



# Combined effects of herbicides and insecticides reduce biomass of sensitive aquatic invertebrates

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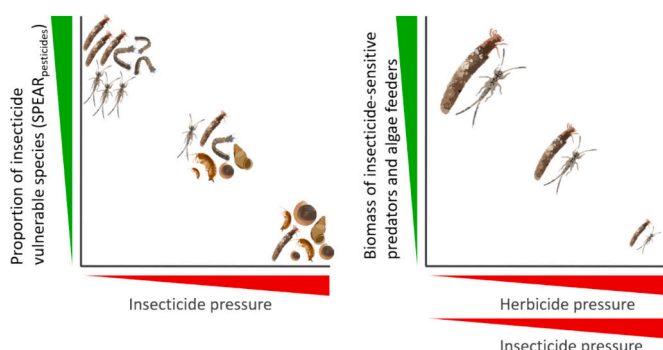
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## HIGHLIGHTS

- In-stream invertebrate biomass determination performed by image analysis.
- Insecticide pressure reduces abundance of SPEAR<sub>pesticides</sub> taxa.
- Herbicide pressure reduces insecticide-sensitive algae feeders' + predators' biomass.
- Combined effect of herbicide mediated food shortage and insecticide pressure.

## GRAPHICAL ABSTRACT



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## ABSTRACT

The structure and biomass of aquatic invertebrate communities play a crucial role in the matter dynamics of streams. However, biomass is rarely quantified in ecological assessments of streams, and little is known about the environmental and anthropogenic factors that influence it. In this study, we aimed to identify environmental factors that are associated with invertebrate structure and biomass through a monitoring of 25 streams across Germany. We identified invertebrates, assigned them to taxonomic and trait-based groups, and quantified biomass using image-based analysis. We found that insecticide pressure generally reduced the abundance of insecticide-vulnerable populations ( $R^2 = 0.43$  applying SPEAR<sub>pesticides</sub> indicator), but not invertebrate biomass. In contrast, herbicide pressure reduced the biomass of several biomass aggregations. Especially, insecticide-sensitive populations, that were directly (algae feeder,  $R^2 = 0.39$ ) or indirectly (predators,  $R^2 = 0.29$ ) dependent on algae, were affected. This indicated a combined effect of possible food shortage due to herbicides and direct insecticide pressure. Specifically, all streams with increased herbicide pressure showed a reduced overall

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biomass share of Trichoptera from 43 % to 3 % and those of Ephemeroptera from 20 % to 3 % compared to streams grouped by low herbicide pressure. In contrast, insecticide-insensitive Gastropoda increased from 10 % to 45 %, and non-vulnerable leaf-shredding Crustacea increased from 10 % to 22 %. In summary, our results indicate that at the community level, the direct effects of insecticides and the indirect, food-mediated effects of herbicides exert a combined effect on the biomass of sensitive insect groups, thus disrupting food chains at ecosystem level.

## 1. Introduction

Benthic macroinvertebrate communities are considered an integral part of aquatic food chains and have long been used to detect ecosystem degradation (Fierro et al., 2016; Pauw et al., 2006; Richter et al., 2003). While macroinvertebrate communities are commonly analysed using ecological metrics such as taxa richness or the Saprobic Index (Kolkwitz and Marsson, 1909), which are also applied for the Water Framework Directive (European Union (2000)), the macroinvertebrate biomass is rarely included in ecological assessments (Bernardini et al., 2000). Biomass is already frequently used as an indicator of environmental and/or anthropogenic stress in terrestrial ecosystems in the field of entomology (Hallmann et al., 2017; Ohler et al., 2023) and for the assessment of freshwater the biomass fish is often considered as an indicator (Pinna et al., 2023), but the macroinvertebrates biomass in aquatic ecosystems is not yet as well understood. Macroinvertebrates biomass can be affected by a wide range of environmental and anthropogenic variables and is considered as an integrating endpoint (Gessner and Chauvet, 2002; Liess and Foit, 2010) reflecting the functionality of a community in its entity.

The biomass of the invertebrate community in streams has not yet provided information on the stressor-biomass-associations, a more nuanced investigation of different taxonomic and trait-based groups may yield valuable insights. The biomass of more generalist invertebrates, such as some taxa from Gastropoda or Crustacea, can be a relevant metric for nutrient turnover in streams, as they tend to benefit from nutrient enrichment increased food resources (Everall et al., 2019). Furthermore, the biomass of Gammaridae as widespread, abundant, large taxa (Chaumot et al., 2015) and the biomass of Gastropoda are known to be insensitive to anthropogenic stressors such as pesticides and nutrients. Nutrients such as phosphate for instance stimulate the growth of fungi, bacteria and algae on aquatic leave litter (Gulis and Suberkropp, 2002) and additionally the growth of phytoplankton in streams both providing food for Gammaridae (Basen et al., 2013; Golterman, 1975; Utsunomiya et al., 2017). While in reference areas, macroinvertebrate biomass appears to be mainly explained by basin- or catchment-level variables rather than local variables (Burgazzi et al., 2023), stressed lowland systems as analysed in our study has been shown to be primarily influenced by local variables (Liess et al., 2021).

Aquatic insects act as an important food source for predators in aquatic ecosystems (Anderson, 2009; Dudgon and Richardson, 1988) and as a link from aquatic to terrestrial ecosystems and are known for containing long chained polyunsaturated fatty acids (PUFAs) acting as an essential nutrition base for terrestrial predators (Hixson and Arts, 2016). One previous study by Twining et al. (2018) showed that the breeding success of a widespread bird species increased with the amount of aquatic insect PUFAs. Also an anthropogenic induced declining of biomass and altering of PUFA profiles of aquatic insects were found to likely affect the food-chain dynamics in a cross-ecosystem context (Ohler et al., 2022; Ohler et al., 2024).

With this study we aimed to explain and understand possible direct and indirect ecological effects in streams exposed to multiple stressors such as combined insecticide and herbicide pressure by analysing the associations between macroinvertebrate biomass and environmental variables including anthropogenic stressors. To this end, we (i) analysed the associations between total macroinvertebrate biomass and environmental variables including anthropogenic stressors based on

individual body length and taxon-specific maximum literature length, (ii) detected effects on aquatic insect's biomass as the nutritional base of higher trophic levels, and (iii) assessed the impact of anthropogenic stress at the functional level considering feeding groups. Biomass was determined using a novel automated image analysis measuring body lengths at individual level as an alternative for the maximum literature length approach. To determine the relationship between biomass and environmental variables, the use of generic biomass of taxa has not proven to reveal specific stressor associations (Liess et al., 2021; Liess and Foit, 2010). Therefore, it may be useful to derive biomass from site-specific body length. This study therefore considers both, site-specific variability of biomass and generic biomass.

## 2. Methods

### 2.1. Study area

We sampled a total of 25 stream sections distributed over four German Federal States (Lower Saxony, Saxony, Saxony-Anhalt and Thuringia) from April to July in 2021 (SI Fig. 1). The streams were selected according to the following criteria: Catchment area smaller than 30 km<sup>2</sup>; Extensive exclusion of waste water treatment plants; A small proportion of urban areas in the catchment area; Telecommunication reception for the automatic sampler. The agricultural monitoring sites are characterised by at least 20 % agricultural land in the catchment area. The non-agricultural monitoring sites represent sections of streams with an environment that is as close to natural as possible, without intensive agricultural use in the catchment area. The term agriculture is restricted to crops and does not include animal grazing. The initial selection comprised 34 stream sections, however, we omitted those stream sections that were affected by drought (lack of flow (<0.05 m/s) or no water). The catchments of the 25 streams were characterised by a gradient of agricultural land use ranging from 0 to 100 % with a median of 72.87 %. Following the definition of Liess et al. (2021), streams with >20 % of agricultural land use in the hydrological catchment area were classified as agricultural streams and streams with <20 % agricultural land use were classified as non-agricultural streams. In the present study, 21 agricultural and 4 non-agricultural streams were investigated. The sites investigated in this study in 2021 are a reduced number of the sampling sites investigated in Liess et al. (2021) in 2018 and 2019, supplemented by four sampling sites in Thuringia and three in Saxony that were not subject to previous investigations.

### 2.2. Pesticide monitoring and analysis

Rainfall event-driven water samples (EDS,  $n = 65$ ) were taken using automated samplers (MAXX TP5, Rangendingen, Germany) to capture the peak concentrations induced by significant rise of water level (approx. 5 cm, depending on the stream bed, Liess et al. (1999)). Sampling was performed during a time period of three months (April to June) with the number of EDS varying between sampling sites with an average of 2 EDS per site (min = 0, max = 6). The activation of sampling due to water level rises yielded a mixed sample of 500 mL with 40 subsamples (every 5 min) collected over a total period of 3 h and 20 min (Liess et al., 2021). Additionally, streams were sampled regularly with 250 mL grab samples ( $n = 231$ ) every three weeks according to WFD standards (EU commission, 2000). 74 pesticides and 24 pesticide

metabolites were selected by prioritization according to active substance-related sale quantities, under consideration of the current environmental quality standards (EQS) and the regulatory acceptable concentrations (RAC) (see list of substances in SI Liess et al. (2021)). All 296 water samples were analysed for 98 substances via target analysis with high pressure liquid chromatograph coupled with a high-resolution tandem mass spectrometer LC-HRMS/MS without enrichment by multiple-reaction-monitoring (MRM, (Halbach et al., 2012; Reemtsma et al., 2013)).

### 2.3. Extrapolating from pesticide exposure to toxic pressure

All pesticide concentrations from EDS and grab samples were converted into invertebrate and algae toxicity by calculating Toxic Units (TU; Sprague (1970)) with the substance-related acute 48 h  $EC_{50}$  (Lethal concentration for 50 % of laboratory experiment individuals) of *Daphnia magna* or *Chironomus riparius* and the 72 h  $EC_{50}$  (effect concentration) of algae species (most sensitive organism selected per substance).  $LC_{50}$  and  $EC_{50}$  values were collected from the Pesticide Property Data Base (PPDB; Lewis et al. (2016)) and the United States Environmental Protection Agency Ecotoxicology (2019). Peak exposure was determined by the maximum single pesticide (invertebrate, algae) toxicity ( $TU_{max}$ ) per site measured across the whole sampling period according to Liess and van der Ohe (2005). To account for additive effects, we further determined  $TU_{sum}$  per site and affected group according to Liess et al. (2021), which yielded very similar results to  $TU_{max}$  (correlation of  $R^2 = 0.87$  for insecticide pressure and correlation of  $R^2 = 0.8$  for herbicide pressure). Therefore, we only applied  $TU_{max}$  in the following analysis for insecticide and herbicide pressure. Due to the monitoring strategy, pyrethroids were analysed at 10 of the 25 streams investigated and were included in the toxicity derivation ( $TU_{max}$ ) of the insecticide pressure.  $TU_{max}$  of four streams were determined by pyrethroids ranging from  $-1.03$  to  $-1.54$ .  $TU_{max}$  derived from insecticides is referred to as insecticide pressure and  $TU_{max}$  derived from herbicides and their respective algae toxicity is referred to as herbicide pressure in the following analysis.

### 2.4. Abiotic variables

To examine the relationships between environmental variables as well as anthropogenic stressors and the aquatic macroinvertebrate biomass, we measured a multitude of relevant abiotic variables. Temperature and oxygen concentration were measured continuously from April to July in a 3-min interval using multi-parameter probes (Log-Trans7-compact measuring system SENSODIVE CTDO2, UIT; Dresden, Germany; O2-Log3055-INT and CTD3100-10 Logger, Driesen + Kern, Bad Bramstedt, Germany). Nutrients ( $NH_4$ ,  $NO_3$ ,  $PO_4$ ) were analysed in EDS and grab samples with UV spectrophotometers (visicolor ECO test kits; Hach DR1900-02L (340–800 nm), Düsseldorf, Germany). Flow velocity (drifting method), pH (Greisinger G 1500, Regenstauf, Germany) and water level were measured every three weeks. Stream hydromorphology (referred to as deficient hydromorphology in the further analyses) was identified once in Mai according to the official procedure by the German Länder-arbeitsgemeinschaft Wasser (LAWA) quantifying all hydromorphological criteria required under the WFD. Agricultural land use was excluded from the analysis due to intercorrelation with insecticide pressure ( $r > 0.7$ ). For more in-depth information regarding each variable and their aggregation, SI Table 1. The aggregated values for each variable can be found in the SI EXCEL workbook 1.

### 2.5. Macroinvertebrate sampling

Macroinvertebrates were sampled after the main pesticide application period (Vormeier et al., 2023) in June 2021 at each site. The macroinvertebrate sampling largely followed the multi habitat sampling approach according to the WFD guideline (Meier et al., 2006a; Meier

et al., 2006b). At each site, 20 subsamples were taken along a 50-meter section with a kick-net each representing 5 % of the present substrates. Each unit (5 %) was sampled by kick sampling ten times in estimated surface of  $0.0625 m^2$  and using a net with a mesh size of 0.5 mm (Meier et al., 2006a). Subsequently, macroinvertebrates were filtered through a sieve tower (8 mm, 4 mm, 2 mm, 0.5 mm mesh size), sorted, and conserved in 90 % ethanol until laboratory determination. Taxa were identified according to the WFD guidelines (European Union, 2000) using a binocular (Zeiss, Stereo Discovery V.20, Carl Zeiss Microscopy GmbH; Jena, Germany).

### 2.6. Automated image analysis – body length and biomass derivation

Macroinvertebrate individuals were photographed taxa-wise. Based on these pictures, a list of all taxa with information on the sample, site and taxa name was created in Python (version 3.8; (van Rossum and Drake Jr, 1995)). Contours of the individual taxa in the pictures were detected with the Open CV package for Python (Bradski, 2000). Ellipses were automatically drawn around the contours identified as individuals (SI Fig. 2). The information on the ellipse characteristics (minor and major axis) was used to identify the pixel number. A conversion factor of 0.04 was used to transform pixel number to mm body length with the help of three reference pictures of cut pond liner (of  $1 \times 1$  cm,  $0.5 \times 2$  cm, and  $2 \times 1$  cm respectively; SI Fig. 3). The scripts with the code in Python are available on GitHub (<https://github.com/LianaLieb/Biomass-macroinvertebrates>). In addition, the perimeter of the ellipse was calculated according to the third approximation of Ramanujan (1914) to estimate the body length of strongly bent individuals of the families Gammaridae, Hydropsychidae, Philopotamidae and Rhyacophilidae. The image based automated body length analysis method was able to delineate contours most precisely based on five ideal prerequisites: Taxa with clear contours, simple shapes, dark colour, homogeneous colour, and dense tissue were recognised mostly accurate (SI Fig. 2). In orders where these prerequisites were only partly met, the size recognition was limited. For instance, Oligochaeta with their sinuous long shape, heterogeneous colour and transparent tissue are mostly identified in several parts (SI Fig. 4). In ethanol conserved Hirudinea are curled up and therefore difficult to photograph. The derived body length of individuals was then used to determine the individual biomass according to taxon-specific body length – dry mass relationships (Bencke et al., 1999).

$$DM = aL^b \quad (1)$$

where  $DM$  is the individual body dry mass (mg),  $a$  and  $b$  are taxon-specific constants delivered by several previous studies (SI EXCEL Workbook 2), and  $L$  is the length of linear body dimension (mm) according to Bencke et al. (1999). For taxonomic levels where no information on the constants could be found, we extrapolated  $a$  and  $b$  from closely related taxonomic levels (genus, family, order). The following linear body dimensions were considered in the referred (SI EXCEL workbook 2) studies: body length, shell height, shell length, and shell width. Aggregating the individual body length and individual biomass on different taxonomic levels per sampling site was done in R (version 4.4.0, R Core Team, 2023). To validate the derived body length, we additionally measured the body length of a subsample of individuals ( $n = 83$ ) from different orders with a microscope (Zeiss, Stereo Discover V20) generated image and measuring tool (Software: Axio Vision from Zeiss). The congruence of the body length between the two methods was determined with an explained variance of  $R^2 = 0.9$  (SI Fig. 5). Furthermore, we validated the biomass by drying individuals at  $80^\circ C$  (Convection dryer, Heraeus UT 6060) for 48 h, then weighted (Sartorius LA620S, scale accuracy = 0.001 g) and compared the dry weight to the derived dry mass of the same individuals. The congruence of the derived biomass compared to the measured biomass was evaluated at individual level ( $R^2 = 0.96$ ; SI Fig. 6). For more detailed information on the applied

parameters and body length – dry mass relationships see SI EXCEL workbook 2. The biomass used refers only to the organic drymass, caddisfly caddis and mollusc shells were not considered.

In addition to deriving the biomass as described above, we calculated the biomass of the collected organisms using the taxon-specific maximum literature lengths of the taxa (SI EXCEL workbook 2) instead of the individual-based lengths in order to compare the associations with the variables and to put the amount of biomass determined in relation to each other.

## 2.7. Aggregations of biomass

The biomass aggregations were selected according to their relevance in food chain functioning (Collier et al., 2002; Fukui et al., 2006; Kraus et al., 2014; MacNeil et al., 1999; Twining et al., 2018) and their sensitivity to pesticides (Liess and van der Ohe, 2005). The macroinvertebrate biomass was calculated at individual level and aggregated at family, order or group taxonomic resolution subsequently. More detailed information on the aggregations and measuring units can be found in the SI Table 1. In addition, biomass was aggregated at the level of five feeding groups (gatherer, grazer, shredder, filterer and predator) and for an in-depth analysis at insecticide-sensitive predators and algae feeders functional feeding groups (SI Table 1) with a group allocation of >5 (Liess et al., 2017). The classification of this two functional feeding groups by insecticide-sensitivity was made based on the SPEAR<sub>pesticides</sub> trait *relative pesticide sensitivity* (Liess and van der Ohe, 2005). The biomass of frequently studied organism groups such as Gammaridae, EPT (Ephemeroptera, Plecoptera, Trichoptera), Gastropoda, aquatic insect larvae (Ephemeroptera, Plecoptera, Trichoptera, Diptera, Megaloptera, Odonata, Coleoptera) and the biomass of SPEAR<sub>pesticides</sub> taxa were also calculated. In addition, the biomass distribution (%) of four orders (Ephemeroptera, Plecoptera, Trichoptera, Diptera), two classes (Bivalvia, Gastropoda) and one subphylum (Crustacea), referred to as groups, with the highest overall biomass share was analysed. Furthermore, streams were grouped in terms of high (>75 percentile) and low (<25 percentile) herbicide pressure and were analysed regarding their group-related biomass composition. The overall biomass of all streams combined was also analysed for the named macroinvertebrate groups (SI Fig. 7). The biomass of various subgroups per stream was analysed as log g/m<sup>2</sup>, this subgroup biomass is a relative proportion of the total sample per site, i.e. relative biomass (subsequently for instance the relative biomass of Trichoptera per site log g/m<sup>2</sup> is referred to as Trichoptera biomass [log] in figures and as Trichoptera biomass in the text).

## 2.8. Statistical data analysis

We analysed the effect of environmental variables including anthropogenic stressors on several biomass aggregations. We also compared biomass-pesticide-association to abundance-pesticide-association in terms of SPEAR<sub>pesticides</sub>. All monitored variables were tested for normal distribution and if necessary, log transformed (SI Fig. 3). We further assessed multicollinearity of all variables using the variance inflation factor (exclusion for VIF > 4; Dodge (2010)), resulting in eleven explanatory variables (insecticide pressure, herbicide pressure, hydromorphology, flow velocity, oxygen, temperature, pH, NH<sub>4</sub>, PO<sub>4</sub>, NO<sub>3</sub>, ratio of stones per stream) considered as potentially important stressors (SI Table 2). All variables and biomass aggregations were evaluated regarding outliers with the z-score test and scaled for standardisation. The relevant variables out of the eleven environmental and anthropogenic variables for each aquatic macroinvertebrate biomass aggregation were then identified using a regression analysis with elastic net regularisation (Zou and Hastie, 2005). In general, the elastic net statistical approach allows for a low sample size to explanatory variables ratio. By using the method leave-one-out-cross-validation (LOOCV) randomness in the training/test data was circumvented. The optimal regularisation was determined by the Root Mean Square Error with the

smallest value. As a second step, we constructed a model containing the maximum four most relevant environmental variables according to the elastic net approach and determined their respective R<sup>2</sup> contribution with the “relaimp” package (version 2.2-3) using the metric “lmj” (Lindeman, Merenda and Gold indices (1980)), which is the R<sup>2</sup> contribution averaged over orderings among regressors. This metric uses both direct effects and effects adjusted for other regressors in the model (Grömping, 2006). Stressors with a relative importance R<sup>2</sup> < 0.05 were not considered. All statistical analyses and visualisations were conducted in R (version 4.4.0; R Core Team, 2023) using the additional packages “vegan”, “plotmo” and “glmnet” (Friedman et al., 2018; Milborrow, 2019; Oksanen et al., 2018).

## 3. Results and discussion

### 3.1. Macroinvertebrate community structure links to insecticide pressure

The pesticide-sensitive bioindicator SPEAR<sub>pesticides</sub> designed by Liess and van der Ohe (2005) and related to insecticide pressure in several previous studies (Knillmann et al., 2018; Kuzmanović et al., 2016; Liess et al., 2021; Münze et al., 2017). The composition and abundance of insecticide-vulnerable macroinvertebrates characterised by SPEAR<sub>pesticides</sub> correlated with insecticide pressure showed a significant reduction (R<sup>2</sup> = 0.43,  $p < 0.001$ ; Fig. 1). SPEAR<sub>pesticides</sub>, however, was not significantly associated to herbicide pressure (R<sup>2</sup> = 0.13,  $p > 0.05$ ) as also identified in previous investigations as well (Liess et al., 2021).

### 3.2. Description of the overall biomass

The biomass composition of the 25 streams was dominated by the biomass of seven groups: Trichoptera, Crustacea, Gastropoda, Ephemeroptera, Diptera, Coleoptera and Bivalvia; in descending order of biomass proportion of the overall biomass of all streams combined (SI Fig. 7). While the relative proportion of biomass in the four groups Trichoptera, Crustacea, Gastropoda and Ephemeroptera showed a wide gradient between the monitoring sites, the gradient in the groups Diptera, Bivalvia and Coleoptera is rather small (SI Fig. 8). The site-specific proportion of the biomass of these groups ranged from a minimum of zero % to a maximum of 95 %, depending on the group (SI Fig. 8).

The highest contribution to the overall biomass of all streams

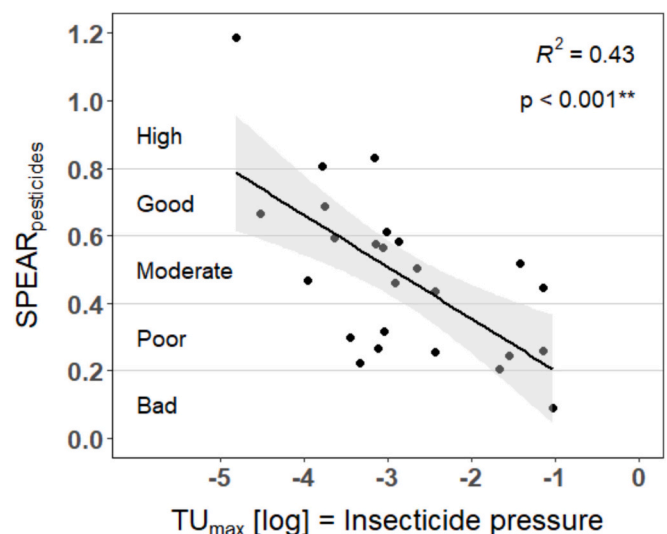


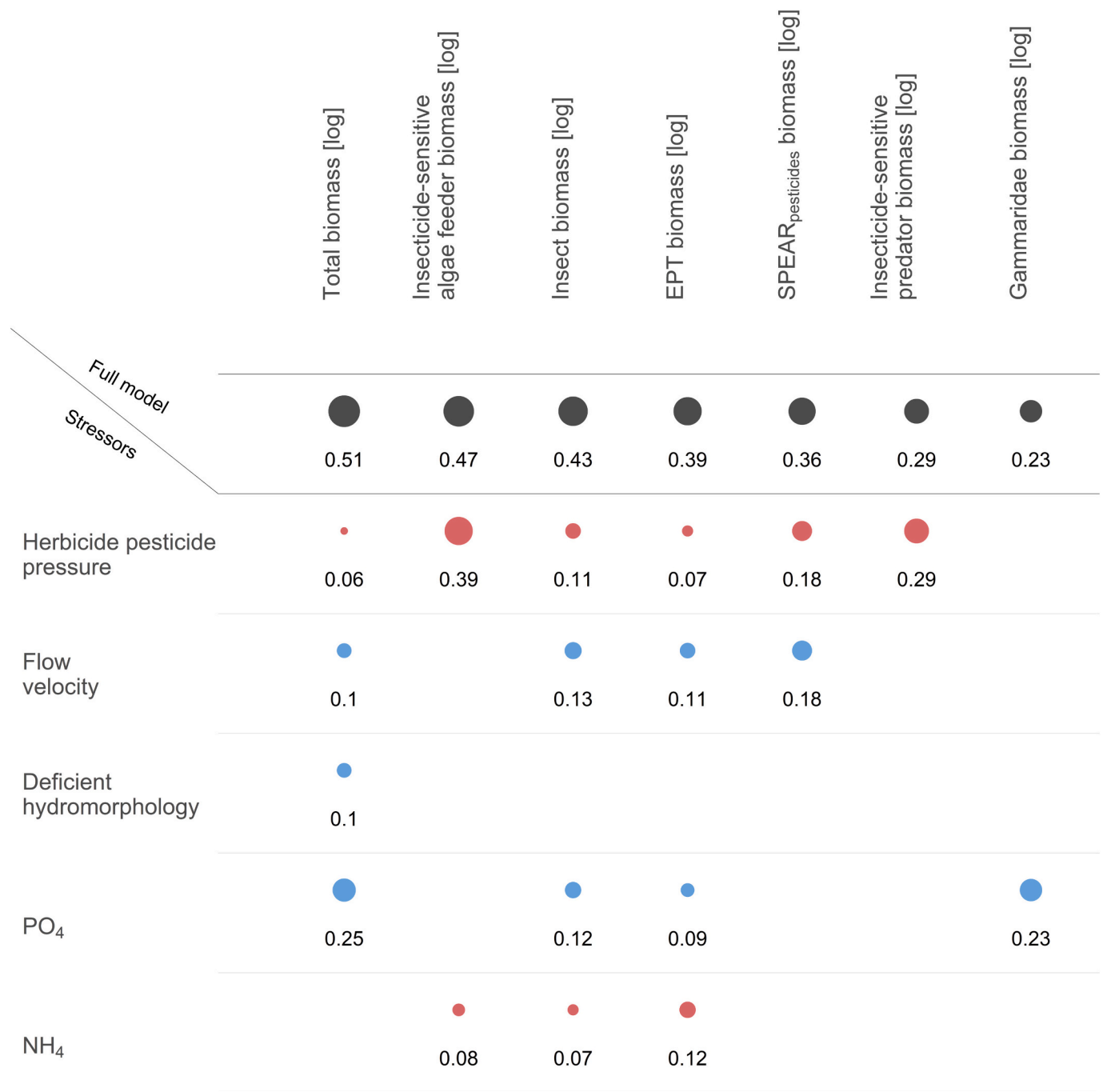
Fig. 1. Linear regression analysis of SPEAR<sub>pesticides</sub> displayed on the y-axis and insecticide pressure (TU<sub>max</sub> log) displayed on the x-axis for the 25 streams investigated in 2021 showing a significant correlation ( $p < 0.001$ ) with an explained variance of R<sup>2</sup> = 0.43.



combined ( $\text{g}/\text{m}^2$ ) were contributed by the following groups: Trichoptera ( $7.99 \text{ g}/\text{m}^2$ ), Crustacea ( $7.5 \text{ g}/\text{m}^2$ ), Gastropoda ( $6.3 \text{ g}/\text{m}^2$ ), Ephemeroptera ( $3.6 \text{ g}/\text{m}^2$ ), Diptera ( $1.7 \text{ g}/\text{m}^2$ ), Coleoptera ( $1.2 \text{ g}/\text{m}^2$ ) and Bivalvia ( $1.1 \text{ g}/\text{m}^2$ ). The average composition of the overall stream biomass showed a fairly balanced biomass proportion among the dominant groups: Trichoptera (27 %), Crustacea (25 %), Gastropoda (21 %) and Ephemeroptera (12 %) (SI Fig. 7). The biomass of the three groups with the highest proportion of total biomass was dominated by one family each: The Lymnaeiidae made up the majority of the

Gastropoda with 86 %, the Gammaridae dominated the biomass of the Crustacea with 85 %, and the Limnephilidae dominated the biomass of the Trichoptera with 87 %. While the biomass of the orders Ephemeroptera and Coleoptera were each mainly characterised by the biomass of two families (Ephemeroptera: Baetidae, Ephemeridae; Coleoptera: Dytiscidae, Scirtidae), the biomass of Diptera was mainly composed of the biomass of the families Simuliidae, Chironomidae and Pediciidae (SI Fig. 7).

A change in biomass can have several causes: (a) an increase in



**Fig. 2.** Relative importance analysis showing the explained variance of the maximum four most relevant stressors per biomass aggregation identified by the elastic net regression. On the y-axis all stressors are displayed showing a significant correlation with at least one of the biomass aggregations. The x-axis shows all biomass aggregations considered in this manuscript. Further biomass aggregations stressor relationships can be found in the SI (SI Figs. 10 and 12). The biomass aggregations were selected according to their relevance in food chain functioning and their sensitivity to pesticides. Red dots represent a reduction of the biomass aggregation with increasing stress, blue dots an increase and black dots represent the full model performance of each biomass aggregation. The number below each dot indicate the explained variance in  $R^2$ . Only explained variances greater than  $R^2 \geq 0.05$  were included in this figure.

abundance, (b) an increase in size of taxa and (c) a combination of (a) and (b). The associations of abundance [log] and biomass of the analysed groups explained a range of 0.17 to 0.48 variance ( $R^2$ ) depending on the group (SI Fig. 9). As the correlation described an average explained variance of 0.2 for most groups, the results suggested a combination of abundance and size relevance (c). The strongest correlations with an explained variance  $>0.4$  were determined for the group of Gammaridae and the group of insecticide-sensitive algae feeders. No correlation was found between total biomass per stream and total abundance per stream.

### 3.3. Associations between aggregations of macroinvertebrate biomass and environmental and anthropogenic variables

#### 3.3.1. Total biomass per stream

In contrast to the effects from insecticides observed on the community structure in terms of SPEAR<sub>pesticides</sub> (Fig. 1), the biomass of all macroinvertebrates per stream (referred to as total biomass) showed no association to insecticide pressure. Instead, a decrease of the majority of various biomass aggregations could be linked to herbicide pressure (Fig. 2; SI Fig. 12).

These were total biomass, shredder biomass, predator biomass, insecticide-sensitive predator biomass, insect biomass, EPT biomass, SPEAR<sub>pesticides</sub> biomass and insecticide-sensitive algae feeder biomass.

The underlying rationale for the relationship between macroinvertebrate biomass and herbicide pressure is that herbicides harm or alter algae communities in streams, resulting in (a) reduced algae biomass and/or (b) changes in algae's community composition. (a) and (b) will then lead subsequently to changes in availability and quality of food for invertebrates, e.g. for functional feeding groups whose diets are partly or entirely based on algae, or indirectly for e.g. predators that consume invertebrates whose diets are based on algae.

The comparison of the biomass-stressor-associations between the use of taxon-specific maximum literature lengths of the taxa (SI Fig. 10) compared to our individual-based measurements (Fig. 2) revealed two major differences: Using the taxon-specific maximum literature lengths, we did not identify any associations between EPT biomass and Gammaridae biomass with the analysed variables. We attribute this to the site-specific biomass-stressor associations relevant to the size of the Gammaridae and thus their biomass. Our results therefore propose that the taxon-specific maximum literature length approach does not properly detect biomass-stressor-associations. As a further example of our conclusion, the biomass-stressor-associations of the total biomass derived from taxon-specific maximum literature length per taxa was only able to detect the strongest stressor-association with  $PO_4$ . Biomass-stressor-associations of biomass aggregations according to pesticide sensitivity, for instance the biomass of SPEAR<sub>pesticides</sub>, were overestimated as the maximum length of some pesticide-sensitive taxa of the order Trichoptera were up to 15 mm longer compared to the actual values identified for individuals from the stream. Individuals may respond to stress by inhibiting growth resulting in a reduced size (Garilli et al., 2015).

A general comparison of the two approaches for the biomass determination per stream ( $g/m^2$ ) showed that biomass based on taxon-specific maximum literature lengths overestimated the real biomass by an average factor of ten (SI Fig. 11). For investigations of biomass in terms of food chain functioning, the taxon-specific maximum literature length approach is accordingly unsuitable. Subsequently, all biomass aggregations refer to biomass derived from the individual lengths of the taxa.

The total biomass was found to be linked to several stressors (Fig. 2). The elastic net regression identified four main variables associated with the total biomass:  $PO_4$ , flow velocity, hydromorphology, and herbicide pressure. A relative importance analysis of these four variables revealed that  $PO_4$  contributed an explained variance of  $R^2 = 0.25$  and both deficient hydromorphology and flow velocity each contributed an

explained variance of  $R^2 = 0.1$  of the full models' explained variance ( $R^2 = 0.51$ ). Herbicide pressure added an explained variance of  $R^2 = 0.06$  to the full models' explained variance.

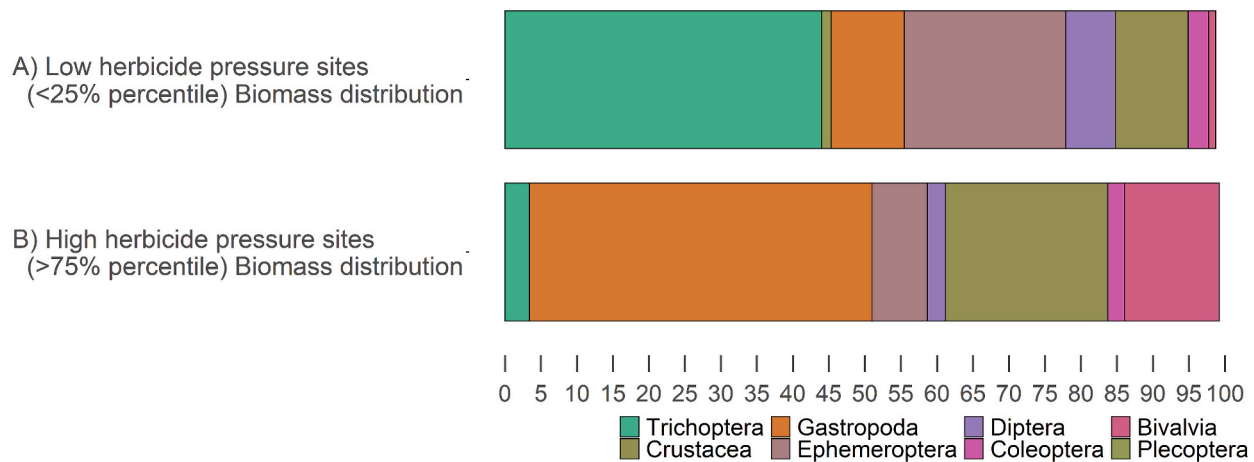
To investigate the effects of herbicide pressure on the biomass in more detail, sites were grouped according to herbicide pressure in order to identify vulnerable groups. In streams with low herbicide pressure ( $TU_{max}$  of herbicide pressure  $< -2.4$ ; six sites), the orders Trichoptera and Ephemeroptera dominated with about 65 % of the overall biomass of the macroinvertebrate communities ( $<25$  % percentile of herbicide pressure; Fig. 3A). In streams with a high herbicide pressure ( $TU_{max}$  of herbicide pressure  $> -1.2$ ; six sites), in contrast, the orders Gastropoda and Crustacea dominated with 70 % the overall biomass ( $>75$  % percentile of herbicide pressure; Fig. 3B). The group-related biomass composition of the streams grouped according to herbicide pressure A and B (Fig. 3) were significantly different in any constellation of A and B with respect to herbicide pressure (paired *t*-test,  $p < 0.05$ ), except for Gastropoda (paired *t*-test,  $p > 0.05$ ). In order to clarify, explain and discuss the relationships between the total biomass per stream and the analysed variables (Fig. 2), an analysis of taxonomic and trait-based biomass groups was carried out.

#### 3.3.2. Taxonomic-based biomass

The aquatic insect biomass is composed of taxa from the orders Ephemeroptera, Plecoptera, Trichoptera, Odonata, Megaloptera, Coleoptera and Diptera. Aquatic insect biomass was positively linked to (i) Flow velocity ( $R^2 = 0.13$ ) and (ii)  $PO_4$  ( $R^2 = 0.12$ ); and negatively linked to (iii) herbicide pressure ( $R^2 = 0.11$ ) and (iv)  $NH_4$  ( $R^2 = 0.07$ ; Fig. 2). The association of the aquatic insects as well as the EPT biomass with slightly different extend to the four variables identified (i) may be due to their lotic preferences (Chanut et al., 2019) and due to some Limnephilidae taxa with higher biomass tending to prefer more rheophilic habitats (Waringer et al., 2020), (ii) is caused by their benefit from a possible  $PO_4$  induced increase aquatic mosses growth (Riis, 2010) or  $PO_4$ -enriched leaves as food source, when algae are scarce (Glime, 2017), (iii) can be explained by herbicide induced algae food limitation and (iv) is due to the sensitivity to the toxicity caused by  $NH_4$  (Frieburg et al., 2010).

Aquatic insects as food source for aquatic predators (Anderson, 2009; Dudgeon and Richardson, 1988) and emerging aquatic insects are essential as a nutritional base of higher trophic levels. Due to their long-chain polyunsaturated fatty acids emerged aquatic insects are particularly attractive predators such as birds (Twining et al., 2018), spiders (Collier et al., 2002; Kraus et al., 2014), and bats (Fukui et al., 2006). Thus, aquatic insect biomass links aquatic and terrestrial ecosystems through the reallocation of energy and biological matter (Ohler et al., 2022; Wieczorek et al., 2015) and is relevant for food chain functioning. Our results suggest that in streams with high herbicide pressure, less essential food in terms of aquatic insect biomass may be available for higher trophic levels in streams and therefore suggest also for higher trophic levels surrounding surface waters.

Gammaridae play an important role in the detritus cycle of aquatic ecosystems (e.g. litter breakdown processes; Gerhardt et al. (2011)) and constitute an important element in food chain functioning (Chaumot et al., 2015) as prey for predators such as fishes (MacNeil et al., 1999) and other invertebrates. As Gammaridae are widespread, abundant and large taxa (Chaumot et al., 2015) with a high proportion of biomass per stream in this study (Fig. 2), their biomass-stressor-associations therefore may partly explain the total biomass-stressor-association with  $PO_4$  described before. The elastic net analysis followed by a regression analysis identified  $PO_4$  as the only environmental parameter linked positively to the Gammaridae biomass ( $R^2 = 0.23$ ). Major anthropogenic sources of  $PO_4$  in streams include runoff from agricultural sites as a result from fertilizer applications for agricultural plant growth; and partially treated or untreated sewage (Everall et al., 2019) or household sewage (Völker et al., 2022). In streams nutrients such as  $PO_4$  stimulate the growth of phytoplankton and benthic microalgae providing one food



**Fig. 3.** Percentage composition of relative biomass of macroinvertebrate groups in 25 streams. A) Sites with low herbicide pressure (<25 % percentile). B) Sites with high herbicide pressure (>75 % percentile). The composition of taxa is significantly different (paired *t*-test,  $p < 0.05$ ) between A–B). Macroinvertebrate groups (Odonata, Hirudinea, Turbellaria, Oligochaeta, Megaloptera, Heteroptera) that contributed each <1.5 % to the overall biomass composition of each A and B were not considered in the figure.

source for Gammaridae (Utsunomiya et al., 2017). Furthermore, the positive effect of nutrient-stimulated bacteria and fungi growth on leaves can make them particularly attractive for consumption by gammarids (Arsuffi and Suberkropp, 1985; Cummins, 1974; Schlieff and Mutz, 2006). While Rastetter et al. (2017) stated that the mortality of Gammaridae increased after exposure to triple superphosphate, a phosphate fertilizer, Crenier et al. (2019) found a significant positive relationship between the biofilm phosphate-content and gammarids growth. Demi et al. (2018) also indicated that phosphate content of leaf litter, is a key driver of the biomass and production of macroinvertebrate consumers. The results from our study, thus, suggest indirect positive effects through  $PO_4$ -enhanced nutrition for Gammaridae.

Further, we revealed that Gastropoda biomass was not linked to any of the anthropogenic and environmental variables studied. This may be linked to the fact that Gastropoda are known to be tolerant toward low substrate and morphological diversity (Berger et al., 2018) and also most pesticides (Liess and van der Ohe, 2005).

### 3.3.3. Trait-based biomass

The biomass of four of the five investigated feeding groups - gatherer, shredder, grazer, predator and filterer - showed a positive correlation with  $PO_4$  ranging from a  $R^2$  of 0.07 to 0.21 (SI Fig. 12). Only the biomass of the grazers showed no association with any of the analysed factors. The biomass of shredders ( $R^2 = 0.21$ ) and filterers ( $R^2 = 0.08$ ) further showed a positive correlation with  $O_2$ . The filterer biomass was also associated with pH ( $R^2 = 0.13$ ). The shredder ( $R^2 = 0.11$ ) and predator biomass ( $R^2 = 0.25$ ) showed a negative correlation with herbicide pressure. To investigate and unravel the association with herbicide pressure, a more in-depth analysis of insecticide-sensitive taxa within the feeding groups was investigated. The biomass of two of those distinct feeding sub-groups were affected by herbicide pressure: The insecticide-sensitive algae feeder biomass and the insecticide-sensitive predator biomass.

The insecticide-sensitive algae feeder biomass composed of taxa categorised as algae feeder (combined grazer and shredder  $\geq 5$ ) and sensitive to pesticides (SPEAR<sub>pesticides</sub> trait *pesticide sensitivity*; Liess and van der Ohe (2005)) were dominated by *Chaetopteryx* sp., and *Lepidostoma* sp. The insecticide-sensitive algae feeder biomass was linked to two anthropogenic stressors. Herbicide pressure ( $R^2 = 0.39$ ) and  $NH_4$  enrichment ( $R^2 = 0.08$ ) were each negatively associated with the insecticide-sensitive algae feeder biomass (Fig. 2). The  $NH_4$  concentrations found in 32 % of the samples (grab sample or EDS) from the 25 streams exceeded the surface water threshold of 0.466 mg/L (Surface

Water Ordinance - OGewV, 2020), potentially causing an ecological effect. The negative association trend with  $NH_4$  enrichment can thus possibly be explained by the sensitivity of several taxa of the EPT orders to the toxicity caused by  $NH_4$  (Friberg et al., 2010). Herbicide pressure is known to negatively affect the growth of algae inhabiting on the surfaces of leaves and stones (Stenström et al., 2021). Taxa categorised as insecticide-sensitive algae feeder in this study rely among others on the growth of algae in streams, as they feed on periphyton by grazing on surfaces (Cummins, 1974; Cummins and Klug, 1979) as well as by feeding directly on leaf tissues covered with algae while converting coarse matter into small particles (Aguilar et al., 2017; Graça et al., 2015; Wallace and Webster, 1996; Webster and Benfield, 1986). Our results therefore suggest indirect effects of herbicide pressure on the biomass of insecticide-sensitive algae feeders through food limitation. Food limitation occurring as a result of herbicide-induced reduction of algae production as a food source for insecticide-sensitive algae feeder. We conclude that the combined direct effects of insecticide pressure and indirect effects of herbicide pressure represent multiple stress under field conditions. The second distinct feeding sub-group negatively associated with herbicide pressure, the insecticide-sensitive predators' biomass, comprise taxa belonging to the orders Megaloptera, Plecoptera and Trichoptera identified according to the SPEAR<sub>pesticides</sub> trait *relative pesticide sensitivity* and their feeding preferences (predator  $\geq 5$ ). The elastic net regression revealed herbicide pressure as solely stressor associated with insecticide-sensitive predators' biomass: ( $R^2 = 0.29$ ; Fig. 2). Insecticide-sensitive predators' prey on insect larvae such as Trichoptera and Megaloptera (Anderson, 2009; Dudgdon and Richardson, 1988), Annelida, Crustaceans and Molluscs largely depending on algae consumption. Our results suggest that herbicide-disturbed algae communities support a reduced number of algae consuming prey species. Accordingly, herbicide pressure may indirectly affect their prey occurrence, growth rate, and body size presumably reducing the predators' food availability.

As a further trait-based biomass aggregation, the biomass of the SPEAR<sub>pesticides</sub> taxa was associated with two factors: Herbicide pressure and flow velocity. Each contributed an explained variance of  $R^2 = 0.18$  to the full models' explained variance ( $R^2 = 0.39$ ; Fig. 2). These biomass-stressor-associations therefore may partly explain the total biomass-stressor-association with herbicide pressure and flow velocity described before.

As the proportion of vulnerable species is generally reduced by insecticide pressure (Fig. 1), an association with insecticide pressure was expected for the biomass of SPEAR<sub>pesticides</sub> taxa. No direct correlation,

however, has been identified in the multiple regression analysis for the SPEAR<sub>pesticides</sub> biomass. Instead, a negative association with herbicide pressure was found which can be explained by several taxa of the SPEAR<sub>pesticides</sub> biomass belonging to the group of insecticide-sensitive algae feeders. The positive association with flow velocity can be explained by several SPEAR<sub>pesticides</sub> taxa belonging to the EPT orders, many of which have a higher preference for rheophilic habitats (Chanut et al., 2019; Meyer and Schröder, 1985). Our results from the analysis of 25 streams suggest that in contrast to the abundance-based SPEAR<sub>pesticides</sub> indicator, the biomass of SPEAR<sub>pesticides</sub> taxa relates to a pollution of streams with herbicides and again herbicide-induced algae food limitation.

#### 4. Conclusion

- The structure of the invertebrate community responded to insecticide pressure with a reduced abundance of vulnerable insects.
- In contrast, insect biomass of insecticide-sensitive algae feeder and predators was determined by the combined effect of herbicide and insecticide pressure.
- The total biomass of macroinvertebrates and Gammaridae biomass were positively associated with PO<sub>4</sub> enrichment in streams.
- For subsequent biomass investigations, we recommend analysing the biomass of algae and community composition of algae in parallel to the macroinvertebrate sampling of streams in order to be able to differentiate results with regard to herbicide pressure.

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#### CRediT authorship contribution statement

**Liana Liebmann:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Verena C. Schreiner:** Writing – review & editing, Data curation. **Philipp Vormeier:** Writing – review & editing, Investigation, Data curation. **Oliver Weisner:** Writing – review & editing, Investigation, Data curation. **Matthias Liess:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

#### 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

I have shared my data in the EXCEL Workbooks.

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