Becker, J.M., **Russo, R.**, **Shahid, N.**, **Liess, M.** (2020):
Drivers of pesticide resistance in freshwater amphipods
Sci. Total Environ. **735** , art. 139264

http://dx.doi.org/10.1016/j.scitotenv.2020.139264

Drivers of pesticide resistance in freshwater amphipods

Becker, Jeremias Martin[1,2#]*; Russo, Renato*[1,2#]*; Shahid, Naeem*[1,2]*; Liess, Matthias*[*1,2#]

[1]UFZ, Helmholtz Centre for Environmental Research, Department of System-Ecotoxicology,
Permoserstraße 15, 04318 Leipzig, Germany

[2]RWTH Aachen University, Institute for Environmental Research (Biology V), Worringerweg 1,
52074 Aachen, Germany

[*] Corresponding author

[#] *These authors contributed equally to this work*

ABSTRACT

Aquatic invertebrates exposed to pesticides may develop pesticide resistance. Based on a meta-analysis we revealed environmental factors driving the magnitude of resistance in the freshwater amphipod *Gammarus pulex* in the field.

We showed that (i) insecticide tolerance of *G. pulex* increased with pesticide contamination in agricultural streams generally by a factor of up to 4. Tolerance increased even at concentrations lower than what is considered safe in regulatory risk assessment (ii) The increase in insecticide tolerance was pronounced at high test concentrations; comparing the LC50 of populations therefore potentially underestimates the development of resistance. (iii) Insecticide resistance in agricultural streams diminished during the spraying season, suggesting that adverse effects of sublethal concentrations in the short term contrast long-term adaptation to insecticide exposure. (iv) We found that resistance was especially high in populations characterized not only by high pesticide exposure, but also by large distance (> 3.3 km) from non-polluted stream sections and by low species diversity within the invertebrate community.

We conclude that the test concentration, the timing of measurement, distance to refuge areas and species diversity mediate the observed response of aquatic communities to pesticide pollution and need to be considered for the sustainable management of agricultural practices.

KEYWORDS

Tolerance
Macroinvertebrates
Gammarus pulex
Insecticide
Esfenvalerate
Clothianidin

ABBREVIATIONS

TU = Toxic unit

GLOSSARY

Tolerance = the term refers to the ability of an organism or population to cope with adverse effects of a given pesticide. Tolerance of a population can be expressed by the effective concentration that affects a given endpoint in x % of its individuals after a given observation time t (ECx_t). Endpoints in this study were immobilization after 48 h constant exposure, and mortality after 48 h following pulse exposure for 1 h.

Resistance = the term refers to an increased pesticide tolerance of exposed organisms or populations relative to the tolerance of non- or lowly exposed reference individuals or populations. Resistance of a population can be expressed as the ratio of its tolerance (ECx) over the median tolerance value across reference populations.

1. INTRODUCTION

The widespread application of pesticides is posing a threat to the biodiversity in freshwater ecosystems worldwide (Beketov et al., 2013; Liess and von der Ohe, 2005; Münze et al., 2015; van der Sluijs et al., 2015). Pesticides exert a pressure on sensitive species to adapt in order to avoid local decline. Several studies have found that non-target species increased their tolerance to pesticides when they were regularly exposed (Becker and Liess, 2017; Bendis and Relyea, 2014; Cothran et al., 2013; Jansen et al., 2015; Shahid et al., 2018, Weston et al., 2013). However, adaptation to pesticides may come at the cost of reduced performance under non-toxic conditions (Siddique et al., 2020; Becker and Liess, 2015; Jansen et al., 2011). Additionally, a direct link between the acquisition of increased tolerance (resistance) to pesticides and the decline in genetic diversity of aquatic non-target communities has been suggested by several authors (Hua et al., 2013a; Coors et al., 2009; Jansen et al., 2011). Studies on fishes (McMillan et al., 2006) and amphipods (Bach and Dahllöf, 2012) confirmed that populations developing resistance to contaminants showed reduced genetic variation (Bach and Dahllöf, 2012; McMillan et al., 2006) that hampers their ability to evolutionarily adapt to future environmental stressors (Bach and Dahllöf, 2012; Blows and Hoffmann, 2005; McMillan et al., 2006; Jansen et al., 2011).

As a result, there is an urgent need to reveal key processes governing the acquisition of resistance in freshwater ecosystems in order to prevent further decline in biodiversity (Laurance and Useche, 2009). In fact, the identification of such processes sparked a discussion as to whether environmental effects of pesticides are generally underestimated during risk assessment (Clark et al. 2015). However, there is very little knowledge concerning the impact of environmental factors on the development of resistance (Becker and Liess, 2017).

We therefore performed a meta-analysis of three recent studies (Russo et al., 2018; Shahid et al., 2018; Becker and Liess, 2017) exploring the influence of various environmental factors on the development of resistance in the freshwater crustacean *Gammarus pulex* under different environmental conditions in the field. The three studies were conducted in the same region and shared the same experimental setup: they compared the insecticide tolerance of *Gammarus pulex* collected in streams that cover a gradient of agricultural pesticide pollution. Despite their similar designs, the studies yielded diverging results that we attributed to differences in the respective environmental conditions that may be potentially relevant to the development of pesticide resistance. The increased statistical power of the present meta-analysis with a merged data set provides detailed insights into the role of relevant environmental factors and the magnitude of their effects.

In this study, we specifically addressed the following questions: (i) At which level of contamination does *Gammarus pulex* develop pesticide resistance? (ii) Is there seasonal variation in insecticide resistance? (iii) To what extent do recolonization from non-polluted refuges and species diversity hinder the development of pesticide resistance?

2. MATERIALS AND METHODS

Study design

The studies of Russo et al., (2018), Shahid et al., (2018) and Becker and Liess, (2017) used for this meta-analysis focused on *Gammarus pulex* (Linnaeus, 1758) as test organism because of its widespread occurrence and its ecological relevance in crucial functions of stream ecosystems (Dangles et al., 2004). In all studies, individuals within the size range of 0.6 – 1.0 cm, that were considered at least two to three months old (Welton and Clarke 1980), have been collected for acute sensitivity tests. The studies covered small to medium streams in central Germany with a wide range of conditions in terms of pesticide pressure (from pristine to highly polluted), seasons (from spring to winter), the macroinvertebrate community (species diversity, total abundance and dominance of *G. pulex*), and the distance to non-polluted stream sections (refuges).

Becker and Liess (2017) tested the acute sensitivity of *G. pulex* from 35 sites to the neonicotinoid insecticide clothianidin. Each site was sampled once in autumn (August – October) 2014 or in spring (April – June) 2015. When enough organisms were available, additionally the sensitivity to the pyrethroid insecticide esfenvalerate was tested. Various endpoints were reported; for this meta-analysis we used immobilization after constant exposure for 48 h. Shahid et al. (2018) reported immobilization after constant exposure for 48 h to clothianidin for 15 sites that have been sampled in winter (December – January) 2015/2016 and again in spring (April – June) 2016. In summer, additionally the sensitivity to esfenvalerate was tested; however, only the data of sensitivity to clothianidin have been previously published. Russo et al. (2018) reported survival after 48 h following pulse exposure to esfenvalerate for 1 h; eight populations were repeatedly sampled in autumn (October) 2015, early spring (March – April) 2015 and in early summer (June) 2015. In summary, one to four toxicity tests from different seasons were available for each of the 48 study sites analysed, with 10 of the sites being sampled in more than one of the studies. Data from 46 of those sites were analysed because assessments of pesticide pollution were not available for all sites (Tab. S3).

Test substances

The neonicotinoid insecticide clothianidin represents one of the most commonly applied classes of insecticides worldwide (Simon-Delso et al., 2015). Concerns over unintended impacts on bees led European Union members to vote in 2018 for a restriction of the three main neonicotinoid insecticides, including clothianidin, for all outdoor uses (EU 2018/784). Ecologically relevant concentrations of clothianidin have been detected in the study area in the studies of Becker and Liess (2017) and of Shahid et al. (2018). For the acute toxicity tests, in both studies a 500 mg L^{-1} stock solution of the neonicotinoid insecticide clothianidin was prepared from granulated DANTOP® (Spiess-Urania Chemical GmbH, Germany) dissolved in distilled water with 12 h stirring. The stock solution was further diluted in artificial *Daphnia* medium (ADaM) (Klüttgen et al., 1994) to obtain the desired test concentrations.

The pyrethroid insecticide esfenvalerate has been reported as highly toxic to *G. pulex* (Hill, 1985), and it has been previously detected in streams at biologically relevant concentrations in the same study area (Münze et al., 2015; Becker and Liess 2017). For the toxicity tests, esfenvalerate was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Hesse, Germany) in powder form. Stock solutions were prepared by dissolving a known weight of esfenvalerate in dimethyl sulfoxide. Neonicotinoids and pyrethroids together have been shown to dominate the overall pesticide toxicity to macroinvertebrates in agricultural streams of the study area (Becker and Liess, 2017), providing pressure for adaptation.

Pesticide pollution in the streams

Because measurements of pesticides were not available for all of the study sites, we quantified pesticide pollution in the streams based on the dominance of vulnerable vs. invulnerable macroinvertebrate taxa, using the $\mathrm{SPEAR}_{\mathrm{pesticides}}$ bioindicator v. 2018.05 (Knillmann et al., 2018, Liess and von der Ohe, 2005). SPEAR values were calculated with the software Indicate 1.1.1 (Dept. System-Ecotoxicology, Helmholtz Centre for Environmental Research UFZ, Leipzig, Germany, 2018; http://www.systemecology.eu/indicate) from macroinvertebrate samples that have been collected at the study sites in 2013 – 2016. Because SPEAR has been designed and evaluated for the season of the main agricultural insecticide application, macroinvertebrate samples collected outside the period March – July were excluded from this analysis. Macroinvertebrate data differed in the level of taxonomic detail between the three studies, therefore we aggregated all data to the family level. In the SPEAR database, the mayfly *Baetis rhodani* (together with *G. pulex* the most abundant species) has been classified as being “not at risk”, whereas the family Baetidae has been classified as “at risk”. However, since Baetidae consisted mainly of *B. rhodani* in our data set, we manually re-classified Baetidae as “not at risk” prior to the analysis.

Altogether, one to seven macroinvertebrate samples per site were available for SPEAR calculations. We calculated SPEAR separately for each sample and converted this value to the maximum toxic unit (TU_{max}) of any pesticide to be expected in water samples during run-off. This conversion is available in Indicate 1.1.1 based on a SPEAR vs TU_{max} regression established in Knillmann et al. (2018). The toxic unit (TU) is a quantification of toxicity that relates the observed concentration *conc* of a pesticide *i* to its acute median lethal concentration for a standard reference organism ($LC50_{reference}$, Tomlin, 2000): $TU_{max} = \max(log_{10}(\frac{conc_i}{LC50_{reference}}))$. Finally, for each site we calculated the median estimated TU_{max} across all samples. This approach is based on the assumption that despite yearly variation in the contamination patterns, averaging the values over time provides a more accurate depiction of the general exposure risk (Schriever et al., 2007). The calculation of SPEAR has been recently improved (Knillmann et al., 2018), therefore SPEAR and estimated TU_{max} values in this analysis deviate from those obtained with SPEAR v. 2016.02 in the previous publications.

The estimated TU_{max} was validated with TU_{max} values observed in a subset of the study sites (Fig. S1a). These TU_{max} values were derived from pesticide concentrations measured in water samples collected after run-off events and in Chemcatcher® passive samplers installed for 1 – 3 weeks during the main pesticide spraying season in 2013 - 2016 (Münze et al., 2017; Shahid et al., 2018; Knillmann et al., 2018, Reiber et al., personal communication). See Münze et al. (2015, 2017) for a description of pesticide measurements and analyses. With Baetidae re-classified as not at risk (see above), the estimated TU_{max} based on SPEAR correlated well with the TU_{max} observed in those sites where pesticide concentrations have been measured ($R^2 = 0.61$, df = 1, res.

df = 25, $F = 39.28$, $p < 0.001$). The correlation was greater than with the toxicity estimated from the older SPEAR version ($R^2 = 0.47$, df = 1, res. df = 25, $F = 22.36$, $p < 0.001$). However, because the aggregated taxonomic data used for the SPEAR calculation contained both vulnerable and invulnerable species in the family of Baetidae, the estimated toxicity constantly underestimated the observed toxicity by one toxic unit. Therefore we adjusted the estimated TU_{max} accordingly by subtracting 1.02 toxic units (mean difference between estimated and observed TU_{max}) to obtain more realistic measures of the overall pesticide exposure in the streams (Fig. S1b).

Pesticide resistance

From the acute toxicity tests, we first compared the shape of dose-response curves for all populations that experienced high or low pesticide pollution in the field, respectively (see details in data analysis). In a second step, we quantified the acute insecticide tolerance for further analyses as the effective concentrations that immobilized (Becker and Liess, 2017; Shahid et al., 2018) or killed (Russo et al., 2018) 75 % of test individuals (EC75). The EC75 was used instead of the EC50 because the shapes of the dose-response curves showed that differences in the pesticide tolerance of populations from highly and lowly polluted streams were pronounced at high test concentrations (Fig. 1).

The insecticide resistance (x-fold tolerance) of a population was then calculated as the ratio of the local EC75 divided by the median EC75 for non-polluted reference populations. Sites with estimated $TU_{max} \leq -4$ were considered as non-polluted (Knillmann et al., 2018). Consequently, resistance < 1 indicates weakening and resistance > 1 indicates increased tolerance compared to those of reference populations. As each of the three studies followed slightly different protocols for the acute toxicity tests (see Tab. S1), we quantified insecticide resistance as local tolerance relative to those of reference populations only within the same study. This way, we standardized the resistance values to make them comparable across pesticides and studies. If a sample was tested for tolerance to both clothianidin and esfenvalerate, we calculated the geometric mean insecticide resistance across the test substances. However, in contrast to pesticide pollution, we did not aggregate resistance values obtained from repeated samples of the same population in order to identify seasonal variation in insecticide resistance.

Environmental drivers of pesticide resistance

Distance to the closest refuge area was measured using digital maps. To identify refuges, we used a procedure related to those by Knillmann et al. (2018). In brief, refuge areas were defined as forested or grassland stream sections with presumably little or no influence of pesticides, with minimum dimensions of 100 m in width and 300 m in length. When the refuge was located upstream, we used the normal distance, while we doubled downstream distances given that organisms had to migrate against the current. If a refuge was located in a tributary that joins downstream of a site, only the distance from the site to the mouth of the tributary was doubled and not the distance from the mouth to the refuge, since the organisms migrate with the current.

Species diversity was calculated from each macroinvertebrate sampling during the spraying season separately using the Shannon index (H’); then the median H’ over repeated measurements from the same site was calculated. Data obtained outside the spraying season were not considered because the macroinvertebrate community composition is likely to change seasonally, impeding the comparison between sites.

Data analysis

All analyses were conducted in R 3.6.2. Dose-response curves for highly and lowly polluted populations were compared using a generalized linear mixed-effects model (GLMM) available with the lme4 package 1.1-21 (Bates et al., 2015). We first summed up the tested and the deceased or immobilized individuals from all tests of the same population and study, and then calculated the proportion of affected individuals (weighted average across tests). Then we fitted a GLMM with a binomial residual distribution and a complementary log-log link function. The average pesticide pollution (estimated TU_{max}) at the sampling site, the test concentration (log-transformed, with half of the lowest non-zero concentration added to avoid infinite numbers), their interaction, and the test substance were incorporated as fixed effects. Study was incorporated as a random effect (intercept) to account for the different test protocols used; additionally, we incorporated a random intercept for each observation (test concentration per test) to handle overdispersion because quasibinomial GLMMs are not yet supported in lme4.

For further analyses, the EC75 was estimated separately for each toxicity test from 5-parameter log-logistic nonlinear regression with the lower and upper boundary set to 0 and 1, available with the drc package 3.0-1 (Ritz et al., 2015). The EC75 was converted to resistance ratio (see above). In two samples obtained from adjacent sites at the same date we observed extraordinarily high insecticide resistance (see results and Fig. S2). To be conservative and avoid that general trends in the presented meta-analysis are driven by rare extreme cases, these resistance values have been excluded as highly influential data points from further modelling. After that, a data set comprising 83 EC75 values from 46 different sites was available for analysis (Tab. S2).

We investigated the effects of pesticide pollution (estimated TU_{max}), seasons, distance to refuge area and species diversity on the (log-transformed) insecticide resistance. These effects were analyzed using a linear-mixed effects model (LMM) with sampling site included as a random factor to deal with pseudo-replication that results from repeated measurements of resistance from the same population. To study the effect of season, we converted sampling dates to days of the year and then implemented this effect in the model using a sinusoidal-cosinusoidal term (see Tab. S7 in the supplemental files). Distance to refuge area was log-transformed, with half of the smallest non-zero distance added to avoid infinite values when sites were refuges themselves. First, we fitted a full model with all possible interactions of the four explanatory variables, and subjected this model to backward selection based on likelihood-ratio tests until only significant highest-level terms remained in the final model.

To quantify the relative contribution of each environmental variable to the overall effect on insecticide resistance, we re-fitted the final model with all environmental variables being standardized (centered and scaled to a standard deviation of one), so that the coefficient values became comparable in size. For each environmental variable, we then summed up the absolute coefficient values of all terms to which the variable contributed (main effects and interactions). The size of the summed up coefficients was considered as an indicator for the relative overall importance of each environmental variable in the model. Additionally, we dropped each environmental variable from the model and compared the log-likelihood of the reduced and the full model. The increase in log-likelihood caused by each environmental variable was considered as an alternative indicator of its importance in the model (partial correlation). Correlations between the environmental variables were analyzed using simple linear regression.

In all (G)LMMs, effects were tested for significance using type III Wald χ^2 tests available with the car package 3.0-6 (Fox and Weisberg, 2019). While resistance values in the main text refer to raw resistance ratios as described above, data points showing resistances in Fig. 1 – 3 have been adjusted to the random effects, i. e. the random intercepts have been subtracted. This way, we averaged over random differences between sites in analogy to the mathematical procedure in (G)LMMs, such that the fixed effects become better visually apparent as "seen" by the model. Marginal and conditional R^2 values were calculated using the MuMIn package 1.43.15 (Barton, 2019). Fitted values with 95 % confidence intervals were extracted using the effects package 4.1-4 (Fox and Weisberg, 2019). Homoscedasticity and normal distribution of residuals were confirmed by means of visual inspection using normal Q-Q plots and residuals vs. fitted values plots.

3. RESULTS

3.1.Insecticide resistance varies with test concentration

Pesticide pollution, quantified as the estimated maximum toxic unit (TU_{max}) derived from SPEAR, ranged from -5.3 to -1.2 (median = -2.9), and thus covered a gradient from high to low observed effects on the freshwater macroinvertebrate composition (Knillmann et al., 2018). First we compared the response of *G. pulex* populations from highly and lowly polluted streams to different test concentrations of the insecticides esfenvalerate and clothianidin. In both groups, immobilization 48 h after the beginning of the test was observed at minimum concentrations of 5 – 10 µg/L clothianidin, and 0.05 – 0.1 µg/L esfenvalerate, respectively (Fig. 1). However, immobilization increased more steeply with test concentrations in populations from lowly polluted sites than in populations from highly polluted sites (χ^2 = 6.20, d.f. = 1, *p* = 0.013, *n* = 546, Tab. S7). Therefore the greatest difference in insecticide tolerance of lowly and highly polluted populations was observed at high test concentrations that immobilized on average 85 % of lowly polluted populations but only 65 % of highly polluted populations (Fig. 1).

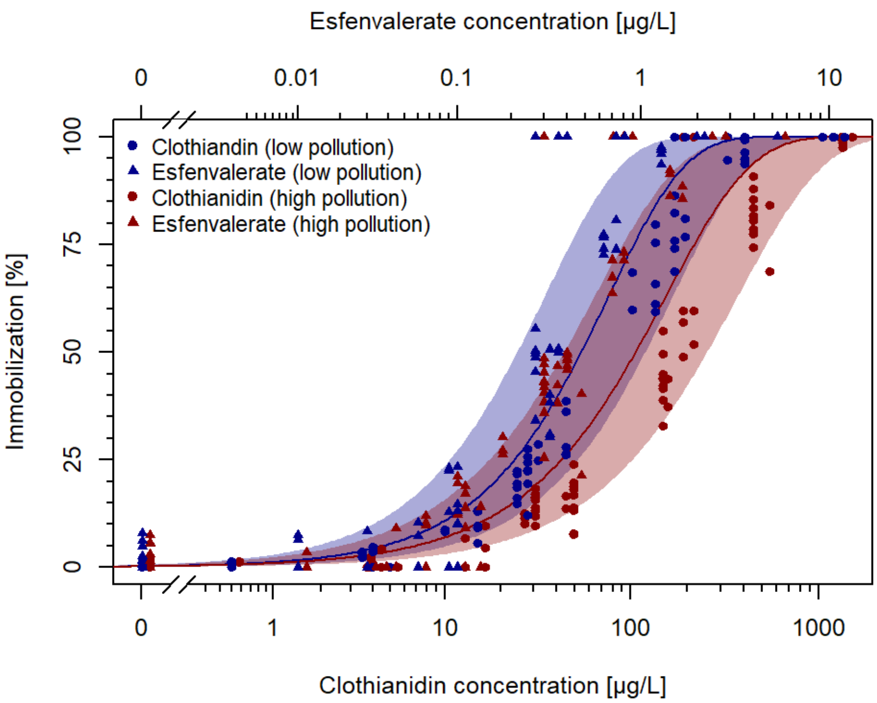

Figure 1. Increased insecticide tolerance in *Gammarus pulex* from highly polluted streams is more pronounced at high test concentrations. Immobilization was measured 48 h after exposure to the nominal concentrations shown on the horizontal axes. Generalized linear mixed-effects model with test concentration (log-transformed), pesticide pollution (estimated TU$_{max}$, derived from the SPEAR$_{pesticides}$ bioindicator) and test substance as fixed effects, and study and observation as random effects. Dose-response curves ± 95 % confidence intervals are averaged across test substances, with estimated TU$_{max}$, set to -4.5 and -1.5, respectively. Data points have been adjusted to the random effects (see materials and methods for details); sites with below-median pesticide pollution (estimated TU$_{max}$ $\leq$ -3.0) are shown in blue, sites with higher pollution in red. Marginal R_GLMM^2 = 0.67, conditional R_GLMM ^{2} = 0.80. See further statistics in Tab. S6.

In those sites for which pesticide measurements were available, pesticide pollution was driven by the toxicity of insecticides with similar modes of action as the test substances. Out of an average of 78 substances analysed per sample (range 22 – 110), neonicotinoids (comprising clothianidin) determined the TU_{max} in 43 out of 79 samples; they most frequently dominated the toxicity in 20 out of 28 sites. Pyrethroids (comprising esfenvalerate) determined the TU_{max} in 11 out of 46 samples in which pyrethroids have been analysed; they most frequently dominated the toxicity in 2 out of 15 sites. Thus, the overall pesticide exposure was dominated by neonicotinoid or pyrethroid insecticides in 22 out of 28 sampling sites (79 %).

3.2. Seasonal variation in insecticide resistance

For further analyses, we calculated the insecticide resistance of a population as the ratio of the local concentration that immobilized 75 % of individuals (EC75) over the median EC75 of lowly polluted populations (see materials and methods). Resistance to clothianidin generally ranged from 0.6 to 4.4, the resistance to esfenvalerate from 0.4 to 4.2. For those populations that were tested with clothianidin and esfenvalerate, we calculated the mean insecticide resistance across both test substances. Therefore insecticide resistance generally ranged from 0.5 to 4.0. However, in two samples from adjacent streams of the same catchment area collected on 25/08/2014 we observed remarkably higher insecticide resistance (MM06 = 8.9, MM07 = 9.7) that resulted mainly from exceptionally high resistance to clothianidin (MM06 = 16.4, MM07 = 19.8) but also from high resistance to esfenvalerate (MM06 = 5.7, MM07 = 4.1). Though both populations showed also high insecticide resistance 15 and 19 months later (MM06 = 3.7 – 4.0, MM07 = 2.9 – 3.4), the resistance was considerably lower than in August 2014. To avoid that effects were driven by a single event, we excluded these two samples from further analyses.

The effects of pesticide pollution, seasons, refuge areas and species diversity on insecticide resistance were analysed using a linear mixed-effects model. We identified interacting effects of pesticide pollution, refuge areas and species diversity (see below) and additionally interacting effects of pesticide pollution and seasons (Tab. S8). Thus, the increase in insecticide resistance with pesticide pollution varied with the season ($\chi^2 = 5.80$, d.f. = 1, $p = 0.016$, $n = 83$, Fig. 2). In highly polluted populations we observed higher insecticide resistance (on average 2-fold increased tolerance) in late winter (February – March) and lower resistance in late summer (August – September), while the insecticide resistance in lowly polluted populations remained low throughout the year.

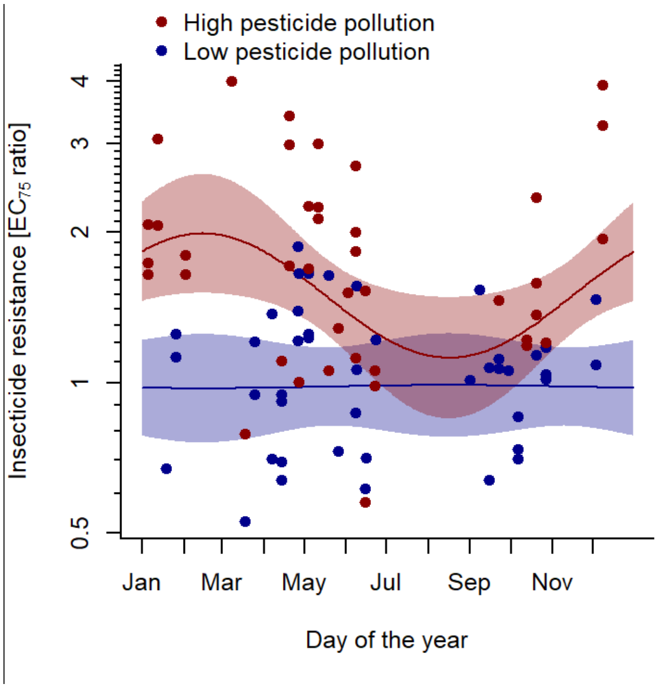

Figure 2. Seasonal variation in insecticide resistance of *Gammarus pulex* from highly polluted and lowly polluted streams. Interacting effects of day of the year (sinus-cosinus transformed) and pesticide pollution (estimated TU_{max}, derived from the $SPEAR_{pesticides}$ bioindicator). Predictions of the linear mixed-effects model average over the additional three-way interaction of pesticide pollution and other environmental factors shown in Fig. 3, and over the random effect of sampling sites. Mean insecticide resistance (tolerance ratio of local EC75 vs. median EC75 from non-polluted reference populations with $TU_{max} < -4$) ± 95 % confidence intervals are shown

when TU_{max} is set to its 20% and 80% quantile, respectively. Data points have been adjusted to the random effect (see materials and methods for details); samples from sites with below-median pesticide pollution ($TU_{max} < -3.0$) are shown in blue, from sites with higher pollution in red. Marginal $R^2 = 0.43$, conditional $R^2 = 0.61$ for the full model. See Tab. S8 for further statistics.

3.3. Refuge areas and species diversity lower insecticide resistance in polluted streams

Distance to the next refuge area ranged from 0 to 25 km, with a median distance of 2.4 km for sites that were not considered refuges themselves (distance to downstream sections were doubled because organisms had to immigrate against the current). The family-based macroinvertebrate species diversity, quantified with the Shannon index H', ranged from 0.83 to 2.34, with a median of 1.67. We observed a significant three-way interaction in the effects of pesticide pollution, refuge distance and species diversity on insecticide resistance ($\chi^2 = 5.79$, d.f. = 1, $p = 0.016$, $n = 83$, Tab. S8): Except for a single site (JB14), high insecticide resistance (≥ 2.5-fold increased tolerance) was only observed when pesticide pollution was high (estimated $TU_{max} \geq -2.4$), refuges were far (≥ 2.3 km) and the local species diversity was low (H' ≤ 1.74). By contrast, insecticide resistance did not significantly increase with pesticide pollution when refuges were close or species diversity was high (Fig. 3).

To assess the relative contribution of each environmental variable in explaining the observed increase in insecticide resistance, we compared the summed up coefficients and the increase in log-likelihood caused by each variable in the model (Tab. 1). On average across both indicators, pesticide pollution contributed for 46 % of the explained variation in insecticide resistance; season contributed for 13 %, refuge distance for 20 % and species diversity for 21 %. All variables together explained 43 % of the total variation in insecticide resistance (marginal R^2). An additional 18 % of the total variation was explained by random differences between the populations (conditional $R^2 = 0.61$).

Species diversity was not affected by pesticide pollution ($R^2 = 0.05$, $F = 2.28$, d.f. = 1, residual d. f. = 44, $p = 0.138$). However, species diversity in sites that were not considered a refuge themselves increased with the proximity to a refuge ($R^2 = 0.25$, $F = 8.50$, d.f. = 1, residual d.f. = 26, $p = 0.007$).

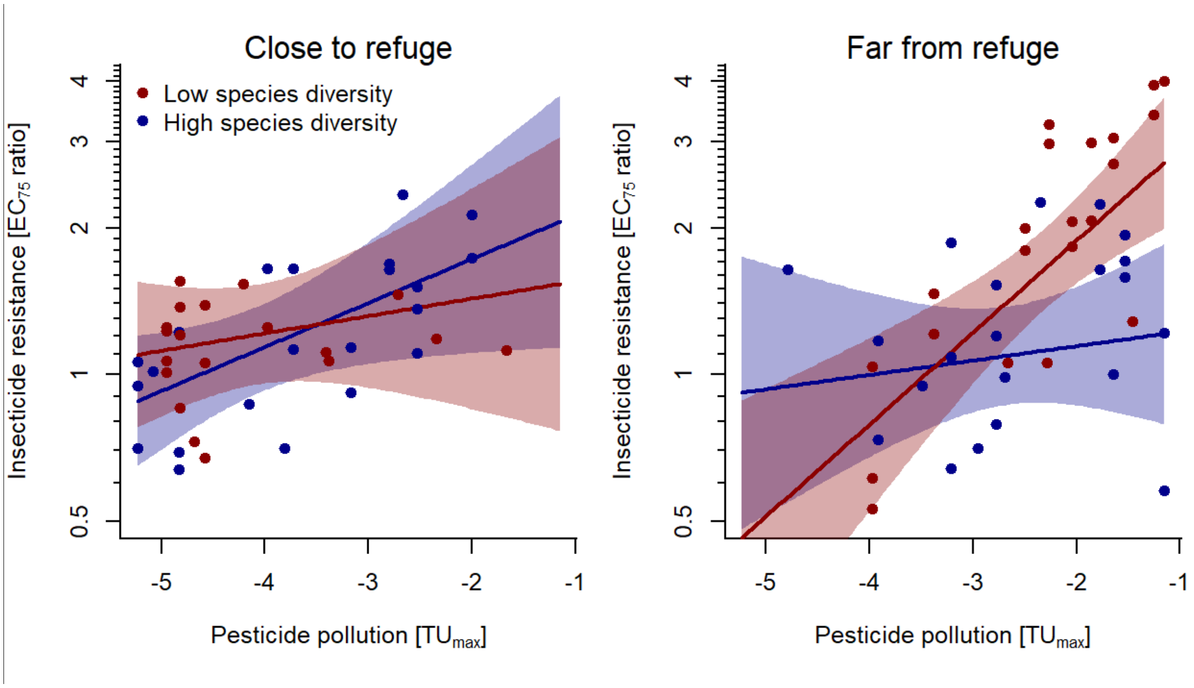

Figure 3. Effects of pesticide pollution, refuge areas and species diversity on the insecticide resistance of *Gammarus pulex* in small streams. Three-way interaction of pesticide pollution (estimated TU_{max}, derived from the SPEAR$_{pesticides}$ bioindicator), distance to the next potentially non-polluted refuge area (log-transformed) and species diversity. Predictions of the linear mixed-effects model average over the additional interaction of season and pesticide pollution shown in Fig. 2, and over the random effect of sampling sites. Mean insecticide resistance (tolerance ratio of local EC75 vs. median EC75 from non-polluted reference populations with $TU_{max} < -4$) ± 95 % confidence intervals are shown when species diversity is set to its 20% and 80% quantile, respectively. Samples from sites with below-median species diversity (H' < 1.67) are shown in red, from sites with higher species diversity in blue. The left panel shows sites with below-median refuge distance (< 0.9 km), the right panel shows sites with higher refuge distance. Data points have been adjusted to the random effect (see materials and methods for details). See Fig. 2 for R^2, and Tab. S8 for further statistics.

Table 1. Relative contribution of each environmental variable to the observed insecticide resistance. The model shown in Fig. 2 and Fig. 3 was re-fitted with explanatory variables being standardized. First, for each environmental variable, the absolute coefficient values from all model terms that contained the variable were summed up. Then the summed up coefficients for each environmental variable were divided by the sum of all summed up coefficients to assess the relative contribution of each environmental variable in explaining the modelled insecticide resistance. Second, all terms that contained a given environmental variable were removed from the model and the difference in the log-likelihood was noted. The increase in log-likelihood caused by the variable was divided by the summed up increase in log-likelihood from all variables to assess the relative contribution in explaining insecticide resistance. Finally, the mean relative contribution from both methods was calculated.

Term	Coefficient	Std. error
Intercept	0.175	0.066
Pesticide pollution	0.249	0.069
Season	0.072	0.042
Refuge distance	-0.104	0.070
Species diversity	-0.010	0.079
Pesticide pollution : season	0.098	0.041
Pesticide pollution : refuge distance	0.063	0.068
Pesticide pollution : species diversity	-0.095	0.078
Refuge distance : species diversity	-0.030	0.079
Pesticide pollution : refuge distance : species diversity	-0.172	0.071

Model	Log-likelihood
Pesticide pollution * refuge distance * species diversity + pesticide pollution * season (full model)	-36.51
Refuge distance * species diversity + season (- pesticide pollution)	-49.82
Pesticide pollution * refuge distance * species diversity (- season)	-40.63
Pesticide pollution * species diversity + pesticide pollution * season (- refuge distance)	-41.26
Pesticide pollution * refuge distance + pesticide pollution * season (- species diversity)	-42.96

Effect	Summed up coefficients	Increase log-likelihood	Average relative contribution [%]

Pesticide pollution	0.676 (45 %)	13.31 (46 %)	46
Season	0.170 (11 %)	4.26 (14 %)	13
Refuge distance	0.368 (24 %)	4.36 (17 %)	20
Species diversity	0.307 (20 %)	6.85 (23 %)	21

4. DISCUSSION

Insecticide resistance in *G. pulex* from streams with below-median pesticide pollution (estimated $TU_{max} \leq -3$) ranged from 0.5- to 2-fold compared to the median tolerance (EC75) in reference streams ($TU_{max} \leq -4$). This range illustrates the natural variation in insecticide tolerance of non-adapted populations. In more polluted populations, insecticide resistance reached up to generally 4.0-fold (9.6 in exceptional cases). Comparable magnitudes of insecticide resistance (based on the LC50 ratio) have been observed also in polluted populations of some other aquatic non-target invertebrates including various insects (on average 3.5-fold, Becker and Liess, 2017), the crustaceans *Daphnia magna* (ca 2.5-fold, Jansen et al., 2015) and *Hyalella azteca* (550-fold across different clades, but only ca. 8-fold within the same clade; Weston et al., 2013), and water boatmen (10-fold; Vinson, 1969).

The results illustrate that despite being subject to governmental regulation, agricultural pesticides exert a considerable pressure on aquatic nontarget organisms to adapt. Increased tolerance was observed even in the estimated TU_{max} range of -2 to -3. This is lower than the threshold considered acceptable according to the most conservative first tier of the regulatory risk assessment in the EU (1/100 of the acute LC_{50} for *D. magna*, resembling $TU_{max} = -2$; EFSA, 2013). The observed adaptation of *G. pulex* (Siddique et al., 2020) and of other aquatic arthropods (Heim et al., 2018; Jansen et al., 2011) to pesticides is typically associated with fitness costs. Resistant populations of *G. pulex* therefore suffer from reduced reproduction even long after the end of pesticide exposure (Siddique et al., 2020) and may be more vulnerable to additional stressors such as pathogens (Jansen et al., 2011) and competition or predation by more tolerant species (Becker and Liess, 2015). Genetic adaptation to toxicants may further result in genetic erosion that is associated with inbreeding depression and the reduced ability to cope with changing environmental conditions (Van Straalen and Timmermans, 2006). Accordingly, our results shed light on the level of protection that the EU framework for governmental regulatory risk assessment provides against potential adverse effects.

4.1. Influence of test concentration on observed insecticide resistance

Differences in insecticide tolerance between highly and lowly polluted populations were more pronounced at high test concentrations, around the average effective concentration that immobilized 75 % of the individuals (EC75). This result confirms observations on *G. pulex* from the same study area in Siddique et al. (2020). Typically, the tolerance of populations to chemicals is compared using the median effective concentration (EC50) because it can be estimated with highest precision (Becker and Liess, 2017; Shahid et al., 2018; Russo et al, 2018; Weston et al., 2013; Vinson, 1969). However, our results suggest that the use of this endpoint may underestimate the actual magnitude of resistance that has developed.

The observed insecticide resistance in highly polluted populations may result from selection (genetic adaptation) at the population level (e. g. Lenormand et al., 1999; Jansen et al., 2015), and/or from physiological adaptation (induced acclimatization) at the individual level (e. g. Hua et al., 2013b). Accordingly, the pronounced difference in tolerance to high test concentrations may be explained by different, non-exclusive mechanisms: (i) Exposure to sublethal insecticide concentrations affects fitness traits of sensitive individuals (Jiang et al., 2020; Crawley et al., 2017). In such exposed populations, the individual fitness may therefore increase with insecticide tolerance (Becker and Liess, 2015). As a result, individuals with moderate to high tolerance will accumulate, while the relative change in the proportion of highly sensitive individuals will be smaller. (ii) Additionally, physiological adaptation may be higher in individuals that show already above-average tolerance before exposure. This disproportional increase in tolerance will result in a broader distribution of tolerance with a higher median tolerance in adapted populations, and consequently in a less steep dose-response curve as observed. This assumption is equivalent to the key assumption of the Stressor Addition Model (SAM) for the prediction of combined effects of pesticides and additional stressors (Liess et al., 2016): Each individual has a stress capacity that is beta-distributed in a population and can be invested to cope with environmental stressors; the tolerance to a specific stressor increases disproportionally with the amount of capacity invested. We conclude that the pronounced resistance to high test concentrations observed fits well in with established concepts of adaptation and should be considered in future resistance studies.

4.2. Seasonal variation in the adaptation to pesticides

Seasonal variation in the insecticide resistance of exposed populations might be overestimated due to the low number of data points in July - September when the lowest resistance occurred. Nevertheless, the increase in insecticide resistance with pesticide pollution was most pronounced in winter and early spring, and significantly diminished in summer.

In the study area, agricultural insecticides are mainly applied from March to September (Fig. S3). Decreasing resistance during the pesticide application season may be surprising as it contrasts reports of selection for higher resistance in mosquitoes during chemical control in summer, and of counter-selection due to fitness costs in winter (Lenormand et al., 1999). However, those mosquitoes are characterized by a much shorter generation time and also were exposed to acute lethal concentrations leading to very high resistance, whereas *G. pulex* individuals are characterized by a longer generation time and were exposed to sublethal concentrations in agricultural streams. The seasonal resistance pattern suggests that in polluted streams, long-term selection for more tolerant individuals is contrasted by short-term weakening from adverse pesticide effects. Pulsed exposure to sublethal concentrations of neonicotinoids and pyrethroids can significantly affect the performance of *G. pulex* for ca. 21 d in the laboratory (Cold and Forbes, 2004; Beketov and Liess, 2008; Galic et al., 2014) and can increase the sensitivity to subsequent exposure events (Russo et al., 2018; Ashauer et al., 2017). This explanation reconciles the contrasting observations of increased (Becker and Liess, 2017; Shahid et al., 2018) vs. decreased (Russo et al., 2018) insecticide tolerance in *G. pulex* from agricultural streams. Notably, decreased tolerance in Russo et al. (2018) was observed only in June, but not in March or October. After the end of the pesticide application season, individuals may recover from short-term adverse effects, while (genetic) adaptation of the populations is maintained till the following spring, so that insecticide resistance may be best observed in winter.

Additionally, in summer *G. pulex* has to cope with harsh conditions such as high temperature and low water levels leading to low oxygen supply, increased density stress and a potential depletion of food sources before the next leaf fall in October. Under such harsh environmental conditions, insecticide resistance has been shown to decrease because individuals may experience a trade-off in physiological acclimatization to different stressors (Liess et al., 2016, Liess et al., 2019) and because fitness costs associated with genetic adaptation are more pronounced (Becker and Liess, 2015; Raymond et al., 2013).

We conclude that the timing of sampling is crucial when assessing the adaptation to pesticides in the field. Our results are in accordance with Weston et al. (2013) who observed higher insecticide resistance in *H. azteca* from Californian streams in early spring (March) compared to midsummer (June).

4.3. Effects of biotic factors on the adaptation to pesticides

Our meta-analysis confirmed what has been previously observed in the separate data sets (Becker and Liess, 2017; Shahid et al., 2018; Russo et al., 2018). Despite high pesticide exposure, insecticide tolerance remains low when sensitive individuals can recolonize a polluted site from close lowly polluted refuge areas (Shahid et al., 2018). Significantly (> 3-fold) increased tolerance in polluted sites was only observed when refuges were ≥ 3.3 km away, suggesting that this may be the effective distance that *G. pulex* migrates downstream within a generation (limit for metapopulation exchange).

Additionally, insecticide tolerance remains low when the population is embedded in a diverse macroinvertebrate community (H’ > 1.6). This observation was predicted by Becker and Liess (2015) based on selection experiments on mosquitoes suggesting that intraspecific competition is a mayor driver of adaptation: In this experiment, intraspecific competition magnified adverse pesticide effects on the fitness of non-adapted individuals that may be cryptic under benign conditions. Predation and interspecific competition decreased intraspecific competition and thus genetic adaptation. Such interactions among antagonistic species are more likely to occur in diverse communities, whereas *G. pulex* may readily adapt to pesticide pollution under high intraspecific competition pressure when species diversity is low.

While pesticide pollution had the highest impact on the local insecticide resistance (46 % of explained variation), season (13 %), the distance to the closest refuge stream section (20 %) and the species diversity (21 %) were also important driving factors. The local species diversity in streams increased with the proximity of refuge areas, probably because refuges provided not only a source for the recolonization of sensitive *G. pulex* individuals, but also for the recolonization of other sensitive taxa. This way, non- or lowly polluted refuges may hinder the development of pesticide resistance both directly through genetic exchange and indirectly through the provision of natural enemies that lower intraspecific competition as a major driver of adaptation.

The analyses outlined above excluded two samples with unusually high insecticide resistance (8.9 – 9.6) collected on 25.08.2014 from streams in the same catchment area. These sites were characterized by high pesticide pollution (estimated TU_{max} = -2.3 - -1.3), large distance to the next refuge area (13 – 18 km) and low species diversity (H’ = 1.04 – 1.07) and thus further supported our conclusions on the effect of biotic factors on the development of insecticide resistance. Repeated measurements from these sites showed that resistance decreased to 3.4 – 4.0 after 15 months and to 2.9 – 3.7 after 19 months; this resulted mainly from a decrease in the extraordinarily high tolerance to clothianidin. This pattern suggests that these populations recovered from a previous unusually high exposure event, as it might be associated with the seeding of winter crops in late summer using seeds coated with neonicotinoids (Simon-Delso et al., 2015).

5. CONCLUSIONS

For a sustainable management of agricultural practices it is paramount to thoroughly understand the process of the development of pesticide resistance and identify environmental conditions that may protect freshwater non-target communities from detrimental effects. The present study showed that pesticide resistance is governed mainly by the level of pollution, but also significantly by seasonal variation, availability of non-polluted refuge sections, and species diversity. We therefore conclude that the influence of these environmental factors must be considered to refine risk assessment and resistance management.

ASSOCIATED CONTENT

Two supplemental files have been provided for further information. The file named "Supplementary Information 1" includes information on the different protocols applied in each study, measured values of abundance, biodiversity, toxicity, availability of refuges, time of exposure, sensitivity of the different sites throughout the sampling campaigns, and identification of respective communities (Tab. S1 – S5). The file named "Supplementary Information 2" provides information on the relation of estimated and measured toxic units, the distribution of insecticide resistance values, and details on the reported analyses of deviance (Fig. S1 – S3, Tab. S6 – S7).

CORRESPONDING AUTHOR

Liess, Matthias. Phone: +49 341 2351578; Email: matthias.liess@ufz.de

AUTHOR CONTRIBUTIONS

Conceptualization: Matthias Liess and Renato Russo.

Formal analysis: Jeremias Martin Becker, Naeem Shahid, and Renato Russo.

Investigation: Naeem Shahid.

Writing - Original Draft: Renato Russo and Jeremias Martin Becker.

Writing - Review & Editing: Matthias Liess.

ACKNOWLEDGEMENTS

This work was funded by Helmholtz long-range strategic research funding (POF III). The funding source was not involved in the study’s design; in the collection, analysis, and interpretation of data; in the writing of the article; nor in the decision to submit the article for publication. We thank Lena Reiber for providing some of the pesticide measurements used for the validation of our SPEAR-based estimated toxic units.

COLORING OF FIGURES

Color should be used in the printed version for Figure 1, Figure 2 and Figure 3.

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