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Macroinvertebrate communities respond strongly but non-specifically to a toxicity gradient derived by effect-based methods[☆]

 Sebastian Heß^{a,b,*}, Delia Hof^c, Matthias Oetken^{c,d}, Andrea Sundermann^{a,b,d}
^a Senckenberg Research Institute and Natural History Museum Frankfurt, Department of River Ecology and Conservation, Clamecystr. 12, 63571, Gelnhausen, Germany

^b Goethe University Frankfurt, Faculty Biological Sciences, Institute of Ecology, Evolution and Diversity, Max-von-Laue-Str. 13, 60438, Frankfurt am Main, Germany

^c Goethe University Frankfurt, Faculty Biological Sciences, Department Aquatic Ecotoxicology, Max-von-Laue-Str. 13, 60438, Frankfurt am Main, Germany

^d Kompetenzzentrum Wasser Hessen, Max-von-Laue-Straße 13, 60438, Frankfurt am Main, Germany

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ABSTRACT

Chemical pollution is one of the most important threats to freshwater ecosystems. The plethora of potentially occurring chemicals and their effects in complex mixtures challenge standard monitoring methods. Effect-based methods (EBMs) are proposed as complementary tools for the assessment of chemical pollution and toxic effects. To investigate the effects of chemical pollution, the ecological relevance of EBMs and the potential of macroinvertebrates as toxicity-specific bioindicators, ecological and ecotoxicological data were linked. Baseline toxicity, mutagenicity, dioxin-like and estrogenic activity of water and sediment samples from 30 river sites in central Germany were quantified with four *in vitro* bioassays. The responses of macroinvertebrate communities at these sites were assessed by calculating 16 taxonomic and functional metrics and by investigating changes in the taxonomic and trait composition. Principal component analysis revealed an increase in toxicity along a joint gradient of chemicals with different modes of action. This toxicity gradient was associated with a decrease in biodiversity and ecological quality, as well as significant changes in taxonomic and functional composition. The strength of the effects suggested a strong impact of chemical pollution and underlined the suitability of EBMs in detecting ecologically relevant effects. However, the metrics, taxa, and traits associated with vulnerability or tolerance to toxicity were found to also respond to other stressors in previous studies and thus may have only a low potential as toxicity-specific bioindicators. Because macroinvertebrates respond integratively to all present stressors, linking both ecological and environmental monitoring is necessary to investigate the overall effects but also isolate individual stressors. EBMs have a high potential to separate the toxicity of chemical mixtures from other stressors in a multiple stressor scenario, as well as identifying the presence of chemical groups with specific modes of action.

1. Introduction

Europe's freshwater biodiversity recovered slightly by the late 2000s, but persistent and new pressures such as climate change, invasive species and chemical pollution seem to prevent further improvement (Haase et al., 2023). Micropollutants and other emerging contaminants occurring at very low concentrations, such as pesticides, pharmaceuticals, industrial chemicals, personal care and household products, may pose an underestimated risk to freshwater ecosystems (Malaj et al., 2014; Schwarzenbach et al., 2006). Because of the large number of chemicals on the market and their potential transformation products,

surface waters are typically exposed to complex mixtures of different substances (Peng et al., 2018; Schwarzenbach et al., 2006). These complex chemical mixtures and their effects challenge the current chemical monitoring practices and risk assessment (Dévier et al., 2011). In the European Water Framework Directive (WFD), a selection of chemicals, such as the priority substances listed in WFD, is quantified by target analysis and the environmental risk is estimated based on the exceedance of environmental quality standards (EQS; European Commission, 2013; 2000). However, the overall ecological effects may be underestimated by using a limited selection of chemicals and neglecting mixture effects (Altenburger et al., 2018; Moschet et al., 2014; Schäfer

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^{*} Corresponding author. Senckenberg Research Institute and Natural History Museum Frankfurt, Department of River Ecology and Conservation, Clamecystr. 12, 63571, Gelnhausen, Germany.

 E-mail address: sebastian.hess@senckenberg.de (S. Heß).

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et al., 2011).

Effect-based methods (EBMs), such as biomarkers, *in vivo* and *in vitro* bioassays, have been proposed as complementary monitoring tools to assess the presence and ecological effects of chemical pollution (Altenburger et al., 2019; Wernersson et al., 2015). In particular, *in vitro* bioassays that address specific modes of action (MoAs) can indicate the presence of certain groups of chemicals such as endocrine disruptors (Neale et al., 2017). EBMs have the potential to detect and quantify toxicity in water and sediment samples (Brettschneider et al., 2019; de Baat et al., 2019; De Castro-Català et al., 2016; König et al., 2017). However, EBMs have rarely been linked to ecological assessment (Moran et al., 2017; Novais et al., 2023; Palma et al., 2016). Therefore, the ecological effects and the ecological relevance of toxicities identified by EBMs are largely unknown.

For the ecological assessment of freshwater systems, benthic macroinvertebrates are one of the most frequently monitored organism groups (Birk et al., 2012). As a result, several metrics have been developed to indicate ecological quality and effects of specific stressors, mostly based on taxonomic composition (Birk et al., 2012). Additionally, approaches using traits, i.e. measurable characteristics of organisms describing morphology, life history, behavior and environmental adaptations (McGill et al., 2006), have the potential to be spatially less influenced and allow a better mechanistic understanding of stress responses (Culp et al., 2011). Since indicators specific to chemical pollution in general or specific pollutants are largely underrepresented (Birk et al., 2012), there is an increasing attention to develop such bioindicators using taxonomic and functional approaches (Beketov and Liess, 2008; Berger et al., 2018; Collins and Fahrig, 2020). The development of toxicity-specific bioindicators could benefit from the indicative potential of EBMs.

In this study, we therefore linked ecological data with toxicities derived from EBMs to investigate changes in macroinvertebrate communities with increasing chemical pollution. Four different *in vitro* bioassays were used to quantify baseline toxicity, mutagenicity, dioxin-like and estrogenic activity of water and sediment samples from 30 river sites in central Germany. The response of the macroinvertebrate community was assessed using taxonomic and functional approaches. By combining these data, we aimed to investigate the macroinvertebrate community response to increasing toxicity, and to identify taxonomic groups, individual taxa and traits that are directly and indirectly affected by toxicity. By addressing these questions, we also provide insights in the potential of macroinvertebrate communities as toxicity-specific bioindicators and the ecological relevance of EBMs.

2. Material and methods

2.1. Study area

We investigated 30 river sites in the Rhine-Main-Metropolitan Region, Germany (Fig. 1). This region has a population of approximately 5.8 million and is characterized by a landscape that is typical of Central Europe. The lowlands are covered by dense urbanization, industry, and intensive agriculture, while the anthropogenic influence decreases towards the mountains and hills, where pastures and forests predominate. The sites were selected across this gradient to cover different types and intensities of anthropogenic influences, including specific sources of pollution such as wastewater treatment plant (WWTP) effluents and runoff from agriculture and streets. The sites (83.4–387 m above sea level) were located in rivers and streams belonging to four different river types (type 5 – small coarse substrate dominated siliceous highland rivers, type 6 – small fine substrate dominated calcareous highland rivers, type 9 – mid-sized fine to coarse substrate dominated siliceous highland rivers, type 19 – small streams in riverine floodplains; Pottgiesser and Sommerhäuser, 2004). The 15 sites in the Gersprenz catchment (513 km²) were sampled in spring 2021. The remaining sites in the Taunus mountain range and the catchments of the Modau (205

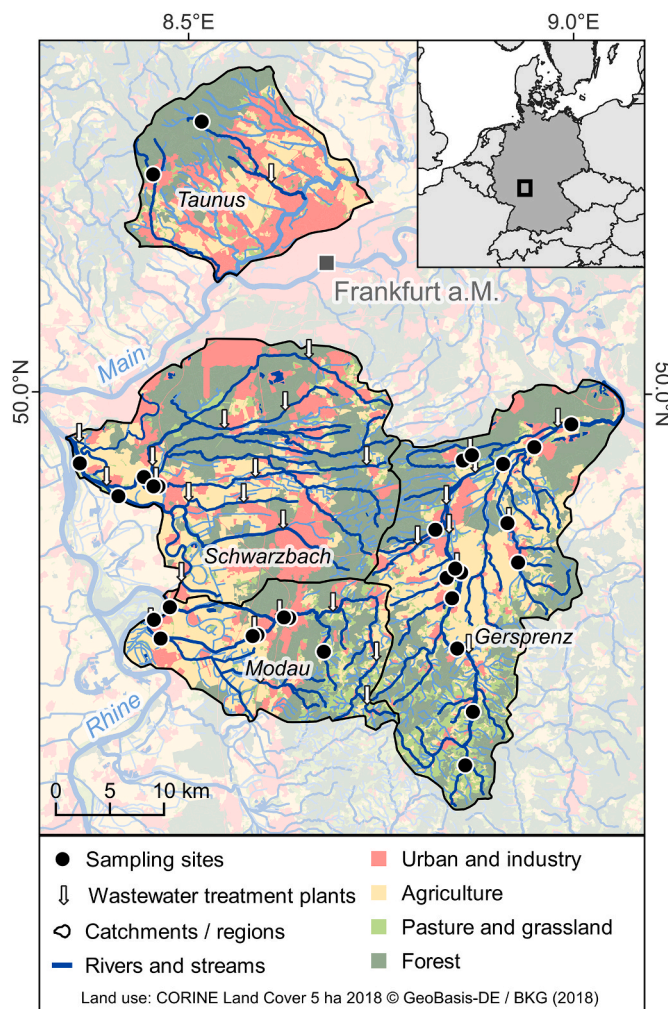


Fig. 1. Location of the 30 sampling sites in the Taunus and the catchments of Gersprenz, Schwarzbach and Modau (Germany).

km²) and Schwarzbach (514 km²) were sampled in spring 2022. Sampling included water and sediment samples, basic physicochemical parameters, and benthic macroinvertebrates.

2.2. Ecotoxicological assessment

At each site, 2 l of water and approximately 5 kg of the top 5 cm of sediment were sampled once. Water samples were filtered with glass microfiber filters (VWR International GmbH, No. 696, European Cat. No. 516–0879, 125 mm, particle retention: 1.5 µm, Darmstadt, Germany). The filtered water samples were extracted with solid-phase extraction (SPE) using Oasis HLB cartridges (6 cc, 200 mg, Waters, Milford, USA) according to Giebner et al. (2018). The resulting extracts were 5000-fold enriched and stored in dimethyl sulfoxide (DMSO) at –25 °C. Sediment samples were freeze-dried (Martin Christ Gefriertrocknungsanlagen GmbH, Alpha 1–4 LSC plus, Osterode, Germany). Sediment extracts were prepared by shaking 10 g freeze-dried sediment of the smallest fraction (<2 mm) in 50 ml methanol followed by 10 min in an ultrasonic bath. The resulting extracts were stored in 500 µl DMSO.

Ecotoxicity of the water and sediment extracts was evaluated using four different *in vitro* bioassays that address non-specific toxicity (baseline toxicity) and specific MoAs indicating certain chemical groups and important sources of micropollutants such as WWTPs (dioxin-like activity, estrogenic activity, mutagenicity; Table 1). The selection of tests was based on data availability and informative value from an initial wider palette of different *in vitro* and *in vivo* tests. Baseline toxicity was

Table 1

Toxicity endpoints of effect-based methods used with mean, minimum (Min) and maximum (Max) values across 30 sampling sites.

Endpoint	Test	Matrix	Mean (Min–Max)	Unit
Baseline toxicity	Microtox assay	Water	220 (40.7–300)	EC ₅₀ REF
		Sediment	41.4 (0.97–200)	EC ₅₀ mg SEQ
Dioxin-like activity	Yeast dioxin screen (YDS)	Water	0.071 (0–0.17)	µg β-Naphthoflavone-EQ/l
Estrogenic activity	Yeast estrogen screen (YES)	Water	0.46 (0–1.69)	ng 17β-estradiol-EQ/l
Mutagenicity	Ames fluctuation test (YG1041 ± S9, YG1042 ± S9)	Water	0.47 (0–1)	–
		Sediment	0.23 (0–1)	–

REF: Relative enrichment factor, SEQ: sediment equivalents, EQ: equivalents.

measured using the microtox assay with *Aliivibrio fischeri* in water and sediment extracts following a modified version of the ISO guideline 11348-3:2007 for 96-well plates (ISO, 2007). Baseline toxicity was quantified as inhibition of bioluminescence and expressed as a 50 % effect concentration (EC₅₀). Non-toxic samples, i.e. < 20 % inhibition, were manually assigned to an EC₅₀ of 200 mg sediment equivalents (SEQ) or a relative enrichment factor (REF) of 300 for water samples. To increase interpretability for later analyses, the resulting EC₅₀ were subtracted from the non-toxic limits. Dioxin-like and estrogenic activities in the water samples were quantified using the Yeast Dioxin Screen (YDS; Stalter et al., 2011) and Yeast Estrogen Screen (YES; Giebner et al., 2018). Dioxin-like and estrogenic activities were expressed as equivalent concentrations of β-naphthoflavone and 17β-estradiol, respectively. Measurements below the detection limit were set to 0. Mutagenicity of sediment and water extracts was determined using the Ames fluctuation test with two strains of *Salmonella typhimurium* (YG1041 and 1042, both with and without S9-mixture) according to the modified ISO guideline 11350:2012 (Hagiwara et al., 1993; ISO, 2012; Shao et al., 2020). A mutation rate of >20 % was used as threshold for mutagenicity and results were dummy coded as 0 (not mutagenic) and 1 (mutagenic). If either one or both of the two strains were mutagenic, the site was considered as mutagenic. All tests were repeated three times with the same extracts and the mean across all replicates was calculated.

2.3. Additional environmental stressors

To account for confounding stressors, basic physicochemical water parameters were assessed in parallel to the water and sediment sampling. For four weeks, pH, electric conductivity (EC) and dissolved oxygen (DO) were measured weekly with a portable multimeter (HQ40d, Hach, Düsseldorf, Germany). Concentrations of ammonium (NH₄-N), nitrite (NO₂), orthophosphate (PO₄-P) were determined twice and total organic carbon (TOC) were measured once during this period with Spectroquant test kits (Merck, Darmstadt, Germany). Concentrations of NO₂-N were calculated based on NO₂ concentrations. Measurements below the limit of quantification were adjusted to half the limit of quantification. The 10th percentile for DO and the mean for the remaining parameters were calculated across all measurements per site (see Table S1 for results).

In addition, morphological degradation was assessed once according to the standard protocol of North Rhine-Westphalia, Germany (Gellert et al., 2014). The degree of degradation of a 100 m segment was evaluated on a scale from unchanged (1) to completely changed (7), compared to reference conditions specific to the morphological river type. The assessment included several characteristics addressing channel development, longitudinal profile, bed structure, cross profile, bank

structure, and adjacent land zone.

2.4. Ecological assessment

Benthic macroinvertebrates were sampled once between March and April according to the German standard protocol following the WFD (Haase et al., 2004). The sampling method consisted of multi-habitat sampling of 20 subsamples representing the substrate coverage at the stream section (AQEM/STAR). The samples were preserved in 96 % ethanol on site. In the laboratory, samples were subsampled and fractionated. Macroinvertebrates of the largest fraction (>2 mm) were identified to the taxonomic levels of the ‘Operational Taxalist for Running Waters in Germany’ (Haase et al., 2006).

To investigate the response of the benthic macroinvertebrate community, we used taxonomic and functional approaches. The taxa abundances at the original identification level and grouped to higher taxonomic levels were used to study changes in taxonomic composition (see Tables S2–3 for taxa lists). In addition, the ecological status class and several commonly used macroinvertebrate metrics were calculated using Perlodes Online (v5.0.9, <https://www.gewaesser-bewertung.de/>). The number of individuals per m² (Abundance), taxonomic richness as number of taxa (#Taxa), Shannon-Wiener diversity index (Shannon), and evenness (Evenness) were calculated as typical descriptors of taxonomic biodiversity. General degradation of ecological quality was assessed with the German Multimetric Index (MMI; Böhmer et al., 2004), the percentage of Ephemeroptera, Plecoptera and Trichoptera in the total abundance (%EPT) and the number of Ephemeroptera, Plecoptera, Trichoptera, Coleoptera, Bivalvia and Odonata taxa (#EPTCBO). In addition, metrics developed to indicate specific stressors were used. The German Fauna Index (GFI) was developed as an indicator for morphological degradation (Lorenz et al., 2004). The German Saprobic Index (GSI; Friedrich and Herbst, 2004; Rolauffs et al., 2004) was calculated as indicator of easily degradable organic matter that leads to increased oxygen depletion. The Average Score Per Taxon (ASPT) and the Biological Monitoring Working Party score (BMWP; Armitage et al., 1983) were calculated using ASTERICS (v 4.01, <https://www.gewaesser-bewertung.de/>) as international descriptors of degradable organic matter. Finally, we calculated the SPEcies At Risk index for pesticides as potential indicator of pesticide driven toxicity (SPEAR_{pest}; Liess and von der Ohe, 2005) using Indicate (v2.3.1, <https://www.systemecology.de/indicate/>).

Functional responses of the benthic invertebrate community were investigated using ten biological traits with in total 51 modalities from the European database “freshwaterecology.info” including Tachet traits (Schmidt-Kloiber and Hering, 2015; Tachet et al., 2010) (Table 2). This selection covers a wide range of frequently used traits addressing life history, morphology and behavior which could be affected by toxicity (Rubach et al., 2011). Trait information was available for 50.0 % of the taxa at the original identification level. Remaining gaps were filled using a stepwise procedure as described in Nguyen et al. (2023) resulting in a total coverage of 97.1 % (coverages of intermediate steps are listed in Table S5). All trait modalities were fuzzy-coded and converted to proportions of the overall affinity of a trait. We calculated four distance-based metrics to describe functional diversity: functional richness (FRic), functional evenness (FEve), functional dispersion (FDis), and functional redundancy (FRed). FRic, FEve, and FDis were calculated based on a weighted Gower dissimilarity matrix of the traits with a Cailliez correction and untransformed community abundances using the ‘dbFD’ function (‘FD’-package, V 1.0–12.1, Laliberté et al., 2014; Laliberté and Legendre, 2010). FRed was calculated using the same input data and the ‘uniqueness’ function (‘adiv’-package, V 2.2, Pavoine, 2020, 2022).

2.5. Data analysis

Principal Component Analysis (PCA) was used to investigate the

Table 2
Biological traits used.

Trait group	Trait
Feeding type	grazers/scrapers (gra); miners (min); xylophagous taxa (xyl); shredders (shr); gatherers/collectors (gat); active filter feeders (aff); passive filter feeders (pff); predators (pre); parasites (par); other feeding types (oth)
Locomotion	swimming/skating (sws); swimming/diving (swd); burrowing/boring (bub); sprawling/walking (spw); (semi) sessil (ses), other locomotion types (oth)
Aquatic lifecycle ^a	full: all life stages are aquatic (full); partial: only some life stages are aquatic (part)
Dispersal	aquatic passive (aquapas); aquatic active (aquact); aerial passive (aerpas); aerial active (aeract)
Life duration	life duration up to one year (≤ 1 year); life duration longer than one year (> 1 year)
Voltinism	semivoltinism: life cycle lasts at least two years (semi); monovoltinism: one generation per year (mono); polyvoltinism: at least two generations per year (poly)
Oviposition	ovoviviparity (ovo); free isolated eggs (fie); cemented, isolated eggs (cie); cemented or fixed clutches (fic); free clutches (frc); clutches in vegetation (vec); terrestrial clutches (tec); asexual reproduction (ase)
Resistance forms	eggs/gemmule/statoblasts (egg), cocoons (coc), housings against desiccation (hou), diapause or dormancy (did), none (non)
Respiration	tegument (teg); gill (gil); plastron (pls); spiracle (aerial) (spi)
Maximal body size (Size (cm))	≤ 0.25 cm (< 0.25); > 0.25 – 0.5 cm (0.25 – 0.5); > 0.5 – 1 cm (0.5 – 1); > 1 – 2 cm (1 – 2); > 2 – 4 cm (2 – 4); > 4 – 8 cm (4 – 8); > 8 cm (> 8)

^a Aquatic lifecycle was calculated based on 'aquatic life stages'-trait (see Supplementary material).

relationship between the different bioassays and to derive major toxicity gradients. Prior to PCA, the bioassay results were standardized to zero mean and unit variance (z-scores) to account for differences in units and scales. The first two principal components (PCs) explained most of the variance ($> 75\%$). Therefore, the site scores on PC1 and PC2 were used as descriptors of toxicity for further analyses. We used Spearman rank correlation to test for co-occurrence of additional stressors along the toxicity gradients derived by the PCA.

To investigate the response of benthic invertebrate communities to the toxicity gradients, we used constrained ordination with four different response matrices: (I) taxonomic and functional metrics, (II) taxonomic composition (grouped), (III) taxonomic composition (original identification level), and (IV) trait composition. For the metrics, Abundance, #Taxa, and FRic were \log_{10} -, #EPTCBO $\log_{10}(x+1)$ -, and % EPT logit-transformed to downweight extremes and improve normality. Afterwards, all taxonomic and functional metrics were standardized to z-scores to account for the differences in units and dimensions. For the taxonomic composition, original and grouped abundances were hellinger-transformed. Trait affinities were transformed into community-weighted means based on taxa abundances. Afterwards, community weighted means were weighted by weights derived with the 'gawdis' function ('gawdis' package, V0.1.5, De Bello et al., 2021) to account for the hierarchical structure of fuzzy-coded traits. All response matrices were tested for spatial autocorrelation using the Mantel test ('mantel.correlog' function with 999 runs, 'vegan' package, v2.6.4, Oksanen et al., 2022) and for linear response using Detrended Correspondence Analysis (DCA). Because DCA indicated a linear response of all response matrices, i.e. first DCA axis ≤ 3.12 , we used partial Redundancy Analysis (RDA) as constrained ordination method. The site scores on the first and second PCs of the PCA were used as explanatory variables. Because the Mantel test indicated a slight positive spatial autocorrelation within the first 15 km (Pearson's $r = 0.1$; Table S7) and to account for temporal and natural longitudinal patterns, region (Fig. 1: Gersprenz, Modau, Schwarzbach, Taunus) and the distance to the source were added as conditions to the models. Variance partitioning indicated only a low joint explained variance ($\leq 3.4\%$) by toxicity and the natural

factors (Fig. S1). Variance inflation factors (VIF) of the explanatory variables and conditions indicated no strong collinearity (all VIF < 2). However, forward selection and permutation tests (999 runs) suggested the removal of PC2. The significances of the models were tested with global permutation tests using 999 runs. To identify the direction and strength of individual response variables, we used the species scores obtained from the RDA (i.e., the coordinate of the tips of the vectors (response variables) on the first RDA axis representing the toxicity gradient).

All statistical analyses were performed in R (v4.2.3; R Core Team, 2023). All multivariate analyses were performed using the 'vegan' package (v2.6-4, Oksanen et al., 2022).

3. Results

3.1. Deriving a toxicity gradient from the effect-based methods

PCA with bioassays revealed a strong joint toxicity gradient explaining 59.3 % of the variability along the first axis (Fig. 2A). Mutagenicity, dioxin-like activity, estrogenic activity, and baseline toxicity of water samples and sediment baseline toxicity increased along PC1. The second axis (PC2) explained 16.3 % of the variability and was mostly associated with sediment mutagenicity. There was a significant correlation of PC1 with TOC (Spearman $\rho = 0.54$, $p = 0.002$), EC (Spearman $\rho = 0.58$, $p = 0.001$), DO (Spearman $\rho = -0.57$, $p = 0.001$), $\text{NH}_4\text{-N}$ (Spearman $\rho = 0.60$, $p = 0.001$) and $\text{PO}_4\text{-P}$ (Spearman $\rho = 0.53$, $p = 0.003$) (Fig. 2B; for correlations of individual bioassays see Table S6). PC2 was not significantly correlated with any additional environmental variable ($p > 0.05$).

3.2. Benthic macroinvertebrate response to the toxicity gradient

We identified a total of 230 macroinvertebrate taxa from 83 families and 127 genera across all 30 sites. Taxonomic richness per site varied between 13 and 72 taxa. The sites covered a large gradient in ecological quality with four sites classified as "good", five as "moderate", 12 as "poor" and nine as "bad" according to the WFD (Fig. 2A).

Taxonomic and functional metrics covaried significantly with the toxicity gradient (PC1) explaining 25 % of the variation in the metrics ($p = 0.001$; Fig. 3A). Changes in metrics were mostly associated with a decrease in ASPT, #EPTCBO, #Taxa, BMWP, FRic, SPEAR_{pest}, MMI, and %EPT with increasing toxicity. Abundance and GSI were positively related to toxicity.

Taxonomic composition also changed significantly along the toxicity gradient (grouped: $R_{adj}^2 = 0.23$, $p = 0.001$; original identification level: $R_{adj}^2 = 0.10$, $p = 0.001$; Fig. 3B and C). Amphipods, such as *Gammarus* spp., and ephemeropterans, such as *Baetis rhodani* and *Rhithrogena semicolorata*-Gr., decreased the most in their abundance with increasing toxicity. At the same time, Oligochaeta (e.g. Naididae/Tubificidae Gen. sp., Oligochaeta Gen. sp.), isopods (*Asellus aquaticus*, *Proasellus coxalis*) and Chironomini Gen. sp. became more abundant with increasing toxicity.

Functional composition was significantly explained by the toxicity gradients ($R_{adj}^2 = 0.18$, $p = 0.001$; Fig. 4). Traits related to respiration, resistance forms, locomotion and feeding changed more strongly than traits such as dispersal, number of reproduction cycles per year (voltinism) and degree of aquatic lifecycle. At toxic sites, tegument respiration (teg), cocoons as resistance forms (coc), burrowing (bur) and sediment gathering (gat) were dominant traits. At low toxicity sites, gill respiration (gil), no resistance forms (non), and ovoviviparity (ovo) were more common.

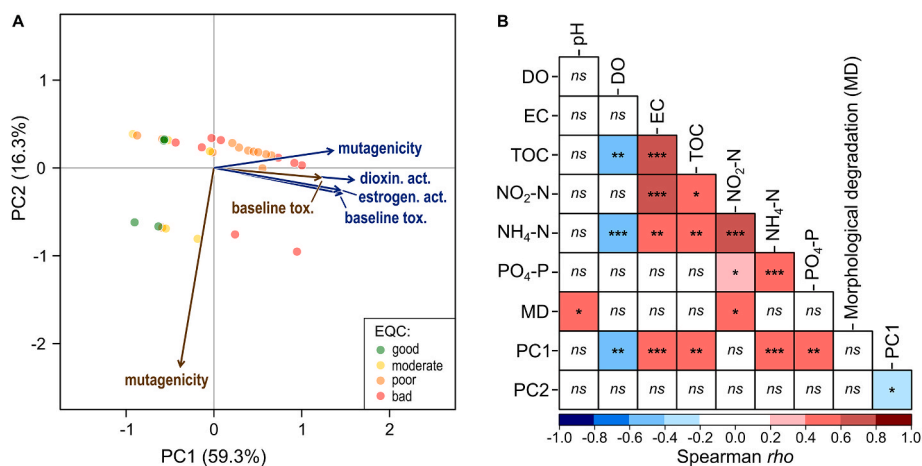


Fig. 2. A - Principal Component Analysis (PCA) with results of six bioassays measuring water (blue) and sediment toxicity (brown). Sites ($n = 30$) are colored according to the ecological quality class (EQC). B - Spearman rank correlation of the first two principal components (PC1 and PC2) with additional environmental variables. Significant correlations (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$) are colored according to the Spearman rho. Non-significant correlations (ns: $p > 0.05$) are colored white. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

4.1. Effect-based methods reveal a strong toxicity gradient associated with a decrease in macroinvertebrate biodiversity

The EBMs indicated a strong toxicity gradient with multiple MoAs. The PCA showed that most of the effects assessed by the four *in vitro* bioassays conducted on water and sediment samples increased along a major toxicity gradient (Fig. 2A). This gradient in toxicity was associated with an increase in baseline toxicity, mutagenicity, dioxin-like and estrogenic activity in water samples, as well as baseline toxicity in sediment samples. Polluted freshwater systems typically contain mixtures of numerous chemicals (Busch et al., 2016; Peng et al., 2018; Rico et al., 2019) and cause effects in multiple EBMs with different MoAs simultaneously (Leusch et al., 2024). Thus, the parallel activity of different MoAs indicated the presence of a complex chemical mixture at our most toxic sites.

The chemical pollution and observed toxicities in our study could be largely related to WWTPs effluents and diffuse pollution from anthropogenic activities. Most WWTPs are not able to completely remove chemicals from the wastewater (Margot et al., 2015; Ternes et al., 2017). Thus, WWTPs release a variety of chemicals, including pharmaceuticals, pesticides and other micropollutants (Beckers et al., 2018; Loos et al., 2013; Münze et al., 2017). These chemical mixtures of WWTP effluents can have a strong negative impact on freshwater systems (Enns et al., 2023; Finckh et al., 2022; Harth et al., 2018; Maltby et al., 2000). Estrogens, such as 17 α -ethinyl estradiol, 17 β -estradiol, and estrone, are primarily introduced into rivers through wastewater and WWTP effluents (Kase et al., 2018). We observed an increase in estrogenic activity along the toxicity gradient with the YES. This bioassay is able to detect estrogens with a high specificity (Di Paolo et al., 2016). Thus, the increasing estrogenic activity along the toxicity gradient indicate WWTP effluents as important source for chemical pollution and associated effects in our study area. In addition, other sources such as runoff from streets, urban and agricultural areas might contribute to the overall toxicity. With an increase of estrogenic activity, we observed an increasing dioxin-like activity. Polycyclic aromatic hydrocarbons (PAHs) can cause dioxin-like activity, which is quantified by the activation of the AhR receptor (Novák et al., 2018) and enter freshwater systems through aerial deposition or surface runoff from urban areas (Ravindra et al., 2008). However, other chemicals, such as pesticides, also bind to the AhR receptor (Neale et al., 2020). Pesticides can originate from WWTP effluents as well as diffuse pollution from agriculture

(Halbach et al., 2021; Le et al., 2017). Rain events in agricultural areas release high pesticide loads into streams, which can also increase baseline toxicity (Betz-Koch et al., 2023). As EBMs can only indicate the presence of certain chemical groups and thus sources of contamination, a subsequent chemical analysis is necessary to identify the individual pollutants causing the toxic effects. In our study, the EBMs indicated that the toxicity gradient may be related to a complex chemical mixture originating from WWTP effluents but also diffuse pollution from anthropogenic activity.

In our study, the macroinvertebrate community significantly changed in taxonomic and functional composition, and decreased in diversity, ecological quality and functional richness with increasing toxicity (Figs. 3 and 4). For instance, MMI, an indicator for general degradation, and taxonomic richness in particular for EPTCBO-taxa also decreased with increasing toxicity. These negative effects of chemical pollution quantified with chemical analysis was observed numerously before (Alric et al., 2022; Heß et al., 2023; Markert et al., 2024). Accordingly, sediment toxicity assessed by *in vivo* bioassays also resulted in a decrease in the US version of the MMI, EPT richness and %EPT (Moran et al., 2017). Furthermore, the ecological potential of reservoirs decreased with a declining ecotoxicological status derived with EBMs (Palma et al., 2016).

The strength of the response emphasizes the role of chemicals as stressors and the ecological relevance of EBM derived toxicities. For example, all of the most toxic sites were classified as “bad” according to the WFD. Taxa such as *Gammarus pulex* and *G. roeselii* were among the most decreasing taxa, despite being previously identified as moderately sensitive or even tolerant to WWTP and micropollutants (Enns et al., 2023; Meyer et al., 2022; Rico and van den Brink, 2015). The disappearance of these taxa illustrates the hostile conditions present at our most toxic sites. Additionally, we observed a decline in both taxonomic and functional diversity. Functional responses can be less responsive than taxonomical ones, as high functional redundancy can buffer effects of taxonomical changes (Alric et al., 2021; Baker et al., 2021; Charvet et al., 2000). Thus, a decrease in functional richness indicates that these buffer capacities were exceeded and that ecological functions may be impaired. For instance, the loss of shredders can lead to a decrease in leaf litter decomposition, which could disrupt the entire nutrient cycle (Feckler et al., 2023; Münze et al., 2017).

The strength of the macroinvertebrate response along the toxicity gradient could emphasize the suitability of EBMs for the ecologically relevant detection of chemical pollution. However, the observed correlation between toxicity and ecological effects does not mean a causal

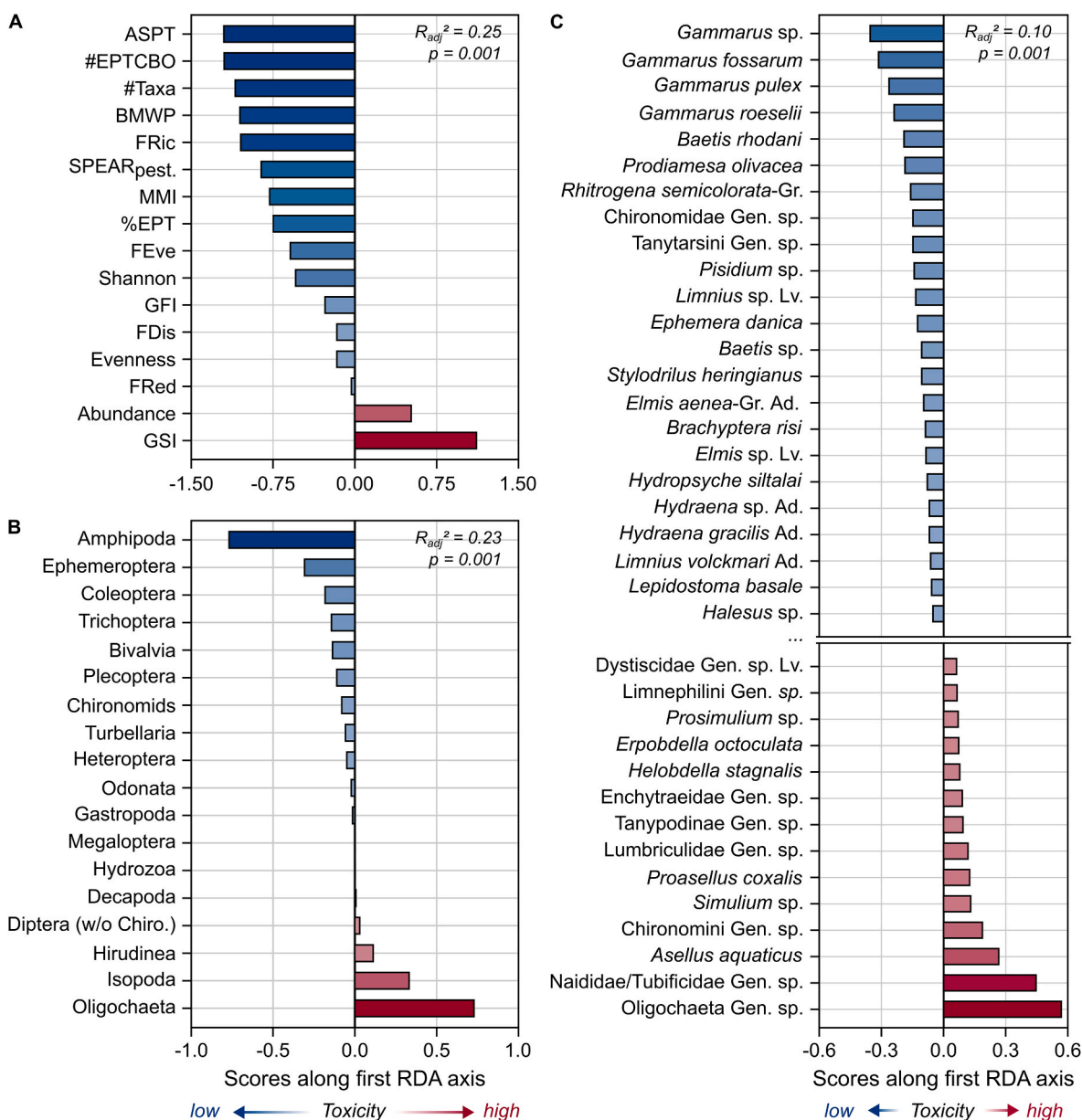


Fig. 3. Scores of taxonomic and functional metrics (A), abundances of taxonomic groups (B) and abundances of the most responding taxa at original identification level (C) on the first RDA axis representing the toxicity gradient. The scores of all taxa at original identification level can be found in the supplementary material. Blue bars indicate negative effect of toxicity, red bars indicate positive effect. $N = 30$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

relationship. For instance, estrogenic activity is quantified by the binding to the estrogen receptor (Di Paolo et al., 2016). Since most macroinvertebrates lack this type of receptor, estrogens may act through a different toxicity pathway or co-occurring chemicals caused the observed ecological effects. In addition, other environmental stressors, such as nutrients, oxygen depletion and salinity, were also moderately correlated with toxicity. Because these stressors are among the most important stressors to freshwater ecosystems (Berger et al., 2017; De Castro-Català et al., 2015; Heß et al., 2023; Markert et al., 2024), they might contribute to the observed ecological decline. The physicochemical changes correlating with toxicity likely originated from similar sources, such as WWTPs and agricultural runoff (Berger et al., 2017; Burdon et al., 2019; Tlili et al., 2017). However, the isolation of effects of individual stressors in multiple stressor contexts is very difficult and we cannot exclude potential interacting or masking effects of physicochemistry and other environmental stressors along the toxicity

gradient. To obtain further evidence for ecological relevance, more studies connecting EBMs and ecological data, including controlled environments such as mesocosm studies, are necessary. Furthermore, since macroinvertebrates tend to respond integratively to all stressors present, it is necessary to find monitoring systems specific for individual stressors. *In vitro* bioassays, that address specific MoAs, can indicate the presence of certain chemical groups independently of other stressors, such as hydromorphological and physicochemical degradation. Thus, the current study highlights the usefulness of EBMs with specific MoAs for detecting the presence of individual chemical groups and identifying potential sources in multiple stressor systems.

4.2. Multiple stressors and integrative bioindication by macroinvertebrates impede toxicity-specific responses

Macroinvertebrate-based bioindicators specific to chemical pollution

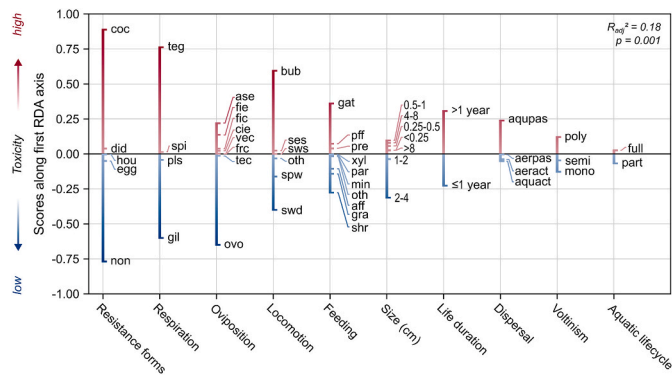


Fig. 4. Scores of trait modalities on the first RDA axis representing the toxicity gradient. Blue bars indicate negative effect of toxicity, red bars indicate positive effect. $N = 30$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

or even chemical groups could be helpful tools to identify stressors in rivers with existing monitoring data. The identification of metrics, taxa and traits related to tolerance and vulnerability to toxicity is key to develop such bioindicators. For instance, $SPEAR_{pest}$ strongly decreased with increasing toxicity suggesting the presence and potential toxic effects of pesticides (Liess et al., 2021). However, indicators of biodiversity (#EPTCBO, #Taxa, FRic) and degradable organic matter (ASPT, BMWP, GSI) also strongly responded (Fig. 3A). Many macroinvertebrate metrics have typically only low stressor specificity and often respond to general degradation (Lemm et al., 2019; Stubbington et al., 2022). Accordingly, severe toxic effects can cause the disappearance of many taxa, including those that are typically sensitive to other stressors, leading to a response of several metrics. In addition, the macroinvertebrate communities are also affected by the presence of other stressors, such as EC, oxygen depletion and nutrients that were moderately correlated with toxicity. An integrative response to these multiple stressors can mask toxicity-specific effects. Thus, the presence of multiple stressors and the integrative response of macroinvertebrates impede the derivation of toxicity-specific bioindicators based on our data.

The change of taxonomic composition revealed taxa that are typically vulnerable and tolerant to anthropogenic stress. Along our toxicity gradient, common and abundant taxa such as gammarids (*G. pulex*, *G. fossarum* and *G. roeselii*) and ephemeropterans (*Baetis rhodani*, *Rhythrogena semicolorata*-Gr.) were decreasing the most with increasing toxicity (Fig. 3B and C). However, less abundant taxa, belonging to Coleoptera, Trichoptera, Plecoptera and Bivalvia, also decreased and can be considered as vulnerable towards toxicity. Accordingly, several Ephemeroptera, Plecoptera, Trichoptera and Coleoptera taxa were determined as sensitive to micropollutants and WWTP effluents (Berger et al., 2016; Enns et al., 2023; Rico and van den Brink, 2015). However, EPT taxa were also demonstrated to be sensitive towards various stressors and are used as indicators for general degradation of water quality (Hering et al., 2004; Juvigny-Khenafou et al., 2021; Markert et al., 2024; Waite and Van Metre, 2017). Oligochaetes, hirudineans, asellids (*Asellus aquaticus*) and Chironomina Gen. sp. were identified as tolerant taxa that became more abundant with increasing toxicity. These taxa are typical of sites polluted by toxicants but also nutrients, salinity, temperature, metals and decreased oxygen levels (Enns et al., 2023; Pallottini et al., 2017). In general, only a few taxa were found to react stressor-specific (Berger et al., 2018). Similar to the response of metrics, the observed changes in taxonomic composition are not exclusive to toxicity, but are also common to other anthropogenic stressors.

Compared to taxonomic composition, functional traits can provide a more mechanistic understanding of the stressor effects and adaptation strategies of the macroinvertebrate community (Culp et al., 2011). Tegument respiration, cocoons as resistance forms, burrowing,

gathering, and longevity were identified as traits favored by toxic conditions. Traits such as gill respiration, lack of resistance forms, swimming locomotion, feeding as shredders, and reproduction with ovoviviparity were related to vulnerability. These traits can be largely related to the taxonomic changes, such as the decrease in gammarids and the increase in oligochaetes, and only partly follow typical adaptation strategies. For instance, ovoviviparity is a reproduction strategy common to tolerant taxa such as *Asellus aquaticus* (Tachet et al., 2010) and is used as an indicator of poor water quality in the French multimetric I2M2 index (Mondy et al., 2012). However, gammarids are also ovoviviparous (Tachet et al., 2010) and were one of the most abundant taxa groups at our low to medium toxic sites. Thus, their strong decrease at our most toxic sites resulted in an overall decrease of ovoviviparity. Overall, the trait response only partially resembles typical toxicity tolerance traits that affect an organism's exposure to toxicants, the intrinsic sensitivity, and the population sustainability (Rubach et al., 2011). Gill respiration was connected with vulnerability, while tegument respiration was connected with tolerance. The differences in assimilation and metabolism rates between these respiration types affect the uptake of toxicants, and, therefore, potential toxic effects (Rubach et al., 2011). Furthermore, the resistance forms, such as cocoons, can facilitate the survival in case of pollution events and increase tolerance to toxicity (Rubach et al., 2011). However, other life history strategies that can increase tolerance, such as multiple short-lived generations per year and high dispersal capacities, responded only weakly to toxicity. Therefore, physiological adaptations affecting the individual tolerance to toxicity might be more important than morphology and life history strategies in our study. However, enzyme activities and other intrinsic physiological adaptations are more difficult to describe (Rubach et al., 2011). Because of this lack of relevant traits and intercorrelations between traits, it is difficult to relate traits specifically to individual stressors such as toxicity (Hamilton et al., 2020). Consequently, trait responses were similar to different stressors such as sediment contamination with metals, PAHs and polychlorinated biphenyls (PCBs) (Archaimbault et al., 2010), micropollutants in water (Meyer et al., 2022), but also nutrients and other stressors (Ieromina et al., 2016; Jiang et al., 2021; Pallottini et al., 2017). Thus, there is a low potential for toxicity- or even chemical-specific macroinvertebrate indicators based on the existing trait information (Collins and Fahrig, 2020). The combinations of many individual trait responses but also taxonomic metrics could provide a potential approach to derive out of many weak responses a stressor-specific response pattern (Alric et al., 2021; Meyer et al., 2022).

5. Conclusions

The current study is part of a limited number of studies that link ecotoxicological and ecological data to investigate the potential of macroinvertebrates as toxicity-specific bioindicators and to evaluate the use and ecological relevance of EBMs. We observed significant changes in the biodiversity, ecological quality, taxonomic and functional composition of macroinvertebrates that suggest strong impacts by toxic pollution and an ecological relevance of the toxicities derived by *in vitro* bioassays. However, the observed changes were rather non-specific for toxicity. Multiple stressors may cover toxicity-specific responses and macroinvertebrates respond integratively to the effects of various stressors. Thus, there is only a low potential to derive toxicity-specific bioindicators in our study. Since biological responses can only provide limited information in this regard, it is even more important to monitor also potential stressors. In this context, using EBMs and linking them to ecological data can be useful for separating the effects of chemical pollution from the overall effects of multiple stressors, as well as identifying the presence of chemical groups with specific MoAs. This information is helpful for the selection of monitoring and mitigation measures, such as more directed chemical analyses of water and sediment samples.

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CRediT authorship contribution statement

Sebastian Heß: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Delia Hof:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Matthias Oetken:** Writing – review & editing, Funding acquisition, Conceptualization. **Andrea Sundermann:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used “DeepL Write” (DeepL SE, Cologne, Germany) in order to check grammar and spelling and improve readability. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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 A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2024.124330>.

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