (0–96 h). **Figure S2.** Concentration–response relationship visualised for the immobility of *Gammarus* spp. individuals after 96 h of exposure to imidacloprid. **Figure S3.** Heatmap visualising the genetic differentiation between *Gammarus fossarum* populations from different sampling sites. **Figure S4.** Dot plots visualising the total lipid content [µg] normalised by the dry weight per gammarid [mg] (A) and the dry weight per gammarid (B) for the second experimental run for *Gammarus fossarum*.

Availability of data and materials
We provide all raw data and the R script on GitHub at https://github.com/rbslandau/schneeweiss_tolerance.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Institute for Environmental Sciences, RPTU Kaiserslautern-Landau, Fortstrasse 7, 76829 Landau, Germany. [2]Department of System-Ecotoxicology, Helmholtz Centre for Environmental Research - UFZ, Permoserstrasse 15, 04318 Leipzig, Germany. [3]Institute for Environmental Research, RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany. [4]Senckenberg Leibniz Biodiversity and Climate Research Centre (SBiK-F), Senckenberganlage 25, 60438 Frankfurt am Main, Germany.

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