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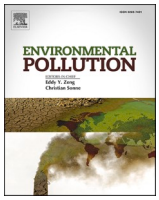

Cryptic species complex shows population-dependent, rather than lineage-dependent tolerance to a neonicotinoid☆

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A B S T R A C T

Cryptic species are rarely considered in ecotoxicology, resulting in misleading outcomes when using a single morphospecies that encompasses multiple cryptic species. This oversight contributes to the lack of reproducibility in ecotoxicological experiments and promotes unreliable extrapolations. The important question of ecological differentiation and the sensitivity of cryptic species is rarely tackled, leaving a substantial knowledge gap regarding the vulnerability of individual cryptic species within species complexes. In times of agricultural intensification and the frequent use of pesticides, there is an urgent need for a better understanding of the vulnerability of species complexes and possible differences in adaptive processes. We used the cryptic species complex of the aquatic amphipod *Gammarus roeselii,* which comprises at least 13 genetic mtDNA lineages and spans from small-scale endemic lineages in Greece to a large-scale widely distributed lineage in central Europe. We exposed eleven populations belonging to four lineages to the neonicotinoid thiacloprid in an acute toxicity assay. We recorded various environmental variables in each habitat to assess the potential pre-exposure of the populations to contaminants. Our results showed that the populations differed up to 4-fold in their tolerances. The lineage identity had a rather minor influence, suggesting that the cryptic species complex *G. roeselii* does not differ significantly in tolerance to the neonicotinoid thiacloprid. However, the observed population differentiation implies that recent pre-exposure to thiacloprid (or similar substances) or general habitat contamination has triggered adaptive processes. Though, the extent to which these mechanisms are equally triggered in all lineages needs to be addressed in the future. Our study provides two key findings: Firstly, it shows that observed phylogenetic differences within the *G. roeselii* species complex did not reveal differences in thiacloprid tolerance. Second, it confirms that differentiation occurs at the population level, highlighting that susceptibility to toxicants is population-dependent. The population-specific differences were within the range of accepted intraspecific variability from a regulatory standpoint. From an evolutionary-ecological perspective, it remains intriguing to observe how persistent stresses will continue to influence tolerance and whether different populations are on distinct pathways of adaptation. Given that the potential selection process has only lasted a relatively short number of generations, it is crucial to monitor these populations in the future, as even brief exposure periods significantly impact evolutionary responses.

1. Introduction

River ecosystems face numerous human-induced stressors, and evidence suggests that they rank among the most endangered ecosystems globally. These threats are associated with significant declines in biodiversity (WWF, 2022), and are attributed to myriad factors, including extensive freshwater resource exploitation, urbanization, pollution, and climate change impacts (Jaureguiberry et al., 2022; Persson et al., 2022). Agricultural land-use poses a specific challenge to aquatic systems as they introduce fertilizers or pesticides through diffuse spraying and runoff (Steffen et al., 2015; Dudley and Alexander, 2017). Although pesticides are aimed at pest control within the terrestrial environments, once they enter the aquatic realm they influence non-target organisms and communities (Malmqvist and Rundle, 2002; Schwarzenbach et al., 2006; Damalas and Eleftherohorinos, 2011; Lakhani, 2015; Major et al., 2018; Kumar et al., 2021). Consequently, the role of

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pesticides in structuring species communities and shaping general biodiversity trends remains poorly understood (Groh et al., 2022; Haase et al., 2023; Sylvester et al., 2023).

Within freshwater ecosystems, the disproportionately high biodiversity (Dudgeon et al., 2006) becomes even more staggering when species complexes are considered, since cryptic species have, until recently, been regarded as single morphospecies (Bickford et al., 2007; Fišer et al., 2018; Struck et al., 2018). The routine use of molecular methods over the past two decades has revealed an enormous number of cryptic species, which are characterized by their concealed genetic diversity and significant roles in enhancing richness and diversity in aquatic environments. The term "cryptic species" refers to two or more species with close or identical resemblance, making their morphological identification challenging or impossible. However, these species can exhibit reproductive isolation and distinct evolutionary lineages (Sáez and Lozano, 2005; Fišer et al., 2018). Cryptic species have scarcely been considered in environmental risk assessments, however, some examples exist. For instance, the cryptic species of the freshwater amphipod *Hyalella azteca* (Saussure, 1858) has been studied in response to environmental stressors such as pyrethroid insecticides (Duan et al., 1997). This study organism has been recognized since the 1990s as a cryptic species complex. A study by Weston et al. (2013) showed that different lineages of the *H. azteca* species complex respond profoundly different to the pyrethroid insecticide cyfluthrin resulting in a 550-fold higher sensitivity of some lineages. The authors were even able to identify the exact point mutation that leads to an increase in tolerance. Moreover, Fung et al. (2021) found elevated activities of detoxification enzymes, particularly cytochrome P450, which were strongly associated with higher resistance levels. Such findings not only challenge the classical risk assessment, considering that *H. azteca* is a widely used test organism in ecotoxicology (Jourdan et al., 2023), but also raises the intriguing question of the extent to which deep-rooted phylogenetic processes influence the ability of individual lineages to respond to environmental changes.

From an ecotoxicological perspective, cryptic species are relevant because they can have different sensitivities to contaminants and thus contribute to higher variability in the outcomes of test methods. This does not only apply to *H. azteca*, but also to many commonly used invertebrate species harbouring cryptic diversity. These include aquatic invertebrates such as the mosquito *Aedes aegyptii* (Linnaeus, 1762), the amphipod *Gammarus pulex* (Linnaeus, 1758), and the bivalve *Mytilus galloprovincialis* Lamarck, 1819 (Jourdan et al., 2023). Thus, variation of experimental test outcomes could potentially be attributed to different genetic lineages and, when not considered, an unreliable-experimental outcome. For instance, an overestimated tolerance could result in the loss of genetic lineages within the cryptic species complex or an incorrect risk assessment of a chemical substance (Feckler et al., 2013; Leung et al., 2016; Fišer et al., 2018; Jourdan et al., 2023). It is therefore highly recommended to gather information about the biogeography of a test species beforehand and to identify genetic lineages before using a species in an ecotoxicological framework.

Amphipod crustaceans are increasingly becoming established as model organisms to investigate cryptic diversity (Fišer et al., 2018). Many species show high cryptic diversity with locally restricted genetic lineages (e.g. Wattier et al., 2020). One of these species complexes is that of *Gammarus roeselii* Gervais, 1835 (often incorrectly referred to as *G. roeseli*). The *G. roeselii* species complex consists of at least 13 phylogenetically separated lineages or molecular operational taxonomic units (MOTUs), with a genetic diversification hotspot originating in the Balkan Peninsula and one genetic lineage (MOTU C) that is widely distributed over Central Europe (Grabowski et al., 2017; Csapó et al., 2020). It is assumed that the earliest phylogenetic split took place 18 Million years ago when the northern Greece clade split from the southern ones presumably due to allopatric separation (Grabowski et al., 2017). Due to different geographical settings and the intensified agricultural land use in some regions, the lineages now occur under different environmental conditions (Kabus et al., 2023). These differences in occurrence can either be explained by differing sensitivities of individual lineages to environmental stressors, or simply due to biogeographical barriers and locally prevailing environmental conditions. In either case, and considering that different genetic lineages within the species complex can have different tolerances to stressors, small-scale genetic lineages might be more prone to changes in the environment (Fišer et al., 2018), and may therefore be at higher risk of extinction. For *G. roeselii* MOTU C, which is now widespread throughout Central Europe, it can be assumed that it has a relatively high resistance to anthropogenic disturbance as it persists in various heavily anthropogenically impaired rivers (Jourdan et al., 2019; Enns et al., 2023; Kochmann et al., 2023; Jourdan et al., 2024). Further, MOTU C shows a rapid adaptive response to pesticides such as the neonicotinoid thiacloprid (Jourdan et al., 2024). Such adaptive processes result in higher tolerances to certain substances, as shown in various gammarids (e.g. *G. fossarum* lineage A and B: Feckler et al., 2012; Feckler et al., 2014; *H. azteca* clade 1 and 8: Leung et al., 2016; *G. pulex*: Becker and Liess, 2017; Shahid et al., 2018a; Shahid et al., 2018b; Siddique et al., 2021; *G. roeselii* presumably MOTU C: Jourdan et al., 2024). Most of these studies, however, tested individual species/lineages. Given that these taxa are often members of large species complexes, it can be assumed that deep phylogenetic differences may lead to different vulnerabilities between cryptic lineages of a species complex. The effect of lineage identity (i.e., MOTU) on tolerance differences in gammarids has only been investigated for *G. fossarum* in relation to the fungicide tebuconazole and the insecticide thiacloprid (Feckler et al., 2012). Feckler et al. (2012) identified significant differences in the sensitivity between two genetic lineages (A and B) of *G. fossarum* to both pesticides. Another study by Feckler et al. (2014) uncovered significant differences in the tolerance and feeding behavior between genetic lineages of *G. fossarum* under ammonium exposure. Accordingly, the results suggest that lineage-specific differences in tolerances are likely.

In this study, we used the *G. roeselii* species complex to test the tolerances of different lineages and their populations to a widespread neonicotinoid, thiacloprid. We hypothesized that the widely distributed MOTU C is characterized by a higher tolerance to thiacloprid, whereas locally isolated MOTUs (A, G, and L) on the Balkan Peninsula show lower tolerances to thiacloprid. Furthermore, we hypothesized that local environmental conditions reflect intra-MOTU-specific differences in tolerances, with populations exposed to higher levels of pollution in their habitat showing higher tolerances to thiacloprid.

2. Material and methods

2.1. Sampling sites and animal keeping conditions

Following the successful genetic characterization of cryptic populations of *G. roeselii* from various freshwater habitats on the Balkan Peninsula in 2021 (Kabus et al., 2023), we re-sampled eleven populations representing four MOTUs in 2022 and re-characterized their mtDNA lineages (see 2.2). The species complex extends from Central Greece over the whole of central Europe, with the distribution hotspot on the southern Balkan peninsula (Grabowski et al., 2017; Kabus et al., 2023), and only MOTU C has extended its range throughout central Europe (Csapo et al., 2020). Three populations each of MOTU A, G and L, and two populations of MOTU C were collected to cover different environmental conditions (Fig. 1 and Supplementary Table S1). The sampling and testing of the animals took place in September 2022 with MOTUs A, G and L being tested at the beginning of September in northern Greece and MOTU C at the end of September in Germany in the same year. We used the same sampling and keeping methods as well as subsequent analysis for all populations. Individuals were sampled via multi-habitat kick-sampling using hand nets (Bioform V2A; mesh size 500 μm). The captured animals were brought to a research station (field station of University of Western Macedonia for populations of MOTUs A,

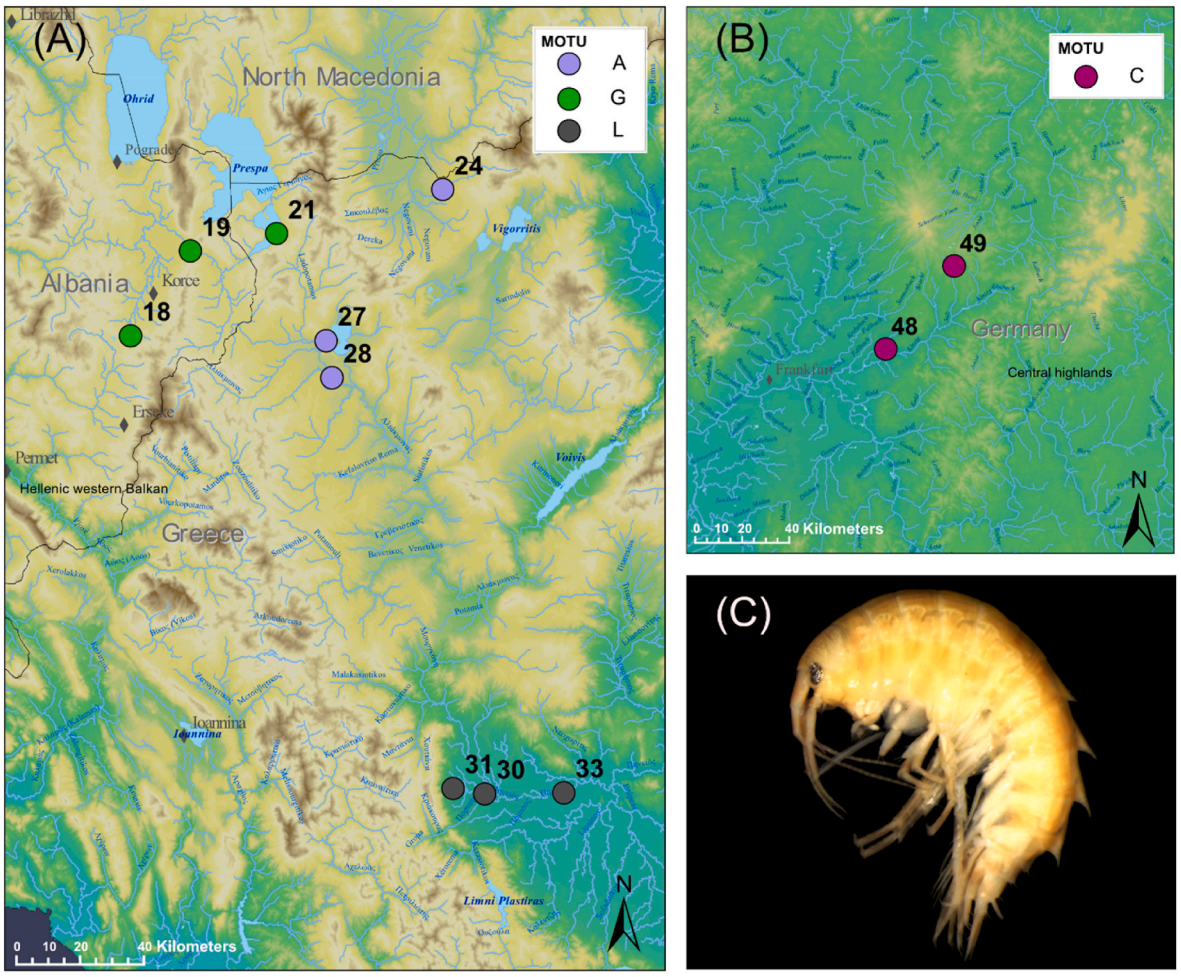

Fig. 1. Map of the sampling sites in the Balkan area and Germany with MOTUs determined by COI barcoding. MOTUs are named following the classification of Grabowski et al. (2017). Sampling sites are numbered according to Kabus et al. (2023) and exact coordinates are given in Table S1. **(A)** Sampling sites in Albania and Greece where MOTUs G, A and L are present. **(B)** Sampling sites in Germany with only MOTU C present. Maps were created using ArcGIS Desktop v.10.8. **(C)** *Gammarus roeselii* MOTU C specimen from site 49 fixed in ethanol.

G and L; Lab at the Goethe University Frankfurt am Main for populations of MOTU C) in aerated cooling boxes. At the research station, the animals were kept in 45 L plastic aquaria in a medium composed of 40 L deionised water, 12 g of $NaHCO_3$, 6.7 g $CaCl_2$ and 5 g Preis-Discus-Minerals (combination of minerals and trace elements; Preis Aquaristik KG, Bayerfeld, Germany). During preliminary testing, this medium proved to be the most suitable for the long-term maintenance of *Gammarus* species and was therefore used here as a maintenance and test medium. Acclimatization to the medium gradually took place over 24 h. Aquaria were equipped with an air-powered foam filter. Ceramic filter tubes served as hiding places in the aquariums, and leaf litter from the respective sampling sites served as food and were given every two days. After a two-day acclimatization period, the animals were used in the acute toxicity assays (see below). Additionally, from each site, 25 individuals were fixed in 96 % EtOH for later genetic evaluation of MOTU identity (see below section Molecular species evaluation). The sampling was done in close cooperation with local partners and the species is not subject to any protection status. Permission was granted by the Hellenic Ministry of Environment and Energy (Permission No: ΥΠΕΝ/ΔΔΔ/7316/280) and the Albanian National Agency of Protected Areas (Permission No: 194, issued on 16.02.2021).

2.2. Molecular species evaluation

To evaluate the MOTU/s for each sampling site we, sequenced the cytochrome *c* oxidase subunit I (COI) of 15 individuals collected from each site (see Supplementary Table S3) and mapped them against individuals from each MOTU delimited by Grabowski et al. (2017). DNA was extracted using the protocol of Montero-Pau et al. (2008). From each individual, two to three dissected pereiopods were put in 96 well plates and a lysis buffer was added. We used 30 μL lysis buffer instead of 50 μL, which showed a better result during DNA extraction. The buffer is composed of 25 mM NaOH, 0.2 mM Na_2EDTA and ddH_2O. The plate was then sealed off and incubated for 30 min at 95 °C. Subsequently, the plate was cooled to 4 °C for 5 min. A neutralizing buffer composed of 40 mM Tris HCL and ddH_2O was added. The COI fragments were amplified using polymerase chain reaction (PCR) with different primer pairs. Primers used were LCO1490 and HCO2198 (Folmer et al., 1994), UCOIF and UCOIR (Costa et al., 2009) and COIGrF and COIGrR2 (Grabowski et al., 2017). The primers used for each individual are listed in Supplementary Table S3. We carried out the PCR in a 30 μL volume within the 96-well plates. The PCR Mastermix was composed of 0.4 μL BSA, 3.6 μL $MgCl_2$ (25 mM), 3.0 μL 10 x Taq-Buffer (BioLabs), 2.4 μL dNTPs (40 mM), 0.4 μL Taq Polymerase (Biolabs), each primer with a volume of 1 μL (10 mM), 14.2 μL of ddH_2O and 4 μL DNA template. The amplification of a 650-bp fragment was carried out using the PCR conditions stated in Mamos et al. (2014) for primer pairs UCOIF/UCOIR and

COIGrF/COIGrR2: Initial denaturation at 94 °C (3 min), 35 cycles of denaturation at 94 °C (20 s), annealing at 50 °C (45 s) and elongation at 65 °C (1 min) and a final extension at 65 °C (2 min). For the primer pair LCO1490/HCO2198 conditions of Weiss et al. (2014) were applied: Initial denaturation at 94 °C (2 min), 36 cycles of denaturation at 94 °C (20 s), annealing at 46 °C (30 s) and elongation at 65 °C (1 min) and a final extension at 65 °C (5 min). PCR products were then checked for successful amplification with a 5 μL aliquot in a GelRed ® (VWR) 1.0 % agarose gel and brought to the Senckenberg Biodiversity and Climate Research Centre (SBiK-F) for sequencing. Thereafter, all sequences were checked using the BLAST search implemented in the software Geneious Prime (Ver. 2022.2.2). These sequences were then aligned with ClustalW and cut to a length of 522 bp. Already published sequences of known MOTUs of Grabowski et al. (2017) and Kabus et al. (2023) were taken as a measure of delimitation and checked using a neighbour-joining tree. The newly acquired sequences are published in the Barcode of Life Data System (BOLD) using their assigned IDs (see Table S3 in the Supplementary in the Appendix). Similarly to Kabus et al. (2023), no sympatric genetic lineages were discovered so at each sampling site only one genetic lineage was present.

The genetic alignment was analysed to identify the genetic diversity and differentiation between the MOTUs and populations using DnaSP (Ver. 5.10, Librado and Rozas (2009)). The nucleotide diversity (Pi), haplotype diversity (Hd), number of segregating sites, and number of haplotypes were calculated between the different populations (Supplementary Table S4).

2.3. Pesticide tolerance experiments

The acute sensitivity of *G. roeselii* sensu lato to the neonicotinoid insecticide thiacloprid (Pestanal®, analytical standard from Sigma-Aldrich, LOT: #BCBW6020) was determined following the OECD guideline for the testing of chemicals (OECD guideline No. 202) and previous testing protocols (Grethlein et al., 2022; Kochmann et al., 2023). Thiacloprid is an insecticide belonging to the group of neonicotinoids that interfere with the transmission stimuli in the nervous system of invertebrates. Neonicotinoids bind to the nicotinoid acetylcholine receptor (nACHR) and prevent hydrolysis of acetylcholine which leads to paralysis due to the accumulation of acetylcholine. This pesticide is used as pest control for Coleoptera or aphids in potatoes and rapeseed oil cultivation (Zimmer and Nauen, 2011; Purhematy et al., 2013). It is highly toxic for invertebrates especially in aquatic systems as it has a high water solubility and is considered persistent (Assessment Report: Directive, 1998; Osterauer and Köhler, 2008). Thiacloprid also affected molecular biomarkers and inflicted damage to the DNA of the earthworm *Eisenia fetida* (Feng et al., 2015). Due to its endocrine activity, the permission to use thiacloprid in agriculture was not renewed for EU countries in 2020 and all remaining stocks needed to be used until February 2021 (Durchführungsverordnung (EU) 2020/23, 2020).

We used 80 mL plastic beakers installed with fine mesh barriers to keep four animals therein separated. Beakers were closed using a lid which had pre-made holes for oxygen exchange. A 1000 mg/L stock solution of thiacloprid was prepared in distilled water. Required test concentrations (45, 110, 260, 620 and 1490 μg/L) were prepared within the *Gammarus* medium. The negative control contained only the *Gammarus* medium. One beaker (i.e., four individuals) corresponded to one replicate. For each concentration and population, we ran six replicates (i.e., 24 individuals). Each beaker was filled with 80 mL of the test concentration. To maintain stable temperature conditions during the 96h test period, all beakers were placed in a temperature-controlled water bath maintained at 20 °C. The mobility of the test organisms was tested every 24 h. When the animals stopped moving, they were gently coaxed with a plastic stick and observed for at least 30 s. If they showed no movement after repetitive coaxing, they were noted as immobile. The mortality rate was never higher than 10 % in any of the negative controls, which led us to classify the tests as valid.

2.4. Measuring environmental conditions

To get the most comprehensive impression of the extent of local water pollution, we recorded a set of environmental parameters at each sampling site. We measured various water parameters *in situ*, including: flow velocity (Dostmann electronic P670), pH (Hach HQ40d multi, PHC201), oxygen saturation (Hach HQ40d multi, LDO101), as well as conductivity (Hach HQ40d multi, CDC401; see Supplementary Table S1 for parameters). Further, we collected water samples from each site and immediately after arrival at the research station, analysed them for nitrate (NO_{3-}), nitrite (NO_{2-}) and phosphate (PO_4^{3-}) using a photometer (FinwellPro for ponds; MDE GmbH & Co. KG, Germany; see Supplementary Table S1).

Additionally, we collected sediment samples and tested them using various *in vitro* assays. The *in vitro* assays evaluate the baseline toxicity and endocrine activity in the sediment samples that account for long lasting, hydrophobic and organic contaminants attached to sediment that accumulate over time (Keiter et al., 2006). These *in vitro* assays capture recent and historical contamination as sediments act as sinks for many environmental pollutants and were used as a proxy for a general anthropogenic impact on the sampling sites. As amphipods are benthic dwellers, they are in close contact with contaminated sediments and are extensively exposed to accumulated pollutants (Nguyen et al., 2012). We took sediment samples and cooled them continuously at 4 °C until we returned to the laboratory at the Goethe University Frankfurt. We performed the following yeast *in vitro* assays to capture endocrine activity of the river sediments: estrogenic activity (Yeast Estrogen Screen = YES; Routledge and Sumpter, 1996), anti-estrogenic activity (YAES), androgenic activity (Yeast Androgen Screen = YAS; Sohoni and Sumpter, 1998), anti-androgenic activity (YAAS) and dioxin-like activity (Yeast Dioxin Screen = YDS; Miller, 1997). The procedure followed the protocols in Giebner et al. (2018). We further used the microtox bioluminescence inhibition assay with *Aliivibrio fischeri* (ISO-Guideline 11348-3, 2007) to assess the general toxicity of the sediments. The assay measures the inhibition of bioluminescence in *A. fischeri*, with effective concentrations causing a 50% reduction in bioluminescence (EC_{50}) calculated for each sediment sample. For the purposes of data presentation and subsequent statistical analyses, including multivariate analysis, we transformed the EC_{50} values by subtracting them from 100. In this transformed dataset, higher values indicate greater toxicity. Since no androgenic activity was detected in the YAS, we excluded it from further analysis. The results for each sampling site can be viewed in Supplementary Table S2. For further information see also Kabus et al. (2023).

2.5. Hydrological, land use and climatic conditions

We further extracted hydro-environmental information for our sampling sites from the dataset of Domisch et al. (2015). The dataset provides information on a spatially continuous and freshwater-specific set of environmental conditions from the years 1970–2000. It includes information on land cover, climate, river topography and geology of the catchment area for a 1 km standardised river network grid created for each pixel by upstream accumulation techniques. The mean of each variable of a 1 km grid cell in the terrestrial dataset is weighted over the distance of the upstream catchment for each corresponding sampling site. We extracted and used the parameters 'flow length' (the cumulative size of the catchment upstream), 'cultivated and managed vegetation' (lc_avg_07), 'urban/built-up land cover' (lc_avg_09), 'annual mean temperature' (Bioclim1) and 'annual precipitation' (Bioclim12) to represent the climatic and anthropogenic influence on each sampling site. The values for each parameter at each sampling site were used in further analyses and can be viewed in Supplementary Table S2.

2.6. Statistical analysis

To study how thiacloprid affects each MOTU and population, we considered the percentage of immobile and dead individuals per replicate and treated them equally, along with the concentration and time, to predict their responses to different concentrations. We used the function 'drm' from the package "drc" (Ritz and Streibig, 2005) in R (Ver. 4.1.0). We ran a four-parameter log-logistic function as the default function to predict dose-response curves for each population and MOTU. The predicted EC_{50} values are displayed in Supplementary Tables S5 and S6. To evaluate differences between the dose-response curves of each population and MOTU, the 'compParm' function from the "drc" package (Ritz and Streibig, 2005) was used and p-values were calculated to determine dissimilarities between each curve (Fig. 2 and Supplementary Tables S7 to S10 and Figure S1 and Figure A1).

To reduce data complexity and aid in interpretation, we conducted a Principal Component Analysis (PCA) on the 18 environmental parameters (Supplementary Table S11). The PCA was made using the function 'dudi.pca' in the R package "ade4" (Dray and Dufour, 2007). The variables were standardized (i.e., z-score transformed) prior to PCA analysis. PC1 and PC2 explained 56.8 % of the variance while PC3 and PC4 explained an additional 21.2 %. PC1 and PC2 were considered in our analysis as evaluated by the broken stick-model using the function 'evplot' and a permutation of Gupta (2019). Applying the 'PCAtest' function within the package "PCAtest" only PC1 resulted in a significant p-value (<0.05). However, we still considered PC2 in our evaluation (p-value $= 0.05$).

By correlating these environmental PCs, the EC_{50} values and genetic diversity metrics, we aimed to evaluate relationships between environmental pollution, population tolerance and genetic diversity. To determine if the EC_{50} values were related to local environmental conditions or genetic characteristics of the respective population, we correlated the EC_{50} values with PCs 1 and 2 (Fig. 4) and number of segregating sites (positions of polymorphism within the DNA, abbr.: segr. sites), haplotype diversity (Hd) and nucleotide diversity (Pi) (Supplementary Fig. S2 and S3 for PCs 3 and 4). We checked for normality using the 'shapiro.wilk' function. Non-normality was assessed for most of the parameters. Consequently, we used Spearman correlations and visualized significant correlations using the 'ggscatter' function within the "ggplot2" package. All statistical analyses were carried out using R (Ver. 4.1.0; R Core Team, 2021)

3. Results

3.1. Population-specific tolerances

After a 96-h exposure to thiacloprid, our models revealed significant population-level differences in the tolerance of *G. roeselii* (Fig. 2). This effect was mainly driven by the high tolerances of population 33 (MOTU L, Greece; EC_{50} 620.39 ± 69.5 µg/L at 96 h; Fig. 2B; Table 1). The most sensitive population was 49 (MOTU C, Germany; EC_{50} 153.03 ± 11.5 µg/L; Fig. 2B; Table 1), which was significantly more sensitive than all other populations, even population 48 from the same MOTU. The tolerance of population 33 was 4-fold higher than that of the most sensitive population 49. Population 18 (MOTU G), which had the lowest genetic diversity, was also significantly more sensitive compared to the other MOTUs (Table 1 and Supplementary Table S4). Population 28 (MOTU A) also had significantly different EC_{50} values to the other populations (Fig. 2B–Table 1). However, these differences were not as strong as those between populations 33 or 49. The EC_{50} values of most populations also differed significantly within the same MOTU, emphasizing population-specific tolerances. Nevertheless, when calculating MOTU-specific differences – and populations are not considered – significant MOTU-specific tolerance differences were found

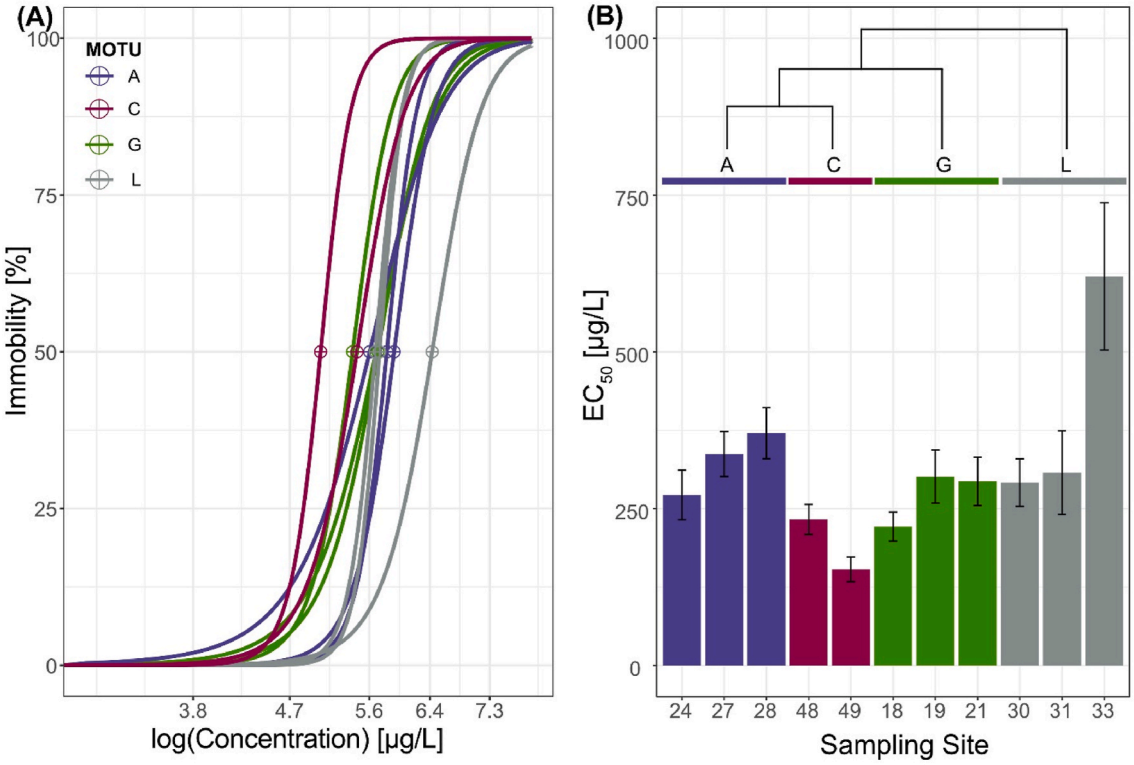

Fig. 2. Population-specific responses of the *Gammarus roeselii* species complex after 96-hour exposure to thiacloprid. (**A**) Concentration-response curves for each population after 96 h exposure time with the EC_{50} values marked by a cross in a circle. (**B**) EC_{50} values for all replicates separated in populations. The whiskers represent the 95% confidence intervals within each population. Colors represent the four MOTUs tested. On the top is a simplified phylogenetic tree of the tested MOTUs. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Pairwise comparisons of the EC_{50} values [µg/L] of all populations after 96 h exposure displayed in Fig. 2. The EC_{50} values (±SE) are displayed in the grey square. p-values: ns > 0.05, * ≤0.05, ** ≤0.01, *** ≤0.001.

	MOTU	site	18									
96h	G	18	221.1 (± 13.7)	19								
		19	***	301.0 (± 25.0)	21							
		21	***	ns	293.3 (± 22.7)	24						
	A	24	*	ns	ns	271.8 (± 23.3)	27					
		27	*	ns	ns	*	337.0 (± 21.3)	28				
		28	***	***	*	***	ns	370.3 (± 24.0)	30			
	L	30	*	ns	ns	ns	ns	*	291.5 (± 22.3)	31		
		31	***	ns	ns	ns	ns	ns	ns	307.2 (± 39.5)	33	
		33	***	*	***	***	***	***	***	***	620.4 (± 69.5)	48
	C	48	ns	*	*	ns	**	***	ns	ns	***	232.7 (± 14.2)
		49	**	***	***	***	***	***	**	**	***	**

(Supplementary Fig. S1 and Table S7 and Table A7). Though, these differences could be attributed to extremes in the individual populations (Fig. 2 and Supplementary Figure S1).

3.2. Exploring influential factors shaping thiacloprid tolerance

To summarize the 18 environmental variables, we performed a PCA. The first 4 PCs explained 77.96 % of the total variance (Fig. 3; Table S4

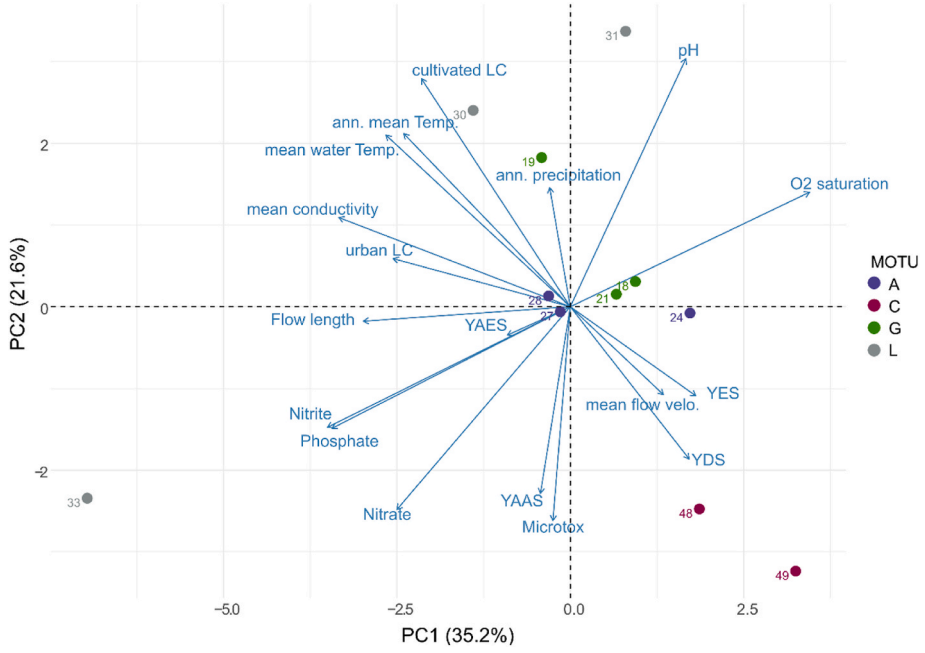

Fig. 3. Principal Component Analysis (PCA) characterizing the sampling sites based on recorded environmental parameters. The length of the arrows indicates the strength of each parameter's influence on the dataset. Dots represent the different sampling sites, colored accordingto their corresponding MOTU. PC1 accounts for 35.2 % and PC2 for 21.6 % of the total variance in the dataset. Abbreviations: YES = Estrogenic activity; YDS = dioxin-like activity; YAAS = Anti-androgenic activity; Microtox = Baseline toxicity; YAES: Anti-estrogenic activity; LC= Land cover; ann. = annual; Temp = temperature; velo = velocity.

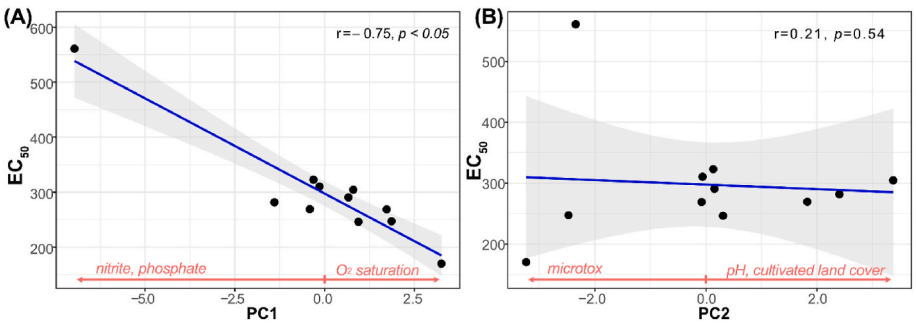

Fig. 4. Correlation between population tolerance to thiacloprid (EC_{50} values) and local environmental conditions, as represented by PC1 and PC2. (A) Spearman correlation of EC_{50} values to PC1 with a highly significant negative correlation. The more negative the PC1 value, the higher the amount of nitrate and phosphate, while the higher the PC1 value, the higher the oxygen saturation. **(B)** Spearman correlation of PC2 with EC_{50} values showed no significant correlation.

in the Supplementary in the Appendix). Site 33 differed substantially in abiotic conditions from all other sites. With a nitrite content of 1.31 mg/L, nitrate 27 mg/L and phosphate 4.6 mg/L, site 33 had significantly higher nutrient concentrations than all other sites, corresponding to extremely low oxygen saturation of 1.13 mg/L (cf., Supplementary Table S1). All populations of MOTU L occurred under warmer conditions (annual mean air temperature: 14.3–15.6 °C). Both populations of MOTU C were found in environmental conditions that have high dioxin-like and estrogenic activity. The populations of MOTU A (24, 27 and 28) and MOTU G (18 and 21) are mostly situated in the centre of the PC plot, suggesting that these populations are not as exposed to extreme environmental conditions.

The EC_{50} values correlate significantly negatively with PC1 (Fig. 4 A; Spearmans-r: −0.75; $p < 0.05$). This correlation is mainly explained by the high EC_{50} value of population 33. However, even if population 33 is classified as an outlier and not considered in the analysis, this correlation remains strong (see Supplementary Fig. S4; Spearmans-r: −0.66, $p < 0.05$). The most sensitive population, population 49, had the most positive value along PC1, suggesting rather pristine conditions and in this case low nitrite and phosphate values (Fig. 4 A). In contrast, PC 2 (and thus the results of the microtox assays, pH and cultivated land area) did not correlate with the EC_{50} values (Fig. 4B–Supplementary Figure S4). Additionally, there is no correlation between the EC_{50} values and the parameters for genetic diversity (Supplementary Table S2).

4. Discussion

Local environmental conditions showed a strong correlation with population tolerance, while MOTU identity, i.e., deeply-rooted phylogenetic differences, exerted a relatively minor influence. In general, our study revealed that populations of the cryptic *G. roeselii* species complex are comparable in their tolerance to the commonly used pesticide thiacloprid. However, some populations showed a significant deviation from this general trend.

4.1. Population vs MOTU-specific differences in tolerance

The patterns we found show that although there are significant differences between the MOTUs, these differences emerge primarily at the population level. This is consistent with previous studies that have shown that the tolerance of gammarid populations to pollutants can vary over a small spatial scale, often even within a river system (Shahid et al., 2018b; Shahid et al., 2018a; Siddique et al., 2020; Siddique et al., 2021; Jourdan et al., 2024). Differences in tolerance arise mainly due to local pollution pressure, with the most sensitive populations occurring in pristine refuge areas. We found a similar pattern here, *G. roeselii* sensu lato sampled in relatively pristine sites (e.g. site 48 and 49, MOTU C) were the most sensitive against thiacloprid compared to populations from anthropogenically influenced sites (e.g. site 33, MOTU L), which is in accordance with our second hypothesis. We therefore conclude that the tolerance tested here is influenced by previous exposure to the same or similar pollution and is different for every sampling site, thereby strongly suggesting population-specific tolerances. Such changes in tolerance can occur extremely rapidly. For example, a multigenerational experiment with *H. azteca* showed that thiacloprid tolerance increased 1.7-fold when pre-exposed to low (sublethal) doses, after only two generations (Jourdan et al., 2024). This demonstrates that adaptive mechanisms take place at contemporary scales and adaptive responses after recent exposure are a major explanation for the tolerance differences that we found within the *G. roeselii* complex. The most tolerant population (33; MOTU L) is situated in the lowlands of Central Greece, a region that is strongly shaped by intensive agriculture (Skoulikidis, 2016). It is therefore likely that the population 33 of MOTU L has been in contact with thiacloprid or pesticides with a similar mode of action, potentially initiating adaptive responses (Schäfer and Piggott, 2018; Zhou and Wang, 2023). The site is also located downstream of the city Tricca, from which further anthropogenic stressors, including inputs from wastewater treatment plants, are introduced. It may therefore also be a general, non-specific adaptive response of the population to these stressors. In contrast to our first hypothesis that the most widespread MOTU C is also the most tolerant, the populations of MOTU C sampled here were likely naïve to neonicotinoid exposure, explaining its low tolerance.

However, we also cannot exclude the possibility that differences in tolerance are due to changes at the deeper genomic level that cannot be covered by evaluating mtDNA differences between populations or MOTUs. If chemical stress lasts long enough, genomic changes can occur (Kim et al., 2015; Brasseur et al., 2023). It is possible that pesticide resistance can be retained after long-term exposure to non-contaminated water and can be seen on the genotype-level (Gamble et al., 2023; Siddique et al., 2024). However, very few studies have actually looked at the genomic signature of possible adaptations to chemical pollution in amphipods. Weston et al. (2013) is an exception, showing that the 550-fold higher pyrethroid tolerance in *H. azteca* is due to a mutation of the voltage-gated sodium channel – the primary target site for pyrethroids. In their study, different populations of four clades, including laboratory strains, were used in acute toxicity testing with a pyrethroid insecticide (Weston et al., 2013). Resistant populations were found in two clades, *H. azteca* clade B and D. However, another population of clade B was much less resistant and comparable to the rest of the tested clades (Weston et al., 2013). This illustrates how important it is to also consider intra-MOTU variability when investigating the differentiation of cryptic species, otherwise there is a risk of confusing population differentiation with species or MOTU differentiation. Studies that show any differences between cryptic species only based on one population per species run the risk of simply having depicted artefacts of population-specific differentiation that they attribute to MOTU differentiation. However, the question of the extent to which populations of

different (cryptic) species have the same variability in their response to stressors is another open and promising avenue of future research.

4.2. Possible mechanisms of tolerance variation

The question remains as to what causes an increased tolerance to thiacloprid. It can be assumed that at least one substance with a similar mode of action triggered the adaptive response in the tolerant population 33. Resistance mechanisms in *G. roeselii* to thiacloprid have been previously explored in Jourdan et al. (2024). These mechanisms encompass modifications to the target site, specifically the nicotinic acetylcholine receptor (Liu et al., 2005; Bass et al., 2011), as well as involvement of metabolic pathways (Bass et al., 2015). Notably, neonicotinoid resistance is often associated with metabolic changes, with cytochrome P450 monooxygenases playing an important role. These enzymes are essential for the metabolism of a diverse array of compounds, including toxicants, hormones, and various endogenous and exogenous substances (Nauen et al., 2022).

4.3. Sensitivity to other stressors and potential trade-offs

We found the most tolerant population 33 at a site with simultaneous high nitrite (1.31 mg/L) and phosphate (4.6 mg/L) concentrations. These measurements are based on single grab samples; pollution peaks after rain events are probably even higher (cf., Betz-Koch et al., 2023), and it shows that this population is able to tolerate extremely harsh conditions. Future studies should address the interesting question of how specific these adaptive responses are and assess the extent to which these populations exhibit similar tolerance to other stressors associated with global change. These could be, for example, other neonicotinoid pesticides but also commonly used pesticides with different modes of action (e.g. pyrethroids, organophosphates or glyphosate). Moreover, it is pertinent to explore additional stressors, including elevated temperatures, rising salinization levels, both individually and in conjunction with pesticides. The crucial aspect is that additional stressors are always present in a multi-stressor context, warranting careful consideration in formulating future research questions. If an adaptive response is substance-specific, this may result in a reduced tolerance to other stressors. This is exemplified by Siddique et al. (2021), who found that populations of *G. pulex* with higher neonicotinoid tolerance had a reduced thermal tolerance. Similar evidence comes from *H. azteca,* where pyrethroid resistant populations also showed reduced thermal tolerance (Heim et al., 2018). In addition, these populations were also more sensitive to, 4, 4′-dichlorodiphenyltrichloroethane (DDT), copper (II) sulfate, and sodium chloride. Furthermore, for the tolerant *H. azteca* populations, a reduced reproductive capacity was found, which was also observed in pesticide-adapted *G. pulex* (Heim et al., 2018; Siddique et al., 2024). However, a contrasting pattern was found in neonicotinoid-tolerant *G. roeselii* (MOTU C) in central Germany, where fecundity even increased, with a simultaneous reduction in embryo size, suggesting an adaptive shift in the reproduction strategy (Jourdan et al., 2024). It would therefore be crucial to test different populations of the same MOTU in a multiple-stressor experiment with e.g. temperature and a pesticide to account for possible trade-offs.

4.4. Biogeographical and conservation aspects

The *G. roeselii* species complex has MOTUs with very different distributions, ranging from localised MOTU L to widely distributed MOTU C. We would have expected to find a tendency toward greater tolerance in MOTU C given its widespread distribution throughout central Europe (Csapo et al., 2020). However, contrary to our expectations, MOTU C exhibited a trend of increased vulnerability. This unexpected outcome may be attributed, at least in part, to the comparatively pristine nature of the sampling sites from which it was sampled. It should be mentioned that our choice of sampling sites of MOTU C is a non-representative sample of the huge distribution range of MOTU C. It can be assumed that we will also find a wide range of tolerances within MOTU C. In a prior experiment, we observed higher tolerance levels in MOTU C at polluted sampling sites (thiacloprid EC_{50}: 337 μg/L; tested at 10°C; Jourdan et al., 2024). This leads us to believe that there are limited MOTU-specific differences in vulnerability, at least for the substance thiacloprid. However, this does not imply the absence of MOTU-specific differences in a broader sense. Adaptation to different ambient temperatures – as is likely within geographically widespread species complexes – may be another factor influencing pollutant tolerance. It remains essential to continue testing tolerances to various environmental stressors to discern distinct vulnerabilities and to protect the evolutionary history of this species complex with all its diversity. Deep phylogenetic splits in the *G. roeselii* complex – with a first phylogenetic separation into a northern and southern group occurring before 18 Mya (Grabowski et al., 2017) – have also left genomic tracks, even if these are neither reflected in their tolerance to thiacloprid nor manifested in the morphology in general. With isolated genetic populations becoming more and more adapted to their specific environments, cryptic species are the precursor to the new, morphologically and functionally distinct species. Given the ongoing biodiversity crisis where countless species are at risk of extinction, our study demonstrates that current anthropogenic activities are having an impact even at a population level in non-target species whose species status remains to be determined and/or discussed.

5. Conclusion

Our results indicate an increased tolerance of non-target amphipod crustaceans against the neonicotinoid thiacloprid due to recent similar chemical pollution. This was reflected in the fact that a high level of tolerance occurred particularly in heavily contaminated habitats where, we assume, pre-exposure to thiacloprid or a substance with a similar mode-of-action. The explanatory efficacy of the genetic signature, represented in this study by distinct genetic MOTU lineages, was relatively lower than the effects on the population-level, and it remains to be investigated whether the found differences are also associated with changes at a genomic level. The transferability of these findings to other substances requires further investigation. In contrast to stressors with longer evolutionary histories within individual lineages, such as climatic variables, it is more plausible to anticipate a distinct vulnerability. Considering that a default safety factor of 10 is typically applied in environmental risk assessment, the differentiation of populations by a factor of 4 which we observed is within the scope of the assumed ecological variability. Nevertheless, the (possibly rapid) population-specific differentiation shows how important it is to continuously monitor adaptive processes to detect variability within species and ongoing differentiation processes. This will be crucial for a better understanding of responses to pesticides not only in species complexes but across biodiversity in the face of a dynamically changing global environment.

CRediT authorship contribution statement

Jana Kabus: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Vanessa Hartmann:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Berardino Cocchiararo:** Writing – review & editing, Resources, Methodology. **Andrea Dombrowski:** Investigation, Formal analysis, Data curation. **Daniel Enns:** Writing – review & editing, Investigation. **Ioannis Karaouzas:** Writing – review & editing, Resources. **Konrad Lipkowski:** Writing – review & editing, Investigation. **Lars Pelikan:** Writing – review & editing, Investigation. **Spase Shumka:** Writing – review & editing, Resources. **Laura Soose:** Writing – review & editing, Investigation. **Nathan J. Baker:** Writing – review & editing, Investigation.

Jonas Jourdan: Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix

Table A1
Sampling locations and environmental parameters found on each site. MOTUs are named after Grabowski et al. (2017). Sites are numbered according to Kabus et al. (2023).

site	MOTU	Location North [dec°]	Location East [dec°]	pH	Conductivity [µS/cm]	O_2 concentration [mg/L]	Water temperature [°C]	Flow velocity [m/s]	Nitrate [mg/L]	Nitrite [mg/L]	Phosphate [mg/L]
18	**G**	40.52544	20.70266	8.46	361.0	8.43	19.50	0.23	0.00	0.02	0.10
19	**G**	40.70808	20.87131	8.50	564.0	9.45	20.90	0.06	0.00	0.00	0.21
21	**G**	40.74545	21.11491	8.25	285.0	7.68	24.00	0.00	0.00	0.01	0.08
24	**A**	40.84016	21.58442	8.60	249.0	8.33	17.15	0.10	0.00	0.02	0.10
27	**A**	40.51528	21.25527	8.23	437.0	7.52	21.30	0.14	3.00	0.02	0.08
28	**A**	40.43537	21.27085	8.43	371.0	8.74	20.60	0.07	12.00	0.12	0.14
30	**L**	39.53077	21.70288	8.29	456.5	8.50	23.80	0.02	5.00	0.09	0.25
31	**L**	39.54235	21.61321	8.67	341.5	8.97	19.40	0.05	0.00	0.01	0.16
33	**L**	39.53334	21.92595	7.75	605.5	1.13	23.40	0.02	27.00	1.31	4.60
48	**C**	50.21297	9.10178	7.88	263.0	8.41	13.40	0.31	15.00	0.05	0.20
49	**C**	50.41665	9.36343	8.10	190.1	9.62	11.70	0.03	8.00	0.02	0.24
			mean	8.29	374.87	7.89	19.56	0.09	6.36	0.15	0.56
			min	7.75	190.10	1.13	11.70	0.00	0.00	0.00	0.08
			max	8.67	605.50	9.62	24.00	0.31	27.00	1.31	4.60
			SD	0.28	124.82	2.22	3.86	0.09	8.26	0.37	1.28

Table A2
Raw chemical and climatic parameters present at each sampling site with the climatic data obtained from Domisch et al. (2015). The corresponding units are displayed in brackets.

site	MOTU	YES [ng/g]	YDS [mg/g]	YAAS	YAES	Microtox assay EC_{50}-100 [mg sediment equivalency]	Flow length [count of grid cells]	Annual precipitation "Bioclim12", [mm]	Annual mean temperature "Bioclim1", [°C]	Cultivated land cover "lc_avg_07", [% cover]	Urban land cover "lc_avg_09", [% cover]
18	**G**	0.74	0.50	245.20	82.80	96.44	27	1818	10.1	56	0
19	**G**	0.00	0.29	0.00	0.00	94.55	2722	1638	9.9	63	1
21	**G**	0.00	14.00	168.80	0.00	77.60	1383	1558	9.9	41	0
24	**A**	2.90	0.40	141.50	0.00	95.88	225	1278	9.4	60	2
27	**A**	0.00	1.57	228.60	26.80	90.96	248	1392	11.0	48	2
28	**A**	1.81	1.01	155.00	28.70	99.04	1851	1552	10.2	66	2
30	**L**	0.00	0.69	0.00	0.00	71.20	459	1570	14.9	70	4
31	**L**	3.95	0.55	0.00	0.00	0.00	56	1602	15.6	63	0
33	**L**	0.00	0.79	243.60	32.10	96.72	3310	1504	14.3	64	3
48	**C**	0.00	1.61	0.00	0.00	98.56	14	1352	9.3	0	0
49	**C**	2.43	23.80	267.60	0.00	97.44	4	1540	8.1	0	0
	mean	1.08	4.11	131.85	15.49	83.49	936	1528	11.2	48	1
	min	0.00	0.29	0.00	0.00	0.00	4	1278	8.1	0	0
	max	3.95	23.80	267.60	82.80	99.04	3310	1818	15.6	70	4
	SD	1.38	7.29	106.45	24.84	27.79	1143.70	140.65	2.43	24.08	1.35

Table A3**IDs for Individuals used in the genetic evaluation**. The corresponding Primers used and BOLD IDs are listed below.

Site	ID	Primer Forward	Primer Reverse	Bold ID	Site	ID	Primer Forward	Primer Reverse	Bold ID
18	Cr18-01-22	COIGrF	COIGrR2	GROAT001-24	27	Cr27-12-22	UCOIF	UCOIR	GROAT069-24
18	Cr18-02-22	COIGrF	COIGrR2	GROAT002-24	27	Cr27-13-22	UCOIF	UCOIR	GROAT070-24
18	Cr18-03-22	COIGrF	COIGrR2	GROAT003-24	27	Cr27-14-22	UCOIF	UCOIR	GROAT071-24
18	Cr18-04-22	COIGrF	COIGrR2	GROAT004-24	27	Cr27-15-22	UCOIF	UCOIR	GROAT072-24
18	Cr18-05-22	COIGrF	COIGrR2	GROAT005-24	27	Cr27-16-22	UCOIF	UCOIR	GROAT073-24
18	Cr18-06-22	COIGrF	COIGrR2	GROAT006-24	27	Cr27-17-22	UCOIF	UCOIR	GROAT074-24
18	Cr18-07-22	COIGrF	COIGrR2	GROAT007-24	27	Cr27-19-22	UCOIF	UCOIR	GROAT075-24
18	Cr18-08-22	COIGrF	COIGrR2	GROAT008-24	28	Cr28-01-22	UCOIF	UCOIR	GROAT076-24
18	Cr18-09-22	COIGrF	COIGrR2	GROAT009-24	28	Cr28-02-22	UCOIF	UCOIR	GROAT077-24
18	Cr18-10-22	COIGrF	COIGrR2	GROAT010-24	28	Cr28-03-22	UCOIF	UCOIR	GROAT078-24
18	Cr18-11-22	COIGrF	COIGrR2	GROAT011-24	28	Cr28-06-22	UCOIF	UCOIR	GROAT079-24
18	Cr18-12-22	COIGrF	COIGrR2	GROAT012-24	28	Cr28-07-22	UCOIF	UCOIR	GROAT080-24
18	Cr18-13-22	COIGrF	COIGrR2	GROAT013-24	28	Cr28-08-22	UCOIF	UCOIR	GROAT081-24
18	Cr18-14-22	COIGrF	COIGrR2	GROAT014-24	28	Cr28-09-22	UCOIF	UCOIR	GROAT082-24
18	Cr18-15-22	COIGrF	COIGrR2	GROAT015-24	28	Cr28-10-22	UCOIF	UCOIR	GROAT083-24
19	Cr19-01-22	COIGrF	COIGrR2	GROAT016-24	28	Cr28-11-22	UCOIF	UCOIR	GROAT084-24
19	Cr19-02-22	UCOIF	UCOIR	GROAT017-24	28	Cr28-12-22	UCOIF	UCOIR	GROAT085-24
19	Cr19-03-22	UCOIF	UCOIR	GROAT018-24	28	Cr28-13-22	UCOIF	UCOIR	GROAT086-24
19	Cr19-04-22	COIGrF	COIGrR2	GROAT019-24	28	Cr28-14-22	UCOIF	UCOIR	GROAT087-24
19	Cr19-05-22	COIGrF	COIGrR2	GROAT020-24	28	Cr28-16-22	UCOIF	UCOIR	GROAT088-24
19	Cr19-06-22	COIGrF	COIGrR2	GROAT021-24	28	Cr28-18-22	UCOIF	UCOIR	GROAT089-24
19	Cr19-07-22	COIGrF	COIGrR2	GROAT022-24	28	Cr28-19-22	UCOIF	UCOIR	GROAT090-24
19	Cr19-08-22	UCOIF	UCOIR	GROAT023-24	30	Cr30-01-22	LCO1490	HCO2198	GROAT091-24
19	Cr19-09-22	COIGrF	COIGrR2	GROAT024-24	30	Cr30-02-22	COIGrF	COIGrR2	GROAT092-24
19	Cr19-10-22	COIGrF	COIGrR2	GROAT025-24	30	Cr30-09-22	LCO1490	HCO2198	GROAT093-24
19	Cr19-11-22	COIGrF	COIGrR2	GROAT026-24	30	Cr30-10-22	LCO1490	HCO2198	GROAT094-24
19	Cr19-12-22	UCOIF	UCOIR	GROAT027-24	30	Cr30-11-22	LCO1490	HCO2198	GROAT095-24
19	Cr19-13-22	UCOIF	UCOIR	GROAT028-24	30	Cr30-12-22	LCO1490	HCO2198	GROAT096-24
19	Cr19-14-22	UCOIF	UCOIR	GROAT029-24	30	Cr30-14-22	LCO1490	HCO2198	GROAT097-24
19	Cr19-20-22	COIGrF	COIGrR2	GROAT030-24	30	Cr30-15-22	LCO1490	HCO2198	GROAT098-24
21	Cr21-01-22	COIGrF	COIGrR2	GROAT031-24	30	Cr30-16-22	COIGrF	COIGrR2	GROAT099-24
21	Cr21-02-22	COIGrF	COIGrR2	GROAT032-24	30	Cr30-17-22	LCO1490	HCO2198	GROAT100-24
21	Cr21-03-22	COIGrF	COIGrR2	GROAT033-24	30	Cr30-18-22	COIGrF	COIGrR2	GROAT101-24
21	Cr21-04-22	COIGrF	COIGrR2	GROAT034-24	30	Cr30-21-22	LCO1490	HCO2198	GROAT102-24
21	Cr21-05-22	COIGrF	COIGrR2	GROAT035-24	30	Cr30-22-22	LCO1490	HCO2198	GROAT103-24
21	Cr21-06-22	COIGrF	COIGrR2	GROAT036-24	30	Cr30-23-22	LCO1490	HCO2198	GROAT104-24
21	Cr21-07-22	COIGrF	COIGrR2	GROAT037-24	30	Cr30-24-22	LCO1490	HCO2198	GROAT105-24
21	Cr21-08-22	COIGrF	COIGrR2	GROAT038-24	31	Cr31-02-22	LCO1490	HCO2198	GROAT106-24
21	Cr21-09-22	COIGrF	COIGrR2	GROAT039-24	31	Cr31-03-22	LCO1490	HCO2198	GROAT107-24
21	Cr21-10-22	COIGrF	COIGrR2	GROAT040-24	31	Cr31-04-22	LCO1490	HCO2198	GROAT108-24
21	Cr21-11-22	COIGrF	COIGrR2	GROAT041-24	31	Cr31-05-22	LCO1490	HCO2198	GROAT109-24
21	Cr21-13-22	COIGrF	COIGrR2	GROAT042-24	31	Cr31-06-22	LCO1490	HCO2198	GROAT110-24
21	Cr21-14-22	COIGrF	COIGrR2	GROAT043-24	31	Cr31-07-22	LCO1490	HCO2198	GROAT111-24
21	Cr21-16-22	COIGrF	COIGrR2	GROAT044-24	31	Cr31-08-22	LCO1490	HCO2198	GROAT112-24
21	Cr21-17-22	COIGrF	COIGrR2	GROAT045-24	31	Cr31-12-22	LCO1490	HCO2198	GROAT113-24
24	Cr24-01-22	UCOIF	UCOIR	GROAT046-24	31	Cr31-14-22	LCO1490	HCO2198	GROAT114-24
24	Cr24-02-22	UCOIF	UCOIR	GROAT047-24	31	Cr31-15-22	LCO1490	HCO2198	GROAT115-24
24	Cr24-04-22	UCOIF	UCOIR	GROAT048-24	31	Cr31-16-22	LCO1490	HCO2198	GROAT116-24
24	Cr24-05-22	UCOIF	UCOIR	GROAT049-24	31	Cr31-17-22	LCO1490	HCO2198	GROAT117-24
24	Cr24-06-22	UCOIF	UCOIR	GROAT050-24	31	Cr31-18-22	LCO1490	HCO2198	GROAT118-24
24	Cr24-08-22	UCOIF	UCOIR	GROAT051-24	31	Cr31-19-22	LCO1490	HCO2198	GROAT119-24
24	Cr24-09-22	UCOIF	UCOIR	GROAT052-24	31	Cr31-20-22	LCO1490	HCO2198	GROAT120-24
24	Cr24-11-22	UCOIF	UCOIR	GROAT053-24	33	Cr33-01-22	LCO1490	HCO2198	GROAT121-24
24	Cr24-13-22	UCOIF	UCOIR	GROAT054-24	33	Cr33-03-22	LCO1490	HCO2198	GROAT122-24
24	Cr24-14-22	UCOIF	UCOIR	GROAT055-24	33	Cr33-04-22	LCO1490	HCO2198	GROAT123-24
24	Cr24-15-22	UCOIF	UCOIR	GROAT056-24	33	Cr33-05-22	LCO1490	HCO2198	GROAT124-24
24	Cr24-17-22	UCOIF	UCOIR	GROAT057-24	33	Cr33-06-22	LCO1490	HCO2198	GROAT125-24
24	Cr24-18-22	UCOIF	UCOIR	GROAT058-24	33	Cr33-07-22	LCO1490	HCO2198	GROAT126-24
24	Cr24-19-22	UCOIF	UCOIR	GROAT059-24	33	Cr33-08-22	LCO1490	HCO2198	GROAT127-24
24	Cr24-20-22	UCOIF	UCOIR	GROAT060-24	33	Cr33-09-22	LCO1490	HCO2198	GROAT128-24
27	Cr27-03-22	UCOIF	UCOIR	GROAT061-24	33	Cr33-10-22	LCO1490	HCO2198	GROAT129-24
27	Cr27-04-22	UCOIF	UCOIR	GROAT062-24	33	Cr33-11-22	LCO1490	HCO2198	GROAT130-24
27	Cr27-05-22	UCOIF	UCOIR	GROAT063-24	33	Cr33-12-22	LCO1490	HCO2198	GROAT131-24
27	Cr27-06-22	UCOIF	UCOIR	GROAT064-24	33	Cr33-14-22	LCO1490	HCO2198	GROAT132-24
27	Cr27-07-22	UCOIF	UCOIR	GROAT065-24	33	Cr33-15-22	LCO1490	HCO2198	GROAT133-24
27	Cr27-08-22	UCOIF	UCOIR	GROAT066-24	33	Cr33-17-22	LCO1490	HCO2198	GROAT134-24
27	Cr27-09-22	UCOIF	UCOIR	GROAT067-24	33	Cr33-18-22	LCO1490	HCO2198	GROAT135-24
27	Cr27-11-22	UCOIF	UCOIR	GROAT068-24	48	Cr48-01-22	COIGrF	COIGrR2	GROAT136-24

Table A3
continued IDs for Individuals used in the genetic evaluation. The corresponding Primers used and BOLD IDs are listed below.

Site	ID	Primer Forward	Primer Reverse	Bold ID
48	Cr48-02-22	COIGrF	COIGrR2	GROAT137-24
48	Cr48-03-22	COIGrF	COIGrR2	GROAT138-24
48	Cr48-05-22	COIGrF	COIGrR2	GROAT139-24
48	Cr48-06-22	COIGrF	COIGrR2	GROAT140-24
48	Cr48-07-22	COIGrF	COIGrR2	GROAT141-24
48	Cr48-08-22	COIGrF	COIGrR2	GROAT142-24
48	Cr48-09-22	COIGrF	COIGrR2	GROAT143-24
48	Cr48-10-22	COIGrF	COIGrR2	GROAT144-24
48	Cr48-11-22	COIGrF	COIGrR2	GROAT145-24
48	Cr48-14-22	COIGrF	COIGrR2	GROAT146-24
48	Cr48-15-22	COIGrF	COIGrR2	GROAT147-24
48	Cr48-16-22	COIGrF	COIGrR2	GROAT148-24
48	Cr48-17-22	COIGrF	COIGrR2	GROAT149-24
48	Cr48-18-22	COIGrF	COIGrR2	GROAT150-24
49	Cr49-02-22	COIGrF	COIGrR2	GROAT151-24
49	Cr49-03-22	COIGrF	COIGrR2	GROAT152-24
49	Cr49-06-22	COIGrF	COIGrR2	GROAT153-24
49	Cr49-07-22	COIGrF	COIGrR2	GROAT154-24
49	Cr49-08-22	COIGrF	COIGrR2	GROAT155-24
49	Cr49-10-22	COIGrF	COIGrR2	GROAT156-24
49	Cr49-11-22	COIGrF	COIGrR2	GROAT157-24
49	Cr49-12-22	COIGrF	COIGrR2	GROAT158-24
49	Cr49-13-22	COIGrF	COIGrR2	GROAT159-24
49	Cr49-14-22	COIGrF	COIGrR2	GROAT160-24
49	Cr49-15-22	COIGrF	COIGrR2	GROAT161-24
49	Cr49-16-22	COIGrF	COIGrR2	GROAT162-24
49	Cr49-18-22	COIGrF	COIGrR2	GROAT163-24
49	Cr49-19-22	COIGrF	COIGrR2	GROAT164-24
49	Cr49-20-22	COIGrF	COIGrR2	GROAT165-24

Table A4
Genetic diversity measures for the Cytochrome *c* Oxidase Subunit I (COI) gene based on 15 individuals for each sampling site. Number of segregating sites, haplotype diversity and nucleotide diversity are used in the further statistical analysis.

Sampling site	MOTU	Number of segregating sites	Haploytpe diversity (Hd)	Nucleotide diversity (Pi)	Amount of haplotypes
18	G	2	0.514	0.001	3
19	G	7	0.905	0.004	9
21	G	7	0.867	0.003	8
24	A	29	1.000	0.017	15
27	A	29	1.000	0.025	15
28	A	30	1.000	0.025	15
30	L	7	0.838	0.003	7
31	L	15	0.838	0.007	6
33	L	13	0.952	0.005	11
48	C	13	0.838	0.004	8
49	C	13	0.924	0.006	10

Table A5
EC_{50} [µg/L] values of each MOTU and corresponding time slot divided in 24h, 48h, 72h and 96h. The Standard errors are displayed in brackets.

	24 h	48 h	72 h	96 h
	EC_{50} (± S.E.)	EC_{50} (± S.E.)	EC_{50} (± S.E.)	EC_{50} (± S.E.)
A	380.1 (±12.8)	346.1 (±12.1)	341.9 (±13.2)	333.6 (±12.7)
G	360.4 (±11.1)	346.4 (±12.8)	300.5 (±12.8)	265.5 (±12.2)
L	474,4 (±27.5)	471.7 (±23.7)	439.6 (±22.3)	375.9 (±20.9)
C	262.0 (±14.5)	235.8 (±11.0)	226.7 (±10.3)	190.0 (±9.9)

Table A6
EC_{50} [µg/L] values of each population/sampling site and corresponding time slot divided in 24h, 48h, 72h and 96h. The Standard errors are displayed in brackets.

Sampling site	24 h EC_{50} (± S.E.)	48 h EC_{50} (± S.E.)	72 h EC_{50} (± S.E.)	96 h EC_{50} (± S.E.)
18	341.5 (±19.0)	315.6 (±22.3)	251.5 (±11.5)	221.1 (±13.7)
19	385.3 (±18.5)	370.3 (±22.4)	323.8 (±27.1)	301.0 (±25.0)
21	355.7 (±19.9)	355.3 (±21.1)	339.6 (±22.3)	293.3 (±22.7)
24	329.0 (±21.2)	301.5 (±20.7)	301.3 (±22.9)	271.8 (±23.3)
27	362.7 (±18.5)	349.1 (±16.1)	336.9 (±18.9)	337.0 (±21.3)
28	453.2 (±20.2)	385.3 (±20.7)	385.3 (±24.6)	370.3 (±24.0)
30	305.8 (±15.0)	338.1 (±20.5)	316.6 (±16.1)	291.5 (±22.3)
31	401.5 (±20.9)	385.3 (±20.7)	368.6 (±18.2)	307.2 (±39.5)
33	704.0 (±660.5)	691.4 (±148.9)	696.0 (±65.0)	620.4 (±69.5)
48	310.0 (±22.1)	284.3 (±15.1)	259.1 (±11.2)	232.7 (±14.2)
49	233.3 (±18.7)	198.1 (±13.6)	198.1 (±13.6)	153.0 (±11.5)

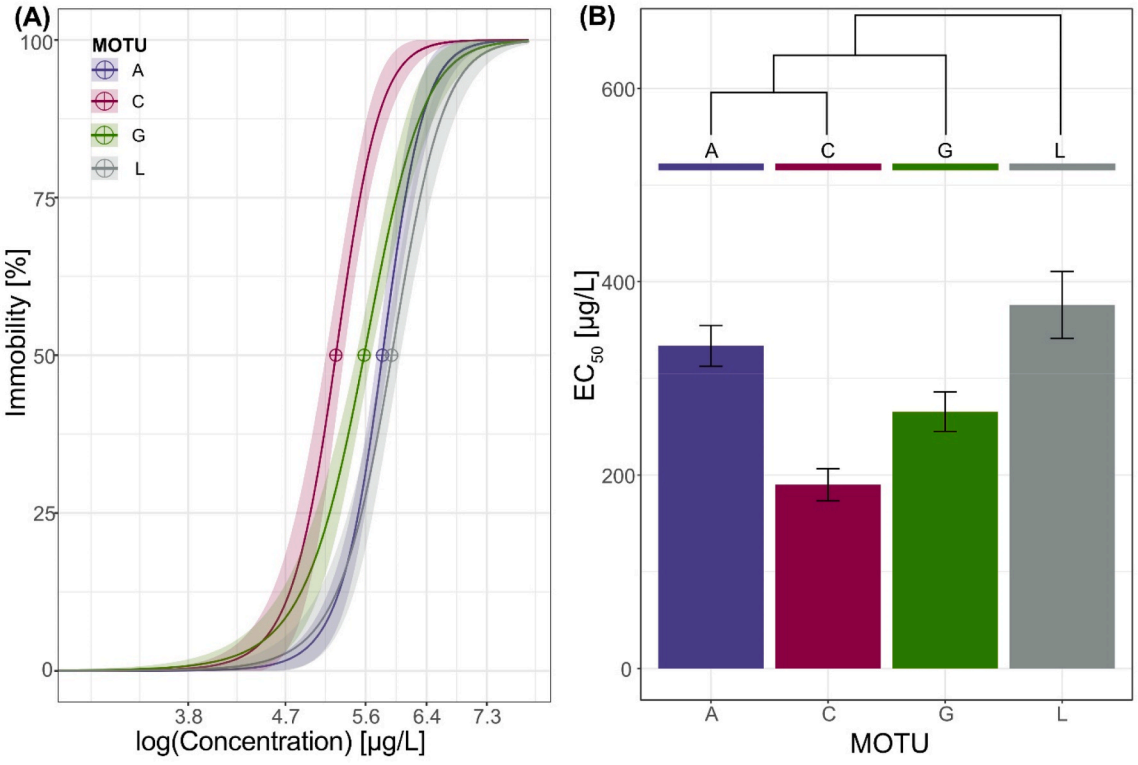

Fig. A1. MOTU-specific EC50 values after exposure to thiacloprid for 96 h. (A) Concentration-response curves with 95 % confidence interval for each MOTU after 96 h exposure and EC_{50} values indicated by the circled cross. (B) EC_{50} with 95 % interval of each MOTU. All MOTUs have significantly different EC_{50} values from each other except for MOTU L to A. See table XY for values. On the top is a simplified phylogenetic tree of the tested MOTUs.

Table A7
Test of significance for the EC50 [µg/L] of all MOTUs after 96 h exposure displayed in Figure A1B. The EC_{50} values are displayed in the grey square with their corresponding Standard error in brackets. The EC_{50} values of all MOTUs are significantly different except for MOTU L to A. p-values:. ≤ 0.1, * ≤0.05, ** ≤0.01, *** ≤0.001.

	MOTU	G	A	L	C
96h	**G**	265.5 (± 12.2)			
	A	***	333.6 (± 12.7)		
	L	***	.	375.9 (± 20.9)	
	C	***	***	***	190.0 (± 9.9)

Table A8
Test of significance for the EC_{50} values between every population for the time slots 24 h and 48 h. The significance was evaluated using the 'compParm' function in the "drc" package in R. The significance levels are: >0.1 = ns (not significant), <0.1 = ., <0.05 = *, <0.01 = ** and <0.001 = ***.

	MOTU	site	24h										
			18	19	21	24	27	28	30	31	33	48	49
48h	G	18		ns	ns	ns	ns	***	ns	*	ns	ns	***
	G	19	*		ns	.	ns	*	**	ns		*	***
	G	21	ns	ns		ns	ns	***	.	ns	ns	ns	***
	A	24	ns	*	.		ns	***	ns	*	ns	ns	***
	A	27	ns	ns	ns	ns		*	.	ns	ns	ns	***
	A	28	**	ns	ns	***	ns		***	ns	ns	***	***
	L	30	ns	ns	ns	ns	ns	ns		***	***	ns	**
	L	31	**	ns	ns	**	ns	ns	ns		ns	**	***
	L	33	.	ns	ns	ns	ns	ns	ns	ns		ns	ns
	C	48	ns	**	**	ns	*	***	.	***	ns		**
	C	49	***	***	***	***	***	***	***	***	ns	***	

Table A9
Test of significance for the EC_{50} values between every population for the time slots 72 h and 96 h. The significance levels are: >0.1 = ns (not significant), <0.1 = ., <0.05 = *, <0.01 = ** and <0.001 = ***.

	MOTU	site	72h										
			18	19	21	24	27	28	30	31	33	48	49
96h	G	18		***	***	*	***	***	*	*	***	ns	**
	G	19	***		ns	ns	ns	*	ns	ns	***	*	***
	G	21	***	ns		ns	ns	*	ns	ns	***	*	***
	A	24	*	ns	ns		*	***	ns	ns	***	.	***
	A	27	*	ns	ns	*		ns	ns	ns	***	*	***
	A	28	***	***	*	***	ns		*	ns	***	***	***
	L	30	*	ns	ns	ns	ns	*		.	***	*	***
	L	31	***	ns	ns	ns	ns	ns	ns		***	***	***
	L	33	***	*	***	***	***	***	***	***		***	***
	C	48	ns	*	*	ns	**	***	ns	ns	***		**
	C	49	**	***	***	***	***	***	**	**	***	**	

Table A10
Test of significance of the EC_{50} values between every MOTU for all time slots. The significance levels are: >0.1 = ns (not significant), <0.1 = ., <0.05 = *, <0.01 = ** and <0.001 = ***.

MOTU	24 h		48 h		72 h		96 h	
	p-value	sig. Level	p-value	sig. Level	p-value	sig. Level	p-value	sig. Level
G/L	<0.001	***	<0.001	***	<0.001	***	<0.001	***
G/A	0.37	ns	0.99	ns	<0.05	*	<0.001	***
G/C	<0.001	***	<0.001	***	<0.001	***	<0.001	***
L/A	<0.001	**	<0.001	***	<0.001	***	0.08	.
L/C	<0.001	***	<0.001	***	<0.001	***	<0.001	***
A/C	<0.001	***	<0.001	***	<0.001	***	<0.001	***

Table A11
Loadings of each environmental variable to the PCA. The higher the value the higher the influence of that variable is on the distribution of the data within the PCA. The three strongest influences are highlighted in grey. For PC1 the highest contributors are nitrite, oxygen saturation and phosphate. For PC2 the strongest contributors are pH, annual precipitation and cultivated land cover.

	PC1	PC2	PC3	PC4
pH	0.4236	0.7714	0.2227	-0.2182
mean conductivity	-0.8481	0.2774	0.1248	0.1181
O2 saturation	0.8767	0.3561	0.0140	-0.0040
mean water Temp.	-0.6756	0.5337	0.1502	-0.1547
mean flow verlocity	0.3410	-0.2732	0.2806	0.8422
nitrate	-0.6353	-0.6305	-0.2003	0.1631
nitrite	-0.8896	-0.3747	-0.0515	-0.0543
phosphate	-0.8734	-0.3781	-0.0674	-0.0609
estrogenic activity	0.4601	-0.2771	0.0814	-0.4945
dioxin-like activity	0.4356	-0.4735	-0.1279	-0.5918
anti-androgenic activity	-0.1093	-0.5808	0.5475	-0.4356
anti-estrogenic activity	-0.2307	-0.0884	0.9110	0.1923
microtox	-0.0636	-0.6650	0.3623	0.0252
flow length	-0.7584	-0.0450	0.0323	-0.2331
annual precipitation	-0.0755	0.3704	0.6149	-0.1278
cultivated land cover	-0.5456	0.7094	0.2376	-0.1712
urban land cover	-0.6497	0.1496	-0.1698	-0.0947
annual temperature	-0.6103	0.5383	-0.2862	0.0837
explained variance [%]	35.23	21.57	11.36	9.81
cumulative variance [%]	35.23	56.80	68.15	77.96

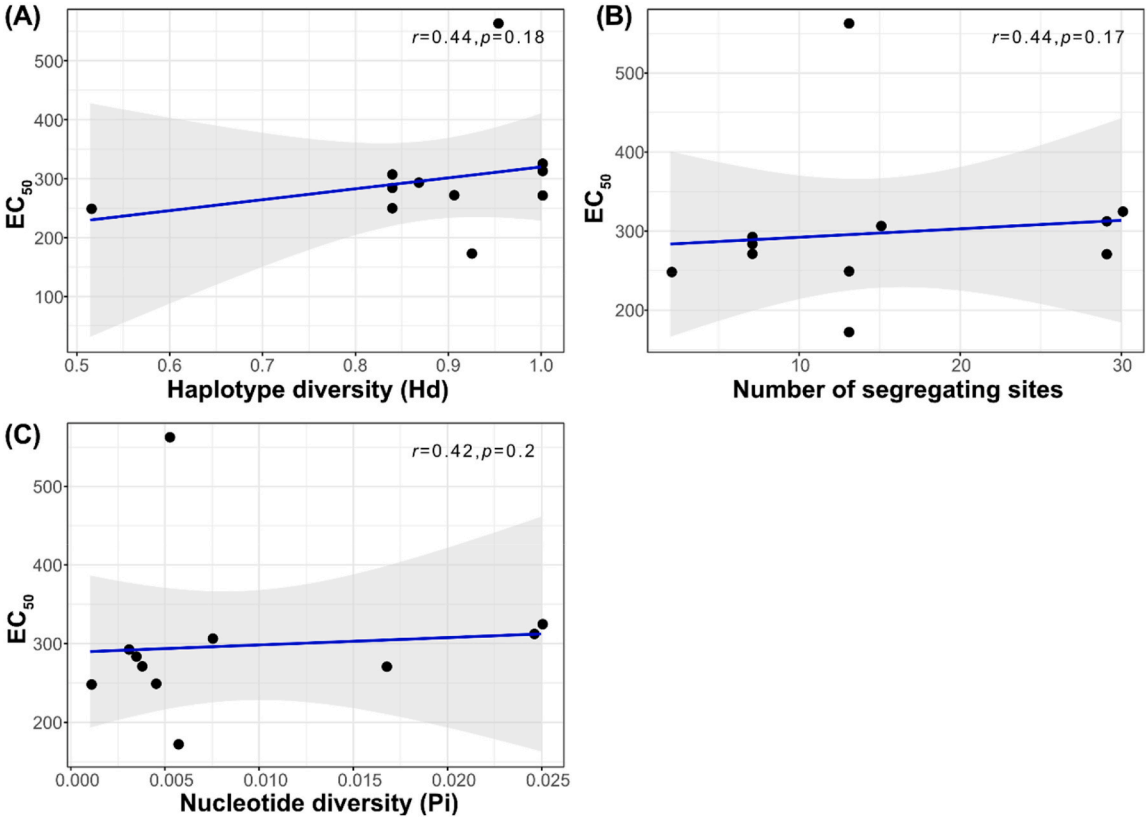

Fig. A2. Spearman correlation of EC_{50} values to parameters indicating genetic diversity. No correlation is found between these parameters. **(A)** Correlation of EC_{50} values and the haplotype diversity for each population. **(B)** Correlation of EC_{50} values and number of segregating sites for each population. **(C)** Correlation of EC_{50} values and nucleotide diversity for each population.

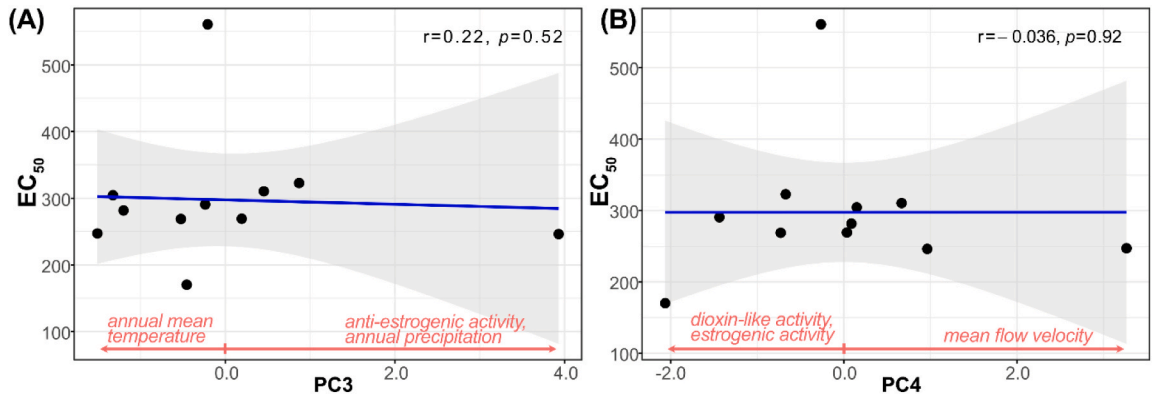

Fig. A3. Spearman correlation of PC3 and PC4 to EC_{50} value. No significant correlation is found between these parameters as also indicated by the Broken-Stick method. **(A)** Correlation of EC_{50} values and PC3 for each population. **(B)** Correlation of EC_{50} values and PC4.

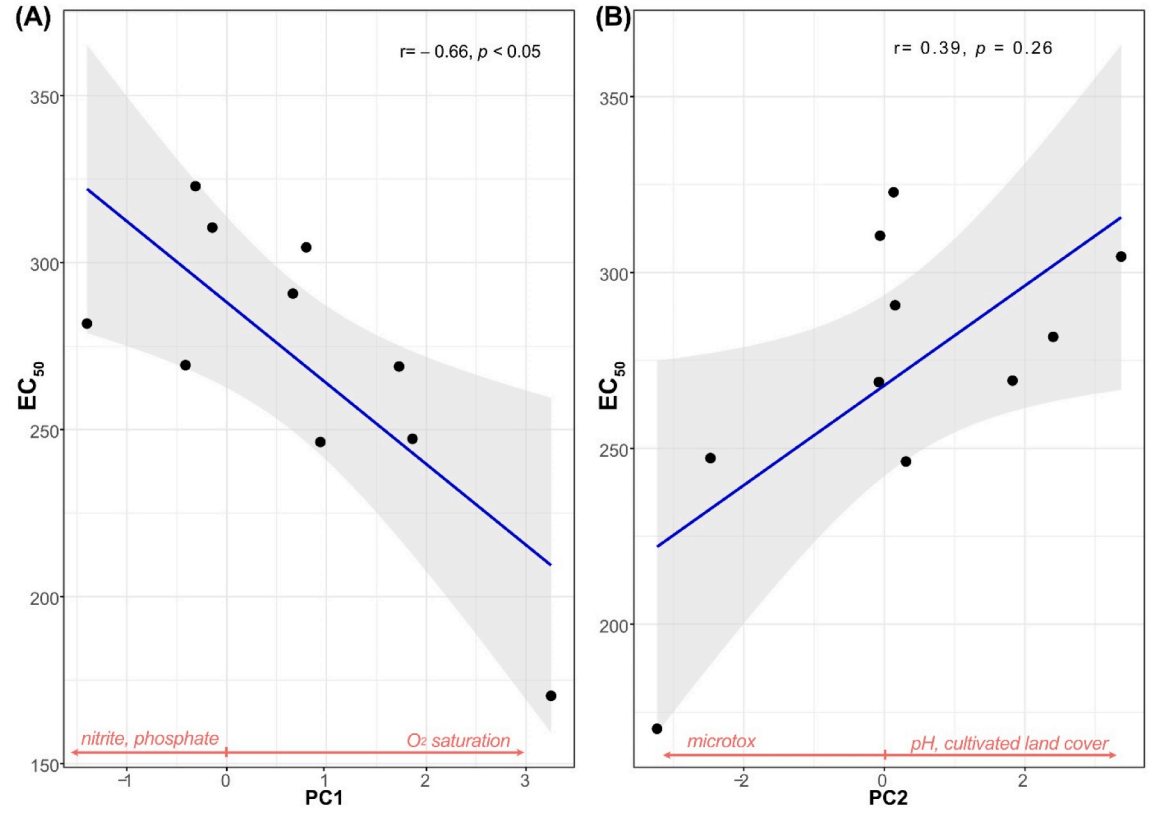

Fig. A4. Correlation of the principal components to the EC_{50} values and PC1 and PC2 without population 33. **(A)** Spearman correlation of EC_{50} values to PC1 with a highly significant negative correlation. The more negative the PC1 value is, the higher the amount of nitrate and phosphate and the higher the PC1 value, the higher the oxygen saturation. **(B)** Spearman correlation of PC2 with EC_{50} values with no significant correlation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.124888.

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