Quantitative metabarcoding reveals the effects of ecological factors and invasive species on functional diversity of freshwater insects

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Abstract

Aquatic insects are the most biodiverse freshwater animals, yet they are less studied than other taxa. Responses of aquatic insects to stressors are rarely assessed and usually measured in terms of taxonomic richness derived from presence-absence data, despite certain mechanisms may cause a reduction in the biomass of certain species, without causing their disappearance. The integration of taxonomic and functional measures with biomass is often disregarded due to methodological difficulties, particularly concerning aquatic insects. Traditional survey methods for insects can require considerable effort, thus metabarcoding can be a useful tool for ecological investigations, but it lacks information on biomass.

We monitored 44 waterbodies in Northern Italy in 2021 – 2022, measuring ecological characteristics, including the presence of the invasive crayfish *Procambarus clarkii*, and collecting insect specimens. We weighted insect specimens before DNA extractions. The relationships between metabarcoding data and biomass were then used as correction factors to retrieve biomass of the seven insect orders in our communities. Then we calculated indices of taxonomic and functional diversity and assessed the effects of ecological parameters on functional traits using fourth-corner analysis.

We found weak negative effects of *P. clarkii* on taxonomic diversity, while functional diversity resulted strongly affected by it. Traits associated with mosquitos were negatively associated by *Rana* tadpole presence. Functional diversity was more sensitive to *P. clarkii* presence than taxonomic diversity, and its effects were different among different functional traits. These results underline the importance of considering functional diversity changes to get a comprehensive assessment of aquatic insects' responses to stressors.

Introduction

Freshwater environments cover less than 1% of Earth's surface but host a disproportionate share of biodiversity, including 6-10% of all described species (Dudgeon et al. 2006; Faghihinia et al. 2021). These habitats provide ecosystem services for billions of people in the form of water quality regulation, recreation, and food resources from aquatic organisms (Ostfeld et al. 2012; Maltby 2013; Hayat & Gupta 2016). Unfortunately, freshwater habitats are suffering faster biodiversity loss than any other biome (Dudgeon et al. 2006; Strayer & Dudgeon 2010; Dudgeon 2019). Human activities have caused a reduction in global freshwater biodiversity (Vörösmarty et al. 2010) and one-quarter of freshwater animals are threatened with extinction. Yet, conservation prioritization remains focused on terrestrial fauna (Sayer et al. 2025).

Biological invasions are a major stressor in freshwater habitats, significantly impacting local biodiversity. Freshwater environments appear to be more sensitive to biological invasions than their terrestrial counterparts (Sala et al. 2000; Strayer 2010; Moorhouse & Macdonald 2015; Sayer et al. 2025). Invasive alien species (IAS) can affect freshwater communities through different mechanisms, such as predation, competition, transmission of diseases, hybridization, and habitat modification (Ficetola et al. 2012; Gallardo et al. 2016; Hata et al. 2019; Manenti et al. 2019; Falaschi et al. 2020; Lejeune et al. 2024) and these alterations determine major impacts on the functions provided by freshwater environments.

The effects of IAS on freshwater communities are complex and multi-layered thus, to understand these impacts, we need to consider the different facets of diversity. The responses of aquatic communities to environmental stressors are frequently assessed focusing on taxonomic diversity (e.g., de Paiva et al. 2021). Alterations in taxonomic diversity are often expected to be aligned with changes in functional diversity (Schmera et al. 2017), since the local extinction of a given species can cause a reduction in the functional diversity of the community, with consequences on ecosystem functions and services (Covich et al. 2004; Cao et al. 2018). However, in certain cases, the link between taxonomic diversity and ecosystem functionality is not straightforward. On the one hand, if multiple species share the same combination of traits ("functional redundancy"), changes in taxonomic diversity might not be accompanied with changes in functional diversity (Fetzer et al. 2015). On the other hand, the extinction of non-redundant species with specialised traits may have small effects on taxonomic diversity but disproportionate impacts on functional diversity, as their unique ecological roles cannot be fulfilled by other species. Therefore, taxonomic diversity may not always represent the best measure to assess ecosystems' responses to environmental stressors and should be complemented with measures of functional diversity (de Bello et al.

2010; Cadotte et al. 2011; Li et al. 2021). Nevertheless, variation of functional diversity is still under-investigated in freshwater habitats (Schmera et al. 2017), challenging to draw general conclusions on the impacts of IAS on freshwater functionality.

Freshwater macroinvertebrates can be heavily affected by biological invasions, with consequences on their functional diversity (Mathers et al. 2020; Guareschi et al. 2021). Aquatic insects represent the most species-rich taxon in freshwaters and dominate the macrozoobenthos (Balian et al. 2008; Dijkstra et al. 2014; Del-Claro & Guillermo 2019). Importantly, they fill most ecological roles and interact with different taxonomic groups, being thus pivotal for ecosystem functioning (Fenoglio et al. 2014; Suter & Cormier 2015). Still, analyses of the impact of IAS on the functional diversity of freshwater insects remain scarce (Mathers et al. 2020). Multiple factors concur in determining this scarcity of studies. First, aquatic insects cannot always be morphologically identified in the field at a high taxonomic resolution. Standard monitoring procedure requires specimen collection and preservation, followed by sorting and laboratory examinations (Santos & Fernandes 2021). This process is labour-intensive and time-consuming, and does not always grant species-level identification as, for some taxa, morphological features are not sufficient to differentiate closely related species, or they are only applicable to a specific life stage (Orlofske & Baird 2013; Luiza-Andrade et al. 2017; Czechowski et al. 2020). Moreover, there is no uniformity in the terminology used to identify functional traits, existing datasets do not always provide information on how a certain trait has been assigned to a specific taxon, and we lack comprehensive databases of species and their associated functional traits (Tachet 2010, Luiza-Andrade et al. 2017).

Metabarcoding is an efficient tool for addressing challenges in aquatic macroinvertebrate monitoring (Martins et al. 2020; Meyer et al. 2021). For instance, it has the potential to detect species that can hardly be identified morphologically. However, its potential for the management and conservation of freshwater insects is not fully exploited (Belle et al. 2019; Czechowski et al. 2020). One key limitation is its difficulty in quantifying species abundance or biomass, which are key measures of response of species and communities to stressors, and heavily affect the functionality of ecosystems. In certain cases, species richness is less sensitive than their biomass to ecological stressors (Svenningsen et al. 2024). Thus, using biomass to weight measures of taxonomic and functional richness may allow a more comprehensive view of community responses than presence-absence data. This is relevant when assessing and monitoring the effects of biological invasions, as IAS sometimes lead to reductions in the abundance or biomass of native species (Gallardo et al. 2016; Kopf et al. 2019) without necessarily causing local extinctions.

Some studies have proposed using the number or the proportion of metabarcoding reads as proxies for the estimation of species abundances or biomasses (Liu et al. 2020), still the relationship between abundance/biomass measures and number of reads is sometimes weak and show high degrees of uncertainties (Lamb et al. 2019). Moreover, primers can sometimes be more efficient in amplifying certain taxa than others, a phenomenon often referred to as ‘primer bias’ or ‘amplification bias’ (Clarke et al. 2014; Elbrecht & Leese 2015; Piñol et al. 2019; Meyer et al. 2021), and primers can show differences in amplification rate across orders (Ficetola et al. 2021). It is thus difficult to get reliable assessments of species abundance and biomass from metabarcoding data (Elbrecht & Leese 2015). A possible approach is the use of mock communities, i.e., defined combinations of different species to simulate the composition of potential communities, to determine correction factors for each species (Krehenwinkel et al. 2017). However, the definition of species-specific adjustments requires considerable effort, and is often impractical due to incomplete species lists in study areas. This is particularly challenging for species-rich taxa, such as insects. Higher taxonomic levels (e.g., orders) can be a suitable target for the development of adjustment factors, given that identification of individuals at the order-level is generally easy.

Here, we decided to unravel the effects of IAS on the communities of aquatic insects, accounting for impacts on taxonomic diversity, functional diversity, and biomass. We focused on the impacts of one of the most problematic freshwater IAS, the red swamp crayfish *Procambarus clarkii* (Nentwig et al. 2018). *Procambarus clarkii* is native to the south-eastern United States and northern Mexico and has become invasive in all continents but Antarctica and Oceania (Loureiro et al. 2015). It is a generalist species capable to tolerate great variations in water temperature and salinity, to exploit various feeding sources and to survive prolonged droughts (Alcorlo et al. 2004; Casellato & Masiero 2011; Bissattini et al. 2015; Peruzza et al. 2015; Kouba et al. 2016). Its presence has been reported to affect presence, densities, and community compositions of macrophytes, vertebrates, particularly amphibians, and macroinvertebrates, including aquatic insects (Ficetola et al. 2012; Carreira et al. 2014; Falaschi et al. 2021; Watanabe & Ohba 2022). *Procambarus clarkii* is an ecological engineer capable of modifying the occupied habitats causing system-wide shifts in freshwater environments (Matsuzaki et al. 2009). Given its plasticity, this crayfish can have different effects on different taxonomic and functional groups, determining contrasting patterns even between geographically close waterbodies (Klose & Cooper 2012).

To measure the impacts of *P. clarkii* on insect communities, we used a multi-step framework, integrating quantitative measurements of biomass with DNA metabarcoding. First, we obtained quantitative samples, representing the variation of insect distribution and biomass across sites with varying invasion status. Second, we assessed whether DNA metabarcoding provides good estimates of the relative biomass of the different insect taxa across sites. Third, we evaluated how the occurrence of IAS and environmental features determine different characteristics of insect communities (biomass, taxonomic and functional diversity, functional traits). We hypothesized that *P. clarkii*, a generalist IAS that can modify trophic webs and ecological conditions in the colonized ecosystems, may be reflected by a reduction in functional diversity of insects, without necessarily affecting their taxonomic diversity. Moreover, we expected *P. clarkii* presence to affect certain functional traits of insects more than others, as its impacts may be particularly relevant on outcompeted species, preys, and species linked to ecological characteristics that are modified by the presence of the crayfish, e.g., water turbidity and macrophyte abundance (Rodríguez et al. 2003; Carreira et al. 2014).

Materials and methods

Study system

We monitored 44 ponds in Lombardy, Northern Italy, between May 2021 and May 2022. The study area is mostly occupied by urban and agricultural areas, but many of the surveyed waterbodies are located within or next to regional parks and other protected areas (Fig. 1). Biological invasions in freshwater environments are particularly relevant in Northern Italy (Gherardi et al. 2008). *Procambarus clarkii* was first observed in the study area in the early 2000s and is nowadays widespread across the whole of Lombardy due to both natural dispersion and subsequent introduction events (Lo Parrino et al. 2019; Melotto et al. 2020; Falaschi et al. 2021).

Each site was monitored from two to seven times between February and May through visual surveys to identify presence/absence of the invasive crayfish, fish, and tadpoles (mostly tadpoles of the brown frogs, *Rana dalmatina* and *R. latastei*). Tadpoles represent an important food source for both the crayfish and large predatory insects (Chovanec 1992; Klecka & Boukal 2012; Del-Claro & Guillermo 2019). Moreover, certain aquatic insects and tadpoles compete for resources (Morin et al. 1988). During the last survey, a pipe-sampling was performed to collect aquatic insects. Pipe-sampling was performed by thrusting a 0.25 m^2 circular sampler into the sediment to seal the sampling area, following Ficetola et al. (2011). During each sampling, we removed all animals through dip-netting in the water column until 10 consecutive empty net sweeps (Werner et al. 2009; Ficetola et al. 2011). For each site, from two to six pipe samplings were performed, proportionally to the waterbody's surface and in order to encompass the different microhabitats within each site. We performed a total of 136 pipe samplings across the 44 monitored sites (mean = 3.18, SD = 1.10 pipe samplings per site). Additionally, we also recorded site surface, maximum depth, hydroperiod (whether the site retained water for the whole study season), and aquatic vegetation (expressed as the percentage of site surface occupied by aquatic micro- and macrophytes).

Insects were preserved in 80% ethanol for subsequent analyses, while amphibian larvae were counted, weighted, and released. All the insects collected in the same site were pooled in the same sample. After data collection, we visually classified the insect specimens of each sample at the order level and measured their dry weights to obtain a biomass measure (scale accuracy: 0.01 g).

Molecular and bioinformatics procedures

We performed 44 DNA extractions from bulk samples collected in each site using the QIAGEN DNeasy Blood & Tissue Kit and following the producer's protocol. For DNA amplification, we used the Inse01 primer (forward: RGACGAGAAGACCCTATARA; reverse:ACGCTGTTATCCCTAARGTA), targeting a mitochondrial 16S mitochondrial rDNA region (Elbrecht et al. 2016; Taberlet et al. 2018). PCRs were performed in a final volume of 20 μL: 2 μL of template DNA, 2 μL of the mixture of forward+reverse primer at a concentration of 5 μM, 0.16 μL of BSA (Thermo Scientific), 10 μL of AmpliTaq Gold 360 Master Mix 2× (Applied Biosystems). The PCR mixture was denatured at 95°C for 10 min, followed by 40 cycles of 30 s at 95°C, 30 s at 52°C and 1 min at 72°C, and a final elongation at 72°C for 7 min. PCRs for each sample were replicated four times (Ficetola et al. 2015; Guerrieri et al. 2022). We included 5-PCR negative controls and 42 bioinformatic blanks. The resulting amplicons were purified using the MinElute PCR Purification Kit (Qiagen) following the manufacturer's protocol. The purified amplicons were sent to Fasteris (Switzerland), where libraries were prepared following the MetaFast protocol and sequenced using the Illumina HiSeq 2500 platform with a paired- end approach (2 × 150 bp).

The sequencing output was processed using the OBITools software (Boyer et al. 2016), following the same protocol as (Meyer et al. 2021). Sequences were dereplicated using the obiuniq program, and we removed bad-quality sequences (i.e. sequences containing "N"), sequences shorter/longer than expected (minimum length = 75 bp, maximum length = 265 bp), and singletons. Potential PCR or sequencing errors were removed using the obiclean program by only keeping sequences tagged as "heads" in at least one PCR replicate. We clustered sequences into Molecular Operational Taxonomic Units (MOTUs) using the *SUMACLUST* program (Mercier et al. 2013) with a threshold of 0.96. This threshold minimizes the probability that different individuals of the same species are assigned to different MOTUs while reducing the risk that distinct species are merged in one single MOTU (Bonin et al. 2023). Then, we built a sequence reference database from EMBL (version 140) using the ecoPCR program (Ficetola et al. 2010) with the Inse01 primer pair, allowing up to three mismatches per primer. We retained only sequences assigned at the species, genus, and family levels. For taxonomic assignment, we used the *ecoTag* program and removed MOTUs with < 90% percentage identity with any matching sequence of the reference database. A minimum number of reads was required to retain a MOTU, and we selected the threshold that allowed us to remove ≥ 99.9% of sequences detected in PCR blanks. MOTUs found in only one sample are often spurious sequences, thus we removed them. MOTUs present in less than two replicates per sample were filtered out, as well as MOTUs present in more than one PCR negative control.

Relationships between biomass and proportion of reads

To evaluate whether the proportion of reads can be considered a good proxy of the proportion of biomass within each sample, we assessed the relationship between the relative abundance (biomass) of each order and the proportion of reads attributed to that order. The relationship was tested using generalized linear models (GLMs). In GLMs, the dependent variable was the proportion of biomass attributed to each order in each sample, measured by weighting the specimens of insects attributed to the different orders. Taxonomic order and the proportion of reads assigned to that order in that sample were the independent variables. For the proportion of reads, we tested three alternative transformations: i) untransformed proportion; ii) proportion of the log-transformed number of reads and iii) Wisconsin double standardization (also named “eDNA index”, Kelly et al. 2019). Models also tested the interaction between taxonomic order and proportion of reads; a significant interaction between order and proportion of reads would indicate that the relationship between actual abundance and proportion of reads is different across taxonomic groups. Such a relationship is expected, for instance, if different taxa show differential amplification success, as previously observed across orders of freshwater insects (Ficetola et al. 2021). The dependent variable (proportion of biomass) followed a zero- and one-inflated beta distribution, thus we run models using a beta error distribution, after transforming zero- and one- values following Smithson & Verkuilen (2006). The Akaike's information criterion (AIC) was used to identify the model structure best representing the data, assessing the three alternative data transformations. Beta regression models were run using the betareg package (Cribari-Neto & Zeileis 2010). The goodness of fit of the GLM was measured using the squared correlation between the linear predictors and link-transformed response (pseudo-R^2).

We then used the relationship between biomass and DNA reads, identified for each order by the best-AIC model, to estimate the relative biomass of each MOTU in each sample. This approach is expected to correct the relative abundance of MOTUs for the amplification bias of each order (Krehenwinkel et al. 2017). Previous works applied species-specific correction factors, estimated from analyses on mock communities (Krehenwinkel et al. 2017; Lamb et al. 2019), but species-specific correction factors are not feasible in our study case because the species list was unknown before running the molecular analyses, and several species of aquatic insects cannot be morphologically identified from their larvae (Siesa et al. 2014). Relative biomass of each MOTU at each site was then multiplied by the total insect biomass/m^2 at that site, to obtain measures of the actual biomasses of each MOTU.

Functional traits

We selected a set of insect functional traits for which a-priori information suggests potential impacts by invasive crayfishes. Traits were selected from the ones categorized in Tachet et al. (2010) and were:

- Body size. *Procambarus clarkii* is a large predator that is expected to show particularly strong impact on the largest aquatic invertebrates (Klose & Cooper 2012). We considered three body size categories: 2.5-10 mm, 10-40 mm, 40-80 mm.
- Length of life cycle. Insects with larvae that spend long time in water before metamorphosis may be exposed for longer to predation (McPeek 1990), thus they can be particularly sensitive to *P. clarkii* presence.
- Aquatic states. Species with discrete life stages, such as most aquatic insects, usually go through ontogenetic niche shifts that can affect their responses to environmental disturbances and modulate interactions with other species (Nakazawa 2015; Kingsolver & Buckley 2020). Previous studies have reported that species with complex life cycles may be particularly sensitive to the presence of *P. clarkii* (Siesa et al. 2014). We assigned to each MOTU all the possible ontogenetic states found in water (possible stages: eggs, larvae, nymphs, and adults).
- Diet. *P. clarkii* is a generalist omnivorous species able to modify food preferences according to food availability (Alcorlo et al. 2004). We expected different effects of *P. clarkii* presence on insects with different feeding preferences due to competitive interactions and to the higher amount of suspended organic matter in invaded wetlands.
- Preferred microhabitat(s). *P. clarkii* reduces the density of macrophytes and leaf mass, potentially reducing shelter availability for aquatic insects (Matsuzaki et al. 2009; Carreira et al. 2014; Carvalho et al. 2016; Watanabe & Ohba 2022). We expected

strong negative relationships between *P. clarkii* presence and biomass of insects exploiting macrophytes and leaf litter as preferred microhabitats.

- Locomotion and relation with the substrate. *P. clarkii* mostly preys at the bottom of wetlands, thus insects living on water surface are expected to be less affected by its presence than those found on the substrate (Watanabe & Ohba 2022).

For all traits, a fuzzy coding approach was used to describe the considered characters of each MOTU (Usseglio-Polatera et al. 2000). Several MOTUs (48%; see results) were only identified at the genus or family level. In this case, we averaged traits of all species grouped within that taxon (Cantera et al. 2025; Guerrieri et al. 2024). All details are available in Supplementary Materials (S1).

Analysis of relationships between *P. clarkii* and insect communities

With DNA metabarcoding data, biodiversity estimates obtained using Hill's numbers can provide more robust estimates of the actual alpha-diversity (Calderón-Sanou et al. 2020; Mächler et al. 2021). Therefore, we estimated taxonomic alpha diversity using the function hill_taxa from package hillR (Li 2018). Namely, we calculated the first three Hill's numbers, corresponding to species richness (Hill's number Q = 0), the exponential of Shannon's entropy index (Q = 1), and the inverse of Simpson's concentration index (Q = 2) (Chao et al. 2014).

To estimate functional diversity, we calculated a matrix of Gower distances (de Bello et al. 2021) between MOTUs based on the assigned functional traits using the func.dist function from the mFD package (Magneville et al. 2022). Three indices of functional alpha diversity applied to distances between taxa were calculated using the alpha.fd.hill function from the same package. Also for functional diversity, we calculated richness, the Shannon index, and the inverse Simpson index based on an extension of the Hill numbers framework (Chao et al. 2019).

We used standard linear models to assess the relationships between the features of insect communities of each site and environmental features. As dependent variables, we considered total insect biomass, taxonomic diversity (estimated using richness, the Shannon and the inverse Simpson indices), and the estimates of functional diversity (richness, the Shannon and the inverse Simpson indices). All dependent variables were log-transformed before the analyses to increase normality. As independent variables, we used site depth, hydroperiod, *P. clarkii* presence, fish presence, tadpole presence, and aquatic vegetation. Correlation between independent variables was checked before analyses and all Pearson correlation coefficients were < |0.66|.

We used the fourth corner analysis to assess whether *P. clarkii* presence and environmental features determine variation of the functional traits of communities (Dray & Legendre 2008). The fourth corner statistic assesses the link between three tables: a table L containing abundance data of the sampled taxa for each site, a table R containing the environmental characteristics of the sites, and a table Q containing the functional traits associated with the focal taxa. We used the fourthcorner function of the ade4 package (Dray & Dufour 2007) with 9999 permutations. We selected the sixth permutation model, that combines the outputs of permutation of entire rows of table L (values of sites) and permutation of entire columns of table L (values of taxa). This permutation model reduces the risk of Type I errors compared to the available alternatives (Dray & Legendre 2008; Ter Braak et al. 2012). To avoid the increased rate of Type I errors caused by testing simultaneously a family of hypotheses, we controlled the false discovery rate at the 0.05 level (Benjamini & Hochberg 1995; Benjamini & Yekutieli 2001).

Results

We collected insect specimens from all the sampled sites. On average, we collected 2.4 g of insects per site (SD = 3.3) from 7 insect orders (Fig. 2). Odonata (dragonflies and damselflies) accounted for the majority of biomass (56.5%), followed by Coleoptera and Diptera (17.1% and 16.4%, respectively). The other observed orders were Hemiptera, Trichoptera, Ephemeroptera, and Megaloptera. DNA metabarcoding yielded a total of 574 raw sequence reads. After clustering and removal of spurious sequences, we obtained 67 MOTUs of aquatic insects, with an average of 7.7 MOTUs per site (range: 1-21; SD = 4.1). Among these MOTUs, 35 (52.2%) were identified at the species level, 22 (32.8%) at the genus level, and 10 (14.9%) at the family level. Odonata was the most represented order in terms of the number of MOTUs (28 MOTUs), followed by Coleoptera and Diptera (19 and 11 MOTUs, respectively; Fig. 2).

Relationship between relative biomass and proportion of reads

We found a very strong relationship between the relative biomass of the different insect orders and the proportion of reads. The relationship was evident for all the considered transformations of metabarcoding data but was particularly strong when using the proportion of the log-transformed number of reads (Table 1). This model explained a very high proportion of the variation in biomass across sites (pseudo-R^2 = 0.66). The likelihood-ratio tests showed that the interaction between the proportion of reads and the taxonomic orders, indicating that the rate of increase in the proportion of reads at higher biomass was different across orders

(Fig. 3). Also in this case, model fit was higher when using the proportion of log-transformed reads as the dependent variable, thus we selected this approach for the following steps. We transformed the estimated slopes from the logit scale to the response scale, then we divided the proportions of log-transformed reads per order by their corresponding order-specific slopes (Krehenwinkel et al. 2017). Finally, we multiplied these values by the total biomass/m^2 of each site to obtain measures of the actual biomass of each MOTU.

Drivers of biomass, taxonomic, and functional diversity

The biomass of aquatic insects showed strong variation across sites and was negatively related to fish presence (β = -1.905, p = 0.012), while it was not related to the other considered variables (Fig. 4). The three indices of taxonomic diversity obtained using Hill's numbers were highly correlated (all r > 0.99), and the same was found for the indices of functional diversity (r > 0.97). Here we report the results for the Shannon index (Q = 1) calculated for both taxonomic and functional diversity; relationships with Q = 0 and Q = 2 are very similar (Table S1). Taxonomic diversity increased with the cover of aquatic vegetation (β = 0.168, p = 0.048). We found a negative effect of *P. clarkii* presence on taxonomic diversity (β = -0.372, p = 0.055), and a positive effect of water depth (β = 0.309, p = 0.092), although neither relationship was significant at the 0.05 level. The model explained a good amount of variation (adjusted R^2 = 0.211).

Functional diversity was strongly correlated with the number of species (r = 0.83). This index showed a very strong, negative relationship with *P. clarkii* presence (β = -0.511, p = 0.008) and tended to increase in the deepest sites, but this relationship was not significant at the 0.05 level ($\beta = 0.329$, p = 0.068). The model explained a good amount of variation (adjusted $R^2 = 0.262$).

Fourth corner analysis

The fourth corner analysis suggested that variation of functional traits was strongly related to environmental variables, which had heterogeneous effects across traits (Fig. 5).

The presence of *Procambarus clarkii* negatively affected insects laying eggs in water (F = 5.574, p = 0.030; all observations were standardized, and p-values are adjusted for false discovery rate), insects exploiting leaf litter (F = 7.307, p = 0.015), as well as insects swimming in the water (F = 5.256, p = 0.026).

Tadpole presence was negatively related to the presence of small insects (F = 5.532, p = 0.027), insects with aquatic nymphs (F = 5.928, p = 0.026; this trait mostly occurs in Diptera and Trichoptera; Tachet et al. 2010), insects feeding on debris (F = 5.094, p = 0.039) and macrophytes (F = 10.646, p= 0.004), and to surface dwellers (F = 10.413, p = 0.005). Conversely, predatory insects and crawlers were positively associated with tadpole presence (F = 8.667, p = 0.009).

Insect body size showed strong relationships with hydroperiod. Small-sized insects (2.5-10 mm) were more common in temporary sites (F = 13.990, p = 0.003), while the opposite was true for intermediate body sizes (10-40 mm; F = 7.628, p = 0.005,). Insects feeding on microphytes (F = 17.164, p = 0.003) and microfauna (F = 5.466, p = 0.017) were associated with temporary sites, while predators were more common in permanent sites (F = 13.510, p = 0.003). Species exploiting macrophytes as their habitats were negatively related to temporary sites (F = 4.590, p = 0.017), while surface-dwellers were positively associated with temporary sites (F = 7.534, p = 0.017).

Aquatic vegetation had a positive relationship with insects with aquatic eggs (r = 3.140, p = 0.022). Moreover, aquatic vegetation showed a relative relationship with filter-feeders (r = -2.777, p = 0.048) and surface-dwellers (r = -3.002, p = 0.020), while there was a positive association between this variable and species exploiting leaf litter (r = 3.649, p = 0.005) and free-swimmers (r = 3.096, p = 0.014). Fish occurrence decreased the abundance of insects living in the leaf litter (F = 5.758, p = 0.022), while none of the considered functional traits showed a significant relationship with pond depth.

Discussion

Our results showed that the complex effects of invasive species and wetland features on insect communities can be unravelled by analysing multiple facets of the diversity of freshwater insects, their biomass, and functional traits, and that integrating multiple characteristics of communities can provide a more complete understanding of the drivers and consequences of biodiversity changes.

The functional diversity of aquatic insects was strongly related to taxonomic diversity, suggesting low levels of functional redundancy in our communities (Petchey et al. 2007). Nonetheless, functional and taxonomic diversity showed distinct responses to environmental drivers. For instance, taxonomic diversity strongly increased in ponds with more aquatic vegetation, while it showed weaker response to the presence of *P. clarkii*. Conversely, the crayfish appeared as the main driver of functional diversity. Previous studies have reported that in certain cases, functional diversity is more sensitive to stressors, such as deforestation, eutrophication, and invasive species, when compared to taxonomic diversity (Sagouis et al. 2017; Cantera et al. 2023). This suggests that insects with different functional traits have distinct responses to *P. clarkii* presence and that taxa characterized by some specific traits are affected disproportionally.

The fourth-corner analysis allowed us to identify the traits that were most strongly affected by *P. clarkii* through both direct and indirect impacts (Fig. 5). The presence of *P. clarkii* heavily reduced the abundance of insects with aquatic eggs, living on the leaf litter within ponds, or swimming in the water column. These relationships can be explained by the direct predation of *P. clarkii* on eggs, which probably represent easily accessible food sources, and on insects swimming or living on the leaf litter. Swimming prey can be easy to locate for *P. clarkii* thanks to its hydrodynamic receptors, which allow the predator to respond quickly to movements (Breithaupt et al. 1995). Furthermore, in our sites insects with aquatic eggs and swimming insects were mainly represented by large aquatic coleopterans and heteropterans (Corixidae and Notonectidae families). The negative impacts on leaf litter insects can be related to both direct predation an indirect impacts on this microhabitat, as *P. clarkii* is known to reduce leaf mass in invaded habitats (Carvalho et al. 2016), with cascading effects on the organisms exploiting leaf litter as microhabitat. The presence of *P. clarkii* did not show any relationship with predatory insects, despite previous works reported negative effects of *P. clarkii* on Odonata larvae, suggesting competitive interactions (Siesa et al. 2014; Bucciarelli et al. 2019). However, this does not mean that predators are not negatively affected by *P. clarkii*. In fact, the fourth corner analysis aims at identifying the traits that are disproportionately affected, and its results should be interpreted jointly with the overall analysis of community diversity. The presence of *P. clarkii* tended to reduce the overall diversity of communities (Fig. 4), and the lack of specific effects on predators indicates that the diversity of these insects declines with a pattern that is comparable to the one of other insects. The fourth-corner analysis is not sensitive to main effects (i.e., the overall effect of an environmental variable on species abundance), thus it does not detect associations between traits and environmental parameters when it affects all the species in the same way (Peres-Neto et al. 2017; ter Braak 2017).

The presence of *P. clarkii* was not the only factor related to the variation of insect traits. Tadpole occurrence was negatively related to insects with small size, aquatic nymphs, filter-feeding or microphyte-feeding, and exploiting the superficial layers of the water columns. In the study area, these are typical traits of small Diptera, particularly Chironomidae and Culicidae. This suggests that the distribution of these insects can be shaped by the presence of amphibians, which can act as both competitors and predators of their larvae. Tadpoles are efficient competitors of mosquitoes’ larvae and are able to reduce their growth and survival rates (Mokany & Shine 2003; Rubbo et al. 2011; Perrin et al. 2023). Their role as competitors may be particularly relevant because tadpoles can selectively feed on protein-rich algae (Kupferberg 1997), potentially limiting the availability of these resources to small filter-feeding larvae. Moreover, tadpoles of certain species have been proved to feed on mosquito eggs and larvae (Kupferberg 1997; Petranka & Kennedy 1999). Small filter feeders, such as mosquitoes, are among the most problematic aquatic insects and their management remains a key issue for wetlands in human-dominated landscapes (Bruno et al. 2024). The analysis of functional traits was able to identify the features of aquatic insects most affected by tadpoles, and confirms that preserving native elements of freshwater biodiversity, such as amphibians, can provide an effective strategy to limit the abundance of mosquitoes at the landscape scale (Perrin et al. 2023), even though more data are needed to obtain quantitative estimates of the actual impacts of tadpoles on mosquito larvae.

Conversely, predatory insects were more frequent in sites with tadpoles, which are among the main prey of large Odonata and aquatic Coleoptera (Bucciarelli et al. 2019; Hanford et al. 2020). In certain cases, the presence of an invasive species can facilitate colonization by other invasive organisms, a phenomenon known as ‘invasional meltdown’ (Simberloff 2006; Montgomery et al. 2012). Given that *P. clarkii* presence has caused a reduction in tadpole densities and even local extinctions of amphibians in the study area (Ficetola et al. 2011; Falaschi et al. 2021), it is possible that the spread of *P. clarkii* may facilitate establishment and spread of mosquitoes, some of which are problematic invasive species (e.g. the tiger mosquito, *Aedes albopictus*). Facilitation by *P. clarkii* has been assessed for some invasive species, e.g., invasive macrophytes (Thouvenot et al. 2017), but its relationships with invasive mosquitoes remains unaddressed.

Fish presence was associated with a general decrease of insect biomass, without clear effects on diversity or on insects with specific traits. Invertebrate biomass is extremely influenced by fish, with fishless ponds hosting up to 200x invertebrate biomass compared to ponds with fishes (Dorn 2008). The lack of significant association between most traits and fish presence is probably due to the insensitiveness of the fourth-corner analysis to main effects. Moreover, it must be remarked that we do not have complete data on the composition of fish communities. Different fish species can affect trophic webs in different ways, and co-occurrence of multiple fish species may determine complex patterns. Still, we found a negative association between fish and insects living in the leaf litter. Some invasive fishes widespread in the study area, such as the carp (*Cyprinus carpio*) are omnivorous species that feed on both plant matter and benthic insects and can act as ecosystem engineers, modifying plan communities through direct (herbivory, uprooting) and indirect (turbidity, excretion) mechanisms (Matsuzaki et al. 2009).

Aquatic macrophytes are exploited for oviposition by several aquatic insects (e.g., Inoda 2011), explaining the observed positive association between egg-laying insects and vegetation. Moreover, aquatic plants represent an important source of organic matter that composes leaf litter (Zhao et al. 2017), thus promoting the presence of species exploiting leaf litter as a microhabitat. Finally, swimming insects, particularly diving beetles, are particularly able to exploit aquatic macrophytes as shelters (Liao et al. 2023; Inoda & Watanabe 2024). Macrophytes can prevent or reduce sediment resuspension (Jones et al. 2012; Wosnie et al. 2020), decreasing food availability for sediment-feeders, explaining the observed negative association between debris-feeding insects and vegetation, and can reduce the abundance of certain surface-dwelling aquatic insects, such as dipterans (Ugya et al. 2019). Drought events seemed to favour small insects, surface-dwellers, and microfagous insects. Aquatic microorganisms have the ability to disperse and rapidly colonize temporary ponds after droughts (Incagnone et al. 2015). For this reason, aquatic insects feeding on micro-organisms are likely to occupy temporary ponds, where their feeding sources are available and there is a low predation risk. Finally, we found no association between water depth and the investigated functional traits. As this ecological variable showed an almost significant positive effect on both taxonomic and functional diversity, its effect is likely to have a similar pattern across all traits.

DNA metabarcoding can quickly provide detailed information on community composition that is fundamental to understand biodiversity responses to environmental stressors. Nevertheless, most metabarcoding-based analyses lack information on species abundance, which is fundamental to assess effects on ecosystem functioning. The frequency of DNA reads showed very strong relationships with the relative biomass of different insect orders, but the relationship between DNA reads and biomass was not the same across taxa, with Hemiptera and Megaloptera showing significantly weaker relationships than the other taxa. This is in agreement with analyses suggesting the existence of amplification bias of the Inse01 primer across insect orders, with Hemiptera showing lower amplification success than Coleoptera and Diptera (Ficetola et al. 2021). Preferential amplification of certain taxa can limit the reliability of quantitative measures derived from metabarcoding (Elbrecht & Leese 2015). Nevertheless, assessing the relationship between DNA reads and biomass can provide correction factors for easily identifiable taxonomic groups, such as orders (Krehenwinkel et al. (2017). Weighting the easily identifiable taxa from bulk samples before extracting DNA can represent a cost-effective strategy to obtain such calibration, overcoming the need to obtain species-specific correction factors, which would be unfeasible when studying highly diverse communities. Importantly, measures of relative abundance can be integrated with measures of absolute abundance (e.g. biomass / m^2, as performed here) to obtain important information on the functional responses of communities to ecological stressors. Nevertheless, the proposed approach has several limitations. In complex communities, several processes concur to amplification bias, such as differences in GC content and competition for primers between co-occurring species during PCR (Nichols et al. 2018). Therefore, differences in amplification rates likely exist also between closely related taxa, and more investigations are needed to obtain reliable quantitative assessments based on metabarcoding data. Approaches bypassing the barcode amplification step (e.g. shotgun sequencing) can provide an alternative solution, even though evidence for the advantages of PCR-free approaches for the overall assessment of biological communities remain incomplete (Ficetola & Taberlet 2023). Finally, we highlight that correction factors based on the weighting of individuals, as performed here, cannot be applied to metabarcoding approaches that do not involve extraction from tissues, such as environmental DNA, even though information on the amplification bias of different taxa remains important for the interpretation of all the metabarcoding results.

Our results remark the importance of functional diversity to understand the effects of stressors acting on freshwater communities. While changes in taxonomic diversity may be more difficult to spot or slower, changes in functional diversity can efficiently measure the community responses to stressors, particularly to invasive species, which are known for their rapid spread in ecosystems (Bradley et al. 2024). We underline the importance of integrating quantitative data, taxonomic diversity, and functional diversity to assess the multiple facets of community responses to ecological stressors. Future research should focus on increasing the reliability of quantitative estimates obtained from metabarcoding data.

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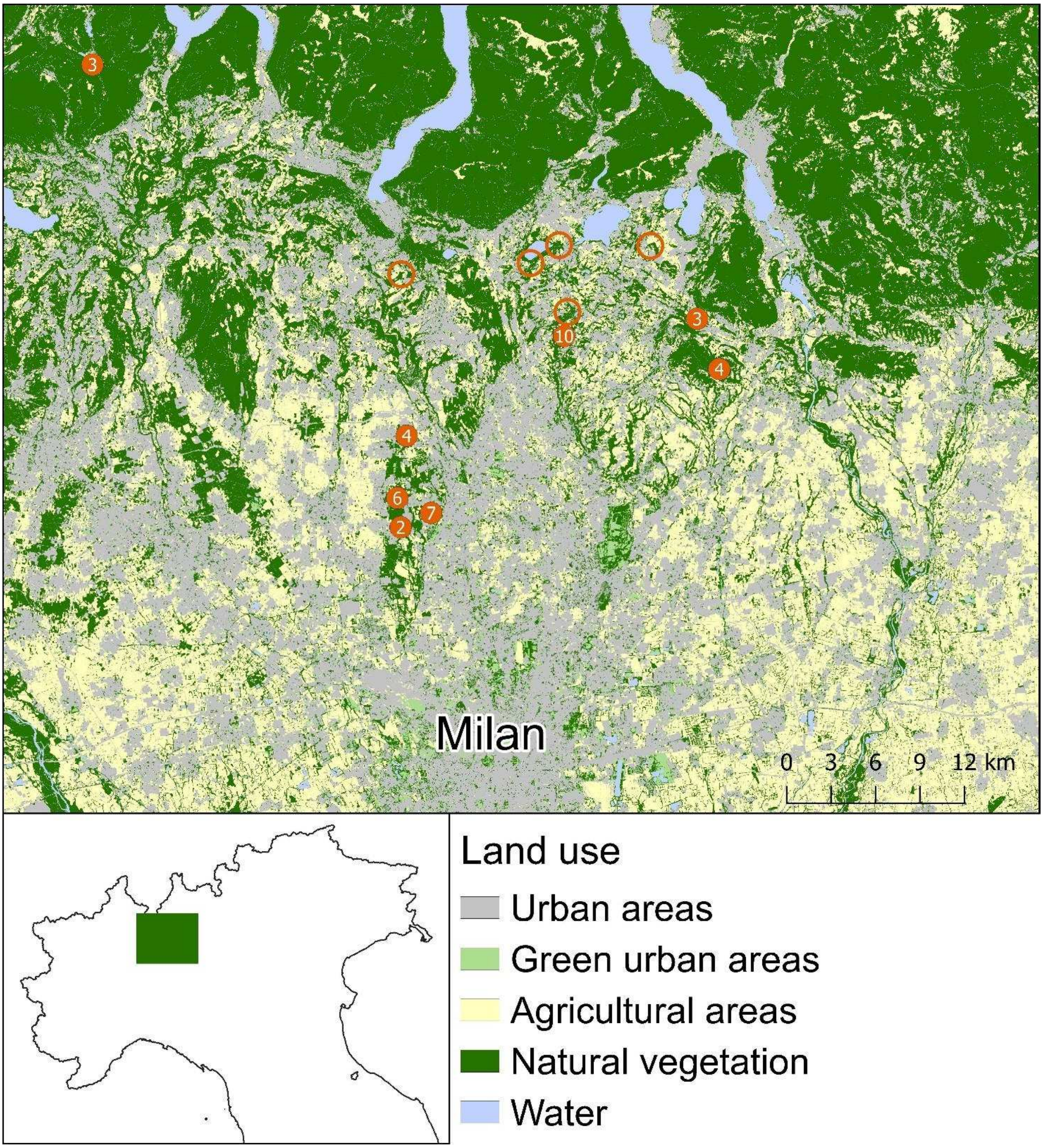


Figure 1: The locations of the monitored sites. Empty circles represent single sites.

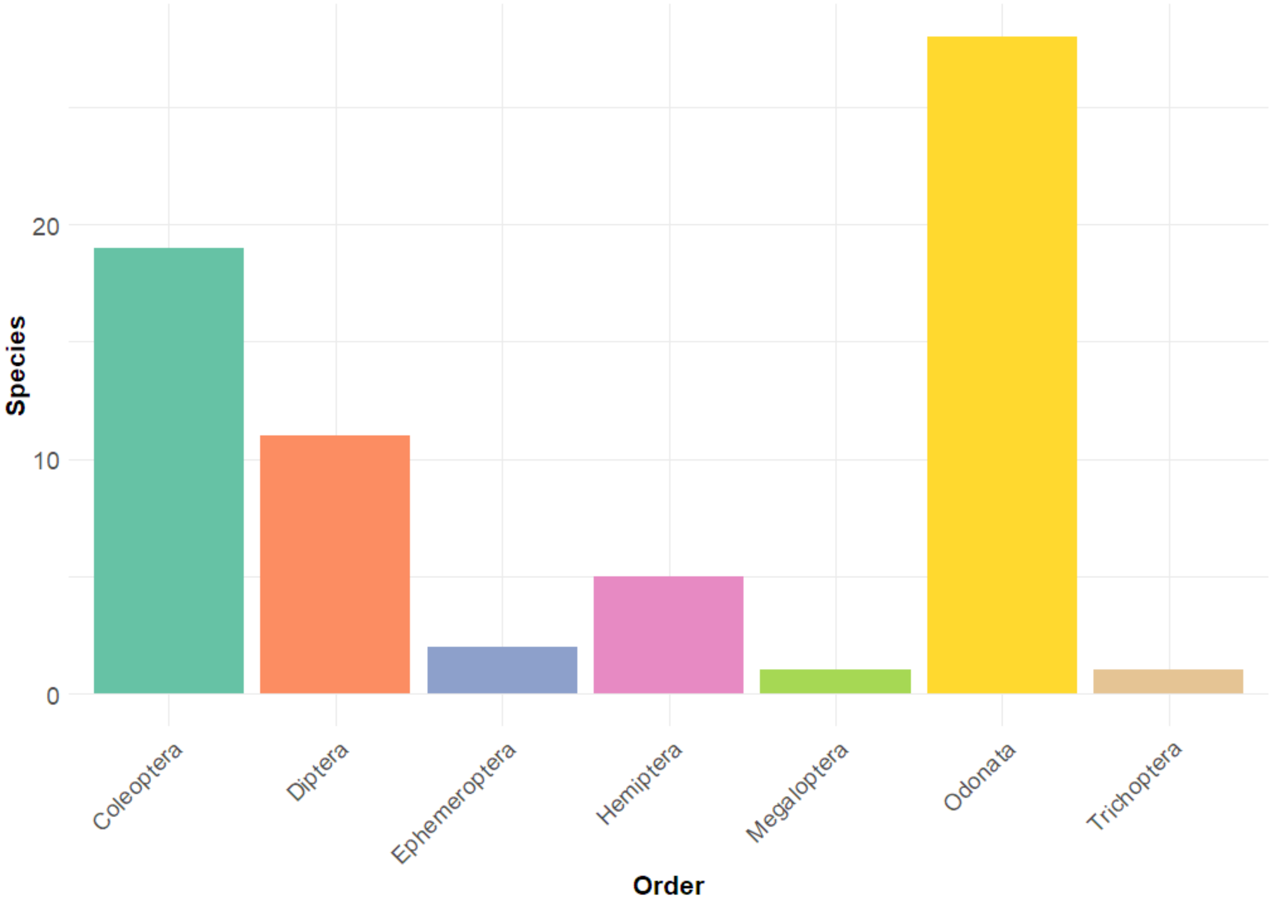


Figure 2: Number of MOTUs recovered, grouped by taxonomic order.

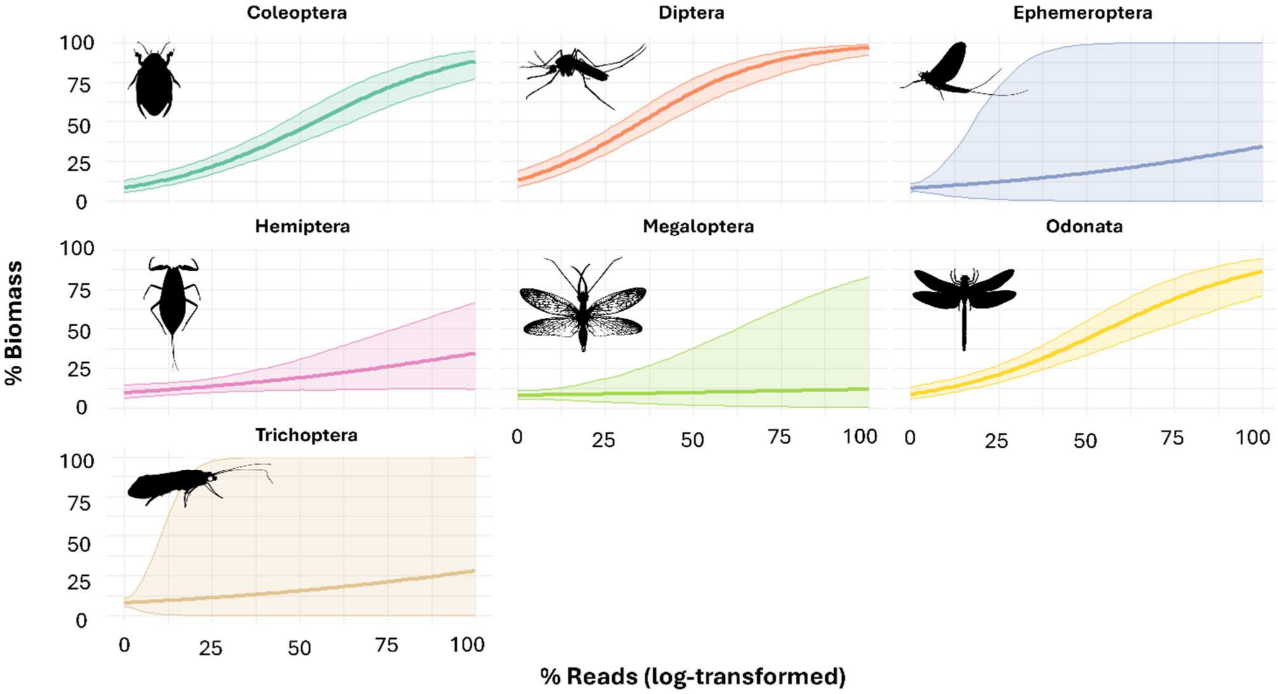
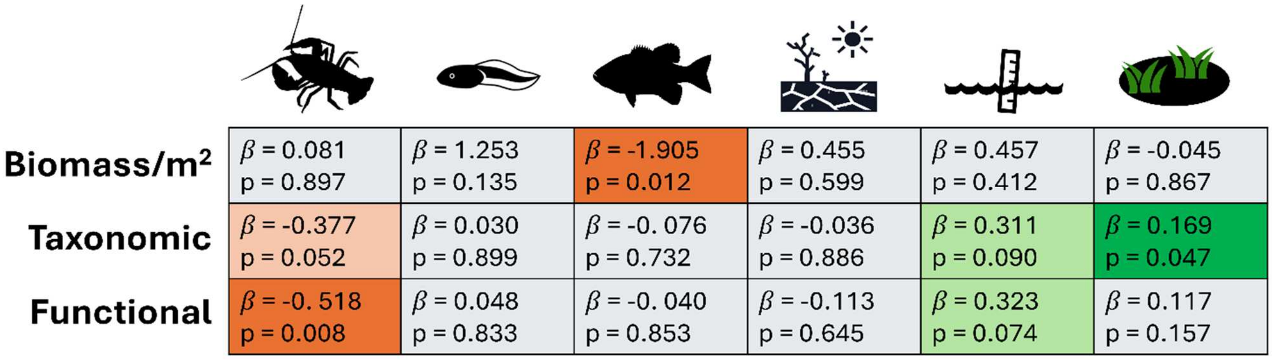
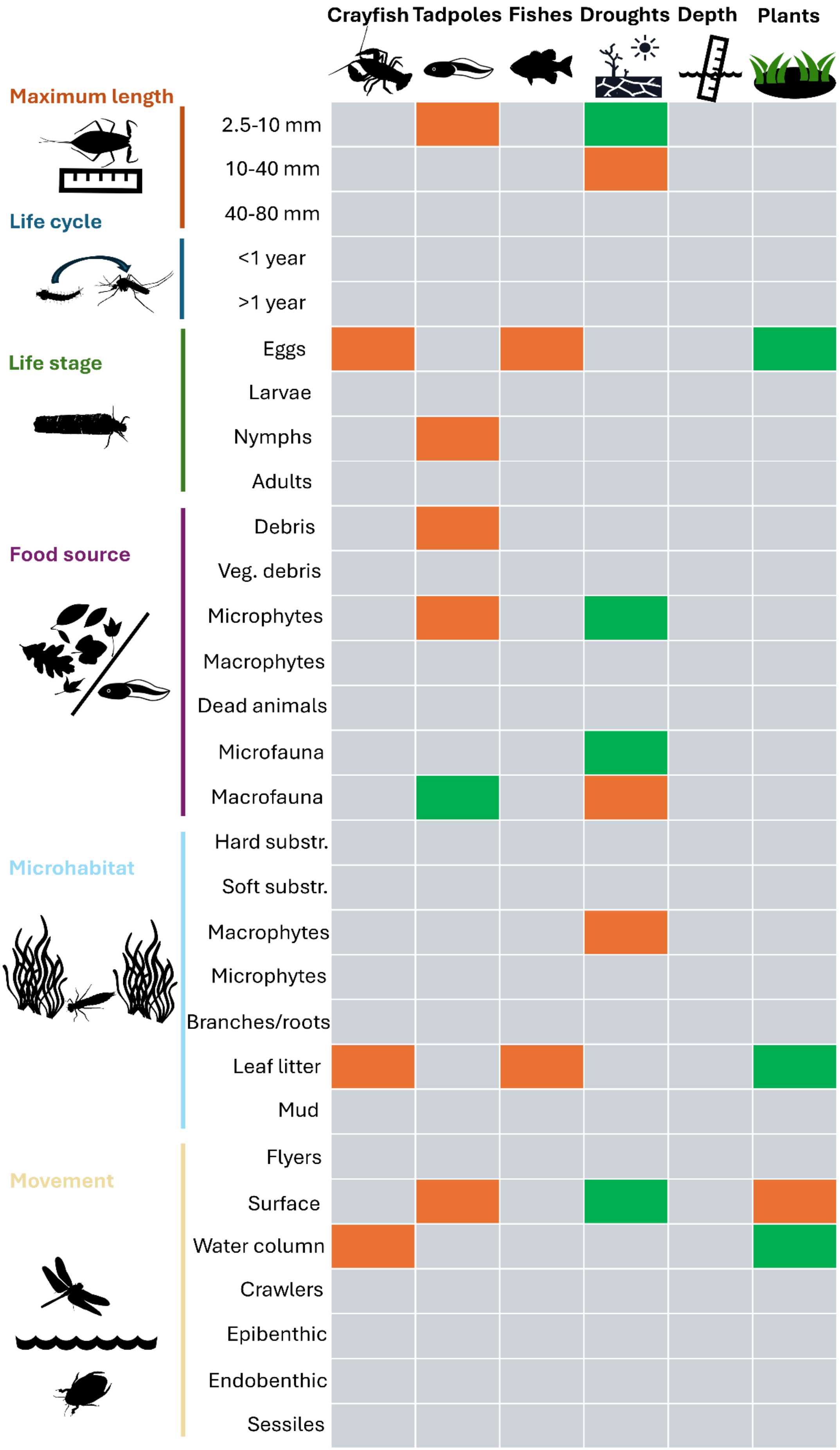


Figure 3: Relationships between relative biomass and relative abundance of log-transformed reads for each taxonomic order. Shaded areas represent the 95% confidence intervals.



Biomass/m^2	β = 0.081 p = 0.897	β = 1.253 p = 0.135	β = -1.905 p = 0.012	β = 0.455 p = 0.599	β = 0.457 p = 0.412	β = -0.045 p = 0.867
Taxonomic	β = -0.377 p = 0.052	β = 0.030 p = 0.899	β = -0. 076 p = 0.732	β = -0.036 p = 0.886	β = 0.311 p = 0.090	β = 0.169 p = 0.047
Functional	β = -0. 518 p = 0.008	β = 0.048 p = 0.833	β = -0. 040 p = 0.853	β = -0.113 p = 0.645	β = 0.323 p = 0.074	β = 0.117 p = 0.157

Figure 4: Effects of environmental variables (crayfish presence, tadpole presence, fish presence, hydroperiod, water depth, and aquatic vegetation) on insect communities' biomass, taxonomic diversity, and functional diversity.



		Crayfish	Tadpoles	Fishes	Droughts	Depth	Plants
Maximum length	2.5-10 mm		🟧		🟩		
	10-40 mm				🟧		
	40-80 mm						
Life cycle	<1 year						
	>1 year						
Life stage	Eggs	🟧		🟧			🟩
	Larvae						
	Nymphs		🟧				
	Adults						
Food source	Debris		🟧				
	Veg. debris						
	Microphytes		🟧		🟩		
	Macrophytes						
	Dead animals						
	Microfauna				🟩		
	Macrofauna		🟩		🟧		
Microhabitat	Hard substr.						
	Soft substr.						
	Macrophytes				🟧		
	Microphytes						
	Branches/roots						
	Leaf litter	🟧		🟧			🟩
	Mud						
Movement	Flyers						
	Surface		🟧		🟩		🟧
	Water column	🟧					🟩
	Crawlers						
	Epibenthic						
	Endobenthic						
	Sessiles						

Figure 5: Results of the fourth-corner analysis, showing associations between functional traits and environmental variables (green = significant positive association; orange = significant negative association).

Transformation	**Chi-squared (Likelihood-ratio test)**	**Pseudo-R^2**	**AIC**
Prop. of log(reads)	Proportion of reads: χ^2= 133.33 *** Order: χ^2= 39.98 *** Interaction: χ^2 = 17.10 ***	0.653	-1453.555
eDNA index	Proportion of reads: χ^2= 126.27 *** Order: χ^2= 54.66 *** Interaction: χ^2 = 24.37 ***	0.629	-1446.496
Prop. of reads	Proportion of reads: χ^2= 125.84 *** Order: χ^2= 68.53 *** Interaction: χ^2 = 30.93 ***	0.629	-1446.068

Table 1: Results of beta regressions between relative biomass and different transformations of relative abundance of reads.