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Estimating Rapid Diversity Changes During Acute Herbicide Contamination Using Environmental DNA

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ABSTRACT

The biodiversity of freshwater ecosystems globally is facing severe threats due to various anthropogenic stressors, such as habitat degradation, introduction of invasive species, and pollution. Assessing the effects of human-induced environmental stressors on population and community persistence requires accurate biodiversity estimates. While environmental DNA (eDNA) metabarcoding has emerged as a promising tool, its effectiveness in capturing rapid biodiversity responses to acute stressors across levels of biological organization (community, population, and intra-specific levels) remains to be investigated. In this study, we tested the efficacy of eDNA metabarcoding in assessing rapid changes in aquatic zooplankton and insect communities by conducting a two-month mesocosm experiment with pulses of glyphosate-based herbicide under contrasting nutrient levels (mesotrophic and eutrophic). We examined the effects of treatments on community assemblages, family richness, and intraspecific diversity, and compared our findings with those obtained through a microscopy approach. Metabarcoding revealed partially congruent ecological findings with microscopy, indicating its potential in assessing rapid community changes. The herbicide induced shifts in community composition and differentially impacted zooplankton and insect family richness (increase in insects, and decrease in crustaceans and rotifers), suggesting a gradient of tolerance to the herbicide among taxa and potential top-down regulation by insect larvae that may counteract the advantage gained by herbicide-tolerant zooplankton. Finally, we showed that nutrient enrichment exacerbated the negative effects of the herbicide on intraspecific diversity, highlighting concerns about genetic erosion. Our findings underscore the complexity of responses to herbicide and nutrient enrichment in freshwater ecosystems. We conclude that eDNA metabarcoding can not only be used to estimate rapid changes in invertebrate communities but also provides additional value by offering a broader perspective on diversity dynamics and potential cascading effects at different scales of biological organization.

1 | Introduction

Preserving biodiversity is essential to ensure the continued functioning of ecosystems. The implementation of large-scale conservation programs often relies on ecological assessments providing reliable estimates of spatio-temporal biodiversity changes (Larigauderie et al. 2012; Thomsen and Willerslev 2015). Traditionally, biodiversity data have been acquired by morphological characterization of captured organisms (e.g., malaise trap, seining, various net types). However, such approaches can be relatively time-consuming, labor-intensive, and more importantly it can be challenging to capture or distinguish between cryptic species or life stages (e.g., larvae) of closely related species (Thomsen and Willerslev 2015; Darling and Mahon 2011). Moreover, anthropogenic activities are causing declines not only in the numbers and abundance of species but also in the genetic diversity of populations within species (International Union of Conservation of Nature 2015; Ceballos, Ehrlich, and Dirzo 2017; Des Roches et al. 2021), which has both ecological and evolutionary significance at the community and ecosystem levels (Hughes et al. 2008).

New rapid and reliable biodiversity assessments able to detect and track diversity at different scales (e.g., genetic, species, community) are crucial, especially in freshwater environments where diversity is declining faster than in terrestrial or marine ecosystems (Grooten and Almond 2018; Pimm et al. 2014; Marques et al. 2019). The advent of molecular methods has resulted in important new genetic tools for species identification and assessment of genetic diversity (Hoffmann and Willi 2008; Vacher et al. 2016; Bohan et al. 2017; Valdez-Moreno et al. 2019). The application of metabarcoding to environmental DNA (eDNA), the DNA shed into the environment by organisms, is particularly promising (Gibson et al. 2014; Barnes and Turner 2016; Carraro et al. 2018; Holman et al. 2018; Pont et al. 2018; Valdez-Moreno et al. 2019; Sales et al. 2021). Several studies have compared metabarcoding with morphological assessments of aquatic invertebrates under natural conditions (Lobo et al. 2017; Cahill et al. 2018; Leasi et al. 2018; Serrana et al. 2019; Sun et al. 2019; Emmons et al. 2023). However, only a few have tested its ability to capture rapid biodiversity changes in response to acute environmental change within short-time scales; in most cases, metabarcoding recovered more taxa than traditional methods and detected overall similar responses to various environmental stresses (Frontalini et al. 2018; Sun et al. 2019; Chen et al. 2023).

Significant efforts have been devoted to reducing the occurrence of both false positives (detection of taxa when not present) and false negatives (absence of detection of taxa when present) (Cowart et al. 2015) in metabarcoding data by minimizing PCR and sequencing artifacts (e.g., erroneous DNA sequences produced by DNA amplification and sequencing), and by the continuous improvement of bioinformatic tools (e.g., Callahan et al. 2016; Edgar 2016). While it can be challenging to estimate the rates of false positive or false negative in metabarcoding datasets, strict protocols can keep these rates relatively low. Current practices include using negative and positive controls, optimizing primer choice and PCR conditions (e.g., increasing the PCR extension time can reduce chimera formation; Qiu et al. 2001), denoising the data (e.g., OTUs and ASVs), and using filtering thresholds based on a minimum read count threshold and/or based on reads in the negative controls. While eDNA metabarcoding is well established for species and community surveys, refined bioinformatics tools have only recently allowed the first eDNA metabarcoding studies on genetic diversity at the intra-population level (Sigsgaard et al. 2016; Tsuji et al. 2018; Adams et al. 2019; Turon et al. 2019; Antich et al. 2023; Thomasdotter et al. 2023). For example, Turon et al. (2019) successfully developed a two-step cleaning approach (denoising step and minimal abundance filtering) of sequences obtained with metabarcoding from marine water samples for phylogeographic inferences. A reduction in the levels of genetic diversity or an alteration of genetic structure frequently precedes population bottlenecks and the eventual extirpation of populations (Wiens 2016; Ceballos, Ehrlich, and Dirzo 2017), and morphological assessments may fail to provide accurate information on the underlying levels of population genetic diversity. Using eDNA metabarcoding at the intra-population level opens new opportunities to assess simultaneously intraspecific genetic diversity for hundreds of individuals and species. This is particularly useful in the context of important and rapid changes in ecosystem structure and functions, such as those induced by biological invasion. For example, Marshall and Stepien (2019) were able to characterize dreissenid mussel community composition, along with relative abundance and intraspecific population-level diversity from two concurrent invasions by quagga and zebra mussels in North America.

Glyphosate-based herbicides (GBH; i.e., herbicides in which glyphosate is the primary active ingredient) are currently the most heavily applied herbicides in modern agriculture and urban weed control worldwide, with a 100-fold increase in usage over the past 50 years (Myers et al. 2016, 2022). The detection of glyphosate in natural environments has raised concerns about its toxicity on non-target organisms (Anderson et al. 2002; Anderson 2005; Byer et al. 2008; Struger et al. 2008; Gill et al. 2018), including in aquatic ecosystems that are contaminated through spray drift, runoff and leaching from agricultural or urban sites (Relyea 2005; Kolpin et al. 2006; Tsui and Chu 2008; Annett, Habibi, and Hontela 2014). The biodegradation of glyphosate releases compounds, such as aminomethyl phosphonic acid (AMPA) and sarcosine, whose degradation leads to the release of bioavailable phosphorus (P) in the environment (reviewed in Hébert, Fugère, and Gonzalez 2019). Therefore, GBHs can be detrimental due to their toxicity and contribution to nutrient enrichment ("fertilizing effect"; Vera et al. 2012; Fugère et al. 2020; Hébert et al. 2021), which can ultimately lead to a cascade of negative ecological consequences on freshwater ecosystem structure and dynamics (e.g., harmful algal blooms, oxygen depletion) (Vera et al. 2010; reviewed in Lozano and Pizarro 2024). On the other hand, the toxic effects of GBHs on certain species may be buffered by natural or pollution-induced tolerance in primary and secondary consumers (Halstead et al. 2014; Hébert et al. 2021). The fertilizing effect of glyphosate is greatest in low-nutrient systems (presumably P-limited) (Fugère et al. 2020), but already eutrophic ecosystems can negatively influence the biodegradation of glyphosate (Carles et al. 2019).

In this study, we used eDNA to assess the effect of a glyphosate-based herbicide and nutrient enrichment on zooplankton and aquatic insect (i) family richness, (ii) community composition,

and (iii) haplotype richness. To do so, we conducted a 2-month mesocosm experiment with three pulse applications of acute doses of herbicide under mesotrophic and eutrophic nutrient conditions (Fugère et al. 2020; Hébert et al. 2021; Barbosa da Costa et al. 2021, 2022). We compared our findings with those obtained with a microscopy approach in the same mesocosm experiment (Hébert et al. 2021). We predicted that: (i) richness and community composition will be negatively influenced by the herbicide and nutrients, (ii) the variation in haplotype richness will be positively correlated to population size but negatively correlated to herbicide and nutrient levels, and (iii) ecological findings associated with richness and community assemblages obtained with metabarcoding will agree with those obtained with microscopy.

2 | Methods

2.1 | Experimental Design

The experiment was conducted in 2016 at the aquatic mesocosm facility (Large experimental array of ponds, LEAP) of McGill University's Gault Nature Reserve (Québec, Canada) (see details in Fugère et al. 2020; Hébert et al. 2021; Barbosa da Costa et al. 2021; Barbosa da Costa et al. 2022). Each Rubbermaid plastic tank (hereafter referred to as mesocosm) was filled with ~1000 L of water from adjacent Lake Hertel. The experiment lasted 8 weeks starting from August 17, 2016 (day 0) to October 12, 2016 (day 56; Figure 1A), and involved a two-level nutrient press treatment (mesotrophic: 15 µg/L phosphorus, and eutrophic: 60 µg/L phosphorus) crossed with a two-level glyphosate-based herbicide (GBH) treatment (0.3 and 15 mg/L; Barbosa da Costa et al. 2021) applied in phase I at both day 6 and 34, and one pulse at 40 mg/L applied at day 43 in phase II (Figure 1). The trophic state (nutrient) was maintained with bi-weekly applications of nitrogen and phosphorus (N:P molar ratio of 33, similar to Lake Hertel; Fugère et al. 2020). The GBH concentrations above refer to the active ingredient glyphosate (standardized to acid equivalents) in Roundup Super Concentrate Grass and Weed Control (PCPA Registration [Reg.] No. 22759), registered to Monsanto Canada Inc. at the time of the experiment. Note that concentrations of the active ingredient, glyphosate, were used as a proxy of the herbicide toxicity. However, because commercial formulations of GBHs contain multiple molecules, including surfactants that may be more toxic than the active ingredient (reviewed in Mesnage and Antoniou 2018), the effects of the herbicide treatment cannot be attributed to a single ingredient (i.e., glyphosate). Glyphosate quantification was performed immediately after applying the first GBH pulse at day 6, then at days 14, 29, and 34 (after the second pulse), and 44 (the day after the pulse at 40 mg/L): 1 L water samples were acidified to a pH < 3 with sulfuric acid, and frozen at −20°C until measurement with liquid chromatography heated electrospray ionization tandem mass spectrometry (Accela 600-Orbitrap LTQ XL, Thermo Scientific, Waltham, MA, USA). As glyphosate degradation was very low, a constant concentration between measurements was assumed (Fugère et al. 2020). Our sample collection focused on eight mesocosms: four controls (two mesotrophic replicates, and two eutrophic replicates) and four treatments (mesotrophic 0.3 mg/L herbicide MMH, mesotrophic 15 mg/L herbicide MHH, eutrophic 0.3 mg/L herbicide EMH, and eutrophic 15 mg/L herbicide EHH) (Figure 1B). Two of the controls did not receive any herbicide through the experiment (MC and EC, Figure 1B), while the two other controls received the 40 mg/L pulse in phase II (MCP and ECP, Figure 1B). For more details on the experimental design, treatments, and methods, see Fugère et al. (2020) and Hébert et al. (2021).

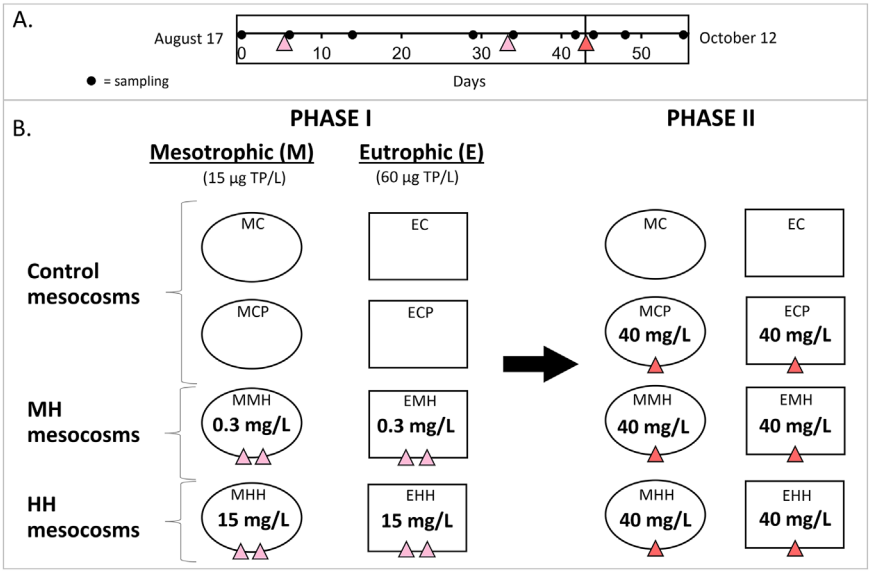

FIGURE 1 | Schematic representation of (A) the timeline and (B) the experimental design. (A) Black circles represent sampling dates (0, 6, 14, 29, 34, 42, 44, 48 and 56), the triangles in the left box represent the "middle" (0.3 mg/L) and "high" (15 mg/L) concentration pulses of the glyphosate-based herbicide gradient (0–0.3–15 mg/L; Barbosa da Costa et al. 2021) applied in phase I at days 6 and 34, and the darker triangle in the right box represents the last pulse (40 mg/L) applied in phase II at day 43. (B) Mesotrophic (15 µg TP/L) mesocosms are represented by circles and eutrophic (60 µg TP/L) mesocosms by squares. The number of herbicide doses is indicated by the triangles, as well as the target concentration of active ingredient (glyphosate) as a proxy of herbicide toxicity. Empty circles and squares represent control mesocosms (i.e., free of herbicide).

2.2 | Sampling and Extraction of eDNA

We used 35-cm-long integrated samplers (2.5-cm-diameter PVC tubing) to collect surface water samples in five random locations per mesocosm and combined them in 1 L Nalgene bottles (Barbosa da Costa et al. 2021). This was repeated on nine sampling dates (days 0, 6, 14, 29, 34, 42, 44, 48, and 56). PVC tubing and bottles were cleaned between each sampling occasion (rinsed three times with tap water and triple-washing with mesocosm water before sampling). All mesocosms had dedicated samplers and bottles to prevent cross-contamination. A total of 250 mL of each combined water sample was filtered on-site using 0.22-μm-pore-size Millipore hydrophilic polyethersulfone membranes (47 mm in diameter; Sigma-Aldrich, St. Louis, USA) for a total of 72 samples (8 mesocosms × 9 time points). All filters were stored at −80°C until extraction.

Extractions of eDNA were conducted using the PowerWater (MoBio) extraction kit as described in Barbosa da Costa et al. (2021). Two blank extractions were included (i.e., only reagents and no filter) to monitor for potential contamination. All DNA extracts were quantified using NanoDrop microvolume Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and diluted to a concentration of 2.5 ng/μL using ultrapure water.

2.3 | Extraction of eDNA and Library Preparation

Libraries were prepared following the two-step PCR protocol "16S Metagenomic Sequencing Library Preparation" (Illumina Inc.) with a few modifications. Each DNA extract, extraction blank, PCR blank, and positive control (mock communities *a* and *b* of 10 and 27 species, respectively; Table S1) was PCR amplified five times using the primers in Leray et al. (2013): mlCOIintF (5′-GGWACWGGWTGAACWGTWTAYCCYCC-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′). The Leray primer set targets a 313 bp fragment within the Folmer region (Folmer et al. 1994) of the Cytochrome c oxidase subunit I (COI) gene and has been widely used for invertebrate metabarcoding (e.g., Harper et al. 2021; Seymour et al. 2021). The COI gene has been the marker of choice in population genetics and phylogeographic studies because it discriminates among closely related species as well as intraspecific information and has an extensive database for arthropods (Hebert, Ratnasingham, and De Waard 2003; Elbrecht et al. 2019; Hajibabaei et al. 2019). The first PCRs were conducted in five replicates, each consisting of a total volume of 12.5 μL:2 μL of forward and 2 μL of reverse primers (final concentration: 0.2 μM), 7.5 μL of 2X KAPA HiFi HotStart ReadyMix (KAPA Biosystems Inc., USA), and 1 μL of diluted DNA extract. The cycling conditions were as described in Leray et al. (2013): 16 initial cycles (denaturation for 10 s at 95°C, annealing for 30 s at 62°C (−1°C per cycle), and extension for 60 s at 72°C), followed by 25 cycles at 46°C annealing temperature. We also included three PCR-negative controls. Amplification success was assessed on a 1% agarose gel electrophoresis using SYBR Safe DNA Gel Stain (Invitrogen). The products of the five PCR replicates were then pooled and purified using ultrapure beads (AMPure XP beads) with 0.875× ratio according to the manufacturer's protocol (in 20 μL of DNA solution). The second PCRs were performed using the Illumina Nextera XT Index kit (24-index, V3) in 25 μL reaction volume containing 2.5 μL of unique pairs of Illumina Nextera tags per sample, 2.5 μL of cleaned PCR1 products, 12.5 μL of 2× KAPA HiFi HotStart ReadyMix (KAPA Biosystems Inc., USA), and 5 μL of ddH20. PCR cycling conditions included 3 min at 95°C, followed by eight cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension of 5 min at 72°C. The final clean-up was done using the same protocol as described before but with a starting volume of 25 μL. After quantification following the manufacturer's instructions (Agilent Technologies Bioanalyzer, DNA 1000 kit), the samples were normalized to the lowest observed concentration (12.5 ng/μL) and pooled in a final library. The library was sent to the Genome Quebec Innovation Center facility (Montreal, Quebec, Canada) for quality check and pair-end sequencing in one run on an Illumina MiSeq platform using a 500-cycle Reagent v2 kit (pair-end 250 bp sequencing). The sequencing run included all negative controls, mock communities, and water samples.

2.4 | Bioinformatics and Statistical Analyses

The bioinformatics pipeline consisted of demultiplexing, quality filtering, trimming raw reads based on quality score, and assigning taxonomy (Figure S1). The DADA2 R package version 1.12.1 (Callahan et al. 2016) was used to identify and trim the low-quality end positions. Then, Cutadapt version 1.18 (Martin 2011) was used to trim the adapters and primers. Sequences were denoised with the amplicon sequence variant (ASV) method in DADA2 which corrects erroneous sequencing from amplification and sequencing errors. The following parameters of the bioinformatic pipeline were selected after optimization on two mock communities (see Tables S1 and S2, and Appendix S1 for the species list and optimization process): a quality filtering score of 23, a maximum number of "expected errors" allowed in forward and reverse reads of four and five respectively, and a minimum abundance of an ASV of four sequences.

ASV were assigned at the family level for the three following main reasons: (1) to minimize the impact of taxonomic errors which are more prevalent at finer taxonomic levels, especially with microscopic groups such as rotifers and microcrustaceans, (2) to increase our statistical power for analyses, and (3) because our primary focus was to understand the herbicide effect on important taxonomic groups (e.g., crustaceans, rotifers, and insects) allowing us to capture broader ecological impacts. We used blastn against Eukaryota COI sequences from NCBI GenBank (Altschul et al. 1990; Camacho et al. 2009). The best BLAST hit (family level) was identified with > 90% identity, *e*-value 0.01, and a minimum query coverage > 95% (relaxed threshold, Brown et al. 2015; Thibodeau, Walsh, and Beisner 2015). ASVs associated with non-zooplankton families, such as microalgae, protists, fungi, fish, mammals, sessile organisms (e.g., polyps), and terrestrial invertebrates (no aquatic/semi-aquatic life stage) were removed (Table S3). Other contaminations such as sequences belonging to the mock communities or species recovered in the controls were also removed.

To investigate genetic diversity within species, the taxonomic assignment at the family level was followed by assignments at the species level using BASTA (Basic Sequence Taxonomy

Annotation; Kahlke and Ralph 2019) which assigns taxonomies to sequences based on the Last Common Ancestor (LCA; Wood and Salzberg 2014). The following parameter values were used: an *e*-value threshold of 1E-80, a minimum number of hits of 1, and a minimum percentage of hits of 60% to assign taxonomies to sequences based on the LCA of the best BLAST hit identified with > 90% identity.

2.5 | Family Richness

All analyses were conducted in R 4.3.1 (R Core Team 2019). A binomial generalized mixed effects model (glmer function of the package "lme4"; Bates et al. 2014) with a logistic link function and mesocosm as a random effect was performed to assess whether metabarcoding detections (1: detected and 0: not detected, i.e., zero read) of the most frequent families (Cyclopidae and Chydoridae) as assessed with microscopy, were related to their abundance (number of individuals/L; zooplankton microscopic data from Hébert et al. 2021), time (number of days since the experiment started), nutrient level (mesotrophic and eutrophic), and (ln + 1) transformed target concentration of the active ingredient (glyphosate, used as a proxy of herbicide toxicity).

We tested the correlation between the standardized (*z*-score) numbers of families obtained with microscopy (data from Hébert et al. 2021) and metabarcoding using a non-parametric Kendall's Tau test (cor.test function in R). Finally, linear mixed models (lmer function; package "lme4") were performed to investigate the effect of time, nutrient level, (ln + 1) transformed target concentration of the active ingredient (glyphosate) (i.e., a proxy of herbicide toxicity) and their interactions, on the number of detected families (rotifers, crustaceans, and insects). The mesocosm ID was set as a random effect. Temporal autocorrelation in the residuals was controlled using the corAR1 function. A top-down strategy was applied for model selection (gradual removal of non-significant interactions from a "beyond optimal" model; Zuur et al. 2009). The residual normality and homogeneity of variance of the models were graphically inspected. The model effects were visualized using the allEffects function in the "effects" R package (Fox and Weisberg 2019). Pairwise comparison between time points was performed using the pairs function of the emmeans package (Lenth 2024) (by default Tukey adjustment of *p*-values for multiple tests). The marginal R^2 (proportion of variance explained by fixed factors) and conditional R^2 (proportion of variance explained by both fixed and random factors) of the optimal model were estimated using the r.squaredGLMM() of the MuMIn package (Bartoń 2024).

2.6 | Community Composition

First, the dynamic patterns of the relative read abundance among and within each taxonomic group (rotifers, crustaceans, and insects) were visualized with barplots. Then, community compositional changes during each phase were visualized using Bray–Curtis dissimilarity index and non-metric multidimensional scaling (NMDS) with the metaMDS function in the "vegan" R package (Oksanen et al. 2022) (distance = "bray", try = 20, trymax = 5000, autotransform = FALSE). NMDS analyses were conducted following the Hellinger transformation on the abundance data (decostand function). The quality of the NMDS solution was evaluated based on the stress value (stress values < 0.05: excellent quality; stress values > 0.2: poor quality; Kruskal 1964). The environmental variable (ln + 1) transformed target concentration of the active ingredient (glyphosate) (i.e., used as a proxy of herbicide toxicity) was fitted to the NMDS using the envfit function (999 permutations) and plotted to the ordination plot only if found significant ($p < 0.05$). Permutational multivariate analysis of variance (perMANOVA; 999 permutations) was performed using the adonis2 function to investigate whether herbicide and nutrient treatment levels (Figure 1), their interactions, and time influenced community composition. Pairwise comparisons were performed using the pairwise.perm.manova function (permutations = 9999, FDR *p*-value adjustment for multiple tests) in the R package "RVAideMemoire" (Herve 2023). Homogeneity of variance was verified using the betadisper and permutest (permutations = 999) functions. Finally, an indicator species analysis was performed to identify families' preferences in the mesocosm treatments using the multipatt function (999 permutations, func = "r.g") in the R package "indicspecies" (De Cáceres and Legendre 2009).

Community compositional changes over time were also illustrated with principal response curves (PRC), which contrast a reference (control) to treated samples (herbicide treatment effect) on the left *Y* axis and the contribution of each family to compositional changes on the right *Y* axis (taxon score). Data were Hellinger transformed as described above, and PRCs were built using the prc function of the vegan package: for each nutrient level (mesotrophic and eutrophic) we contrasted the mesocosms (CP, MH, and HH) to the control "C" which never received any herbicide treatment across the experiment.

2.7 | Intraspecific Genetic Diversity Analyses

Intraspecific genetic diversity was estimated as the number of unique ASV sequences assigned to a particular species, for those that were consistently detected across libraries using BASTA. The non-parametric Kendall's tau test was used to investigate the correlation between haplotype richness (*z*-transformed number of ASV sequences) of selected rotifer, insect, and crustacean species and their respective abundance (*z*-transformed number of individuals estimated with microscopy assessments, data from Hébert et al. 2021). Finally, LMMs were performed to evaluate the effect of time, (ln + 1) transformed active glyphosate concentrations, nutrient levels, and their interaction on haplotype richness (ln + 1 transformed number of ASV sequences) of the selected species during phase I. Mesocosm identity was set as a random effect. Temporal autocorrelation in the residuals was controlled using the corAR1 function. A top-down strategy was performed for model selection (Zuur et al. 2009). The residual normality and homogeneity of variance of the models were visually inspected. The model effects were visualized using the allEffects function in the "effects" R package (Fox and Weisberg 2019). Pairwise comparison between time points was performed using the pairs function of the emmeans package (Lenth 2024) (by default Tukey adjustment of *p*-values for multiple tests). The marginal R^2 (proportion of variance explained by fixed factors) and conditional R^2 (proportion of variance explained by both fixed and random factors) of the optimal model

were estimated using the *r*.squaredGLMM() of the MuMIn package (Bartoń 2024).

3 | Results

3.1 | Relationship Between Metabarcoding and Microscopy Data

The number of raw reads in the treated mesocosms ranged between 23,406 and 279,643, and between 16,729 and 234,555 after the last filtering step in DADA2 (Table S4). We excluded 62 non-zooplankton families and terrestrial invertebrates that do not have any aquatic stage in their life cycle (Table S3). The final dataset (1152 reads with between one and 24 reads per sample, and between one and ten families per sample) comprised four families of crustaceans (Cladocera: Chydoridae, Macrothricidae, Sididae, and Copepoda: Cyclopidae), four families of Rotifera (Monogononta: Asplanchnidae, Brachionidae, Lecanidae, and Synchaetidae), and eight families of insects (Diptera: Baetidae, Chaoboridae, Chironomidae, Culicidae, Limoniidae, Sciaridae; and Odonata: Lestideae, and Libellulidae). While Macrothricidae was only detected with metabarcoding, five zooplankton families recovered by microscopy were not recovered by metabarcoding (crustaceans: Daphniidae, rotifers: Conochilidae, Testudinellidae, Trochosphaeridae, and Trichocercidae) (Hébert et al. 2021).

The binomial GLMMs revealed that the detection success of the crustacean Chydoridae and Cyclopidae using metabarcoding was not associated with their abundance (number of individuals counted under microscopy) (Chydoridae: estimate = 0.283, SE = 0.383, $p = 0.461$, and Cyclopidae: estimate = −0.160, SE = 0.596, $p = 0.788$; Table S5). However, the detection success of Cyclopidae was higher under eutrophic conditions (Mesotrophic estimate = −2.610, SE = 0.960, $p = 0.006$) and was negatively influenced by the glyphosate treatment (estimate = −0.356, SE = 0.158, $p = 0.025$) (Table S5). We found no effect of any of the variables on the detection success of Chydoridae ($p > 0.05$; Table S5). The number of crustacean families detected with metabarcoding was also not correlated with their diversity estimates from microscopy ($z = 1.419$, $p = 0.156$, $\tau = 0.151$). However, the number of rotifer families detected with metabarcoding was positively correlated with their abundance estimates from microscopy ($z = 4.894$, $p = 9.897\text{e-}07$, $\tau = 0.514$).

3.2 | Family Richness Dynamics

During phase I, we observed an overall stronger and faster response to the application of herbicide on the number of zooplankton families in the high herbicide (HH) mesocosms, regardless of the nutrient levels (Figure 2). For example, after the first pulse of herbicide the crustaceans lost two families in the mesotrophic mesocosms treated with 0.3 mg/L herbicide (MMH), while they stayed stable for 8 days and then lost one family in the eutrophic mesocosm treated with 0.3 mg/L glyphosate (EMH) (Figure 2). With a higher concentration of glyphosate (15 mg/L), they lost three families in both the mesotrophic mesocosm (MHH) (immediately) and eutrophic mesocosm (EHH) (two families in 8 days, one additional family in 15 days) (Figure 2). After the second pulse, the number of rotifer and crustacean families remained stable in the treated mesocosms (except the loss of one family each in MMH, Figure 2), while the number of insect families increased from one (MMH) to three (EHH) families (except in EMH where it remained stable at one family, Figure 2). These observations were supported by the LMMs which revealed an overall negative effect of herbicide concentration on

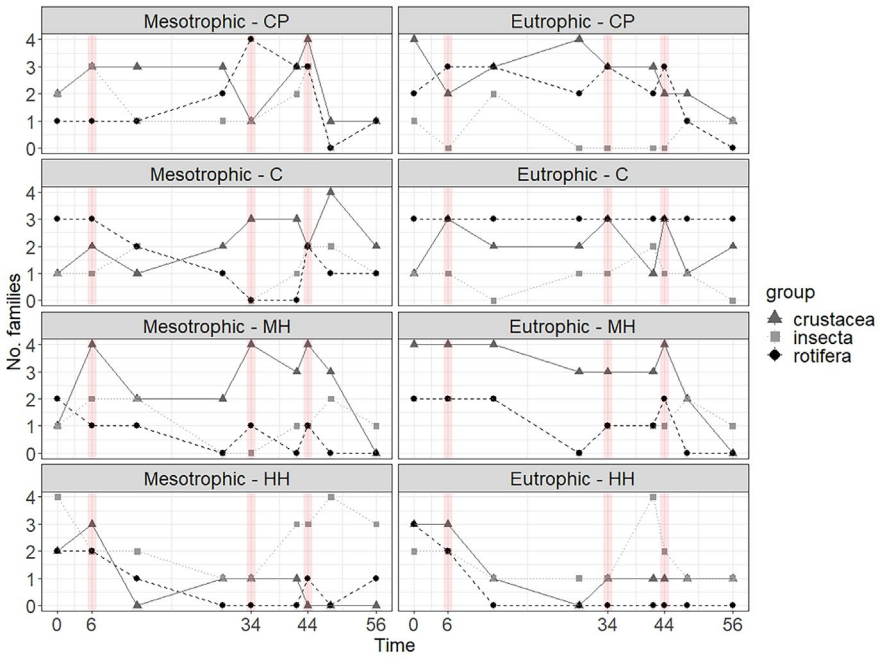

FIGURE 2 | Dynamics of the estimated number of rotifer (black dot), crustacean (dark gray triangle), and insect (light gray square) families through time, obtained with metabarcoding, in each treatment. "C" = Control, "CP" = Control with 40 mg/L pulse at day 43, "MH" = 0.3 mg/L herbicide treatment, "HH" = 15 mg/L herbicide treatment. Times 6, 34, and 44 are highlighted by a red bar as the sampling days with first, second and third pulse, respectively.

zooplankton families (crustacean: estimate = −0.177, SE = 0.071, $p = 0.018$; rotifers: estimate = −0.161, SE = 0.049, $p = 0.003$), an overall positive effect on insect families (estimate = 0.084, SE = 0.037, $p = 0.032$), but no effect of nutrient level ($p > 0.05$; Table S6). Rotifer family richness was also higher at days 0 and 6 than at day 29 (pairwise comparison: $\text{estimate}_{0\text{-}29} = 1.574$, $\text{SE}_{0\text{-}29} = 0.372$, $p\text{-adjust}_{0\text{-}29} = 0.002$; $\text{estimate}_{6\text{-}29} = 1.340$, $\text{SE}_{6\text{-}29} = 0.380$, $p\text{-adjust}_{6\text{-}29} = 0.014$), while insect family richness was higher at day 6 than day 29 ($\text{estimate}_{6\text{-}29} = 1.205$, $\text{SE}_{6\text{-}29} = 0.389$, $p\text{-adjust}_{6\text{-}29} = 0.041$) but lower at day 42 than days 29 and 34 ($\text{estimate}_{29\text{-}42} = -1.271$, $\text{SE}_{29\text{-}42} = 0.390$, $p\text{-adjust}_{29\text{-}42} = 0.028$; $\text{estimate}_{34\text{-}42} = -1.195$, $\text{SE}_{34\text{-}42} = 0.353$, $p\text{-adjust}_{34\text{-}42} = 0.021$). During phase II, none of the three taxonomic groups were significantly influenced by the glyphosate treatment or nutrient level ($p > 0.05$), but time was significant (Table S6): the number of rotifer families immediately decreased after the application of the 40 mg/L GBH pulse ($\text{estimate}_{44\text{-}48} = 1.250$, $\text{SE}_{44\text{-}48} = 0.313$, $p\text{-adjust}_{44\text{-}48} = 0.004$; $\text{estimate}_{44\text{-}56} = -1.125$, $\text{SE}_{44\text{-}56} = 0.367$, $p\text{-adjust}_{44\text{-}56} = 0.021$), the number of crustacean families was lower only at the end of the experiment ($\text{estimate}_{44\text{-}56} = 1.625$, $\text{SE}_{44\text{-}56} = 0.597$, $p\text{-adjust}_{44\text{-}56} = 0.041$) and the number of insect families was not influenced by time (p-adjust > 0.05) (Table S6).

3.3 | Community Composition Dynamics

Rotifers dominated the eutrophic controls (C and CP) almost consistently during the whole experiment, while the dominant taxonomic groups in the mesotrophic controls fluctuated mostly between crustaceans and rotifers (Figure 3A). The mesocosms treated with 0.3 mg/L herbicide (MH) were dominated by crustacean families during phase I, and by insect families after application of the 40 mg/L herbicide pulse. The mesocosms treated with 15 mg/L (HH) were dominated by insects, regardless of nutrient level and phase of the experiment (Figure 3A). We observed variations in the relative number of reads within the three groups over time, even in absence of herbicide pulses (Figure 3B–D).

Community dissimilarity was influenced by herbicide treatment and nutrient level in both phases of the experiment, the interaction time—herbicide treatment in phase I ($F = 2.123$, $p = 0.020$), and the interaction herbicide—nutrient level in phase II ($F = 2.794$, $p = 0.012$) (Table S7). We observed a high overlap between time points and pairwise differences in community composition between time points were non-significant after correction for multiple tests ($p > 0.05$, phases I and II) (Table S7). We also observed a high overlap between the controls (C and CP; Figure 4) and pairwise comparison of herbicide treatments confirmed that C and CP showed similar communities through the experiment ($p_{\text{phase I}} = 0.198$, $p_{\text{phase II}} = 0.297$) (Table S7). In phase I, the controls (C, CP, and C + CP) were characterized by four rotifers families: the Asplanchnidae ($\Phi = 0.494$, $p = 0.004$), Lecanidae ($\Phi = 0.445$, $p = 0.050$), Synchaetidae ($\Phi = 0.478$, $p = 0.004$), and Brachionidae ($\Phi = 0.433$, $p = 0.016$), respectively (Figure 4A, Table S8). In phase II, C was characterized by both Asplanchnidae rotifers ($\Phi = 0.801$, $p = 0.001$) and Sididae crustaceans ($\Phi = 0.524$, $p = 0.034$) (Figure 4B, Table S8).

MH and HH mesocosms differed from controls ($p < 0.05$) and among each other ($p = 0.0004$) in phase I (Figure 4A, Table S7), while the only significant difference in phase II was between HH and C ($p = 0.014$) (Table S7) Figure 4B). In phase I, the MH mesocosms were characterized by the Sididae crustacean family ($\Phi = 0.436$, $p = 0.008$), and the HH mesocosms by the Chironomidae insect family ($\Phi = 0.701$, $p = 0.001$) (Figure 4, Table S8). In phase II, both MH and HH were characterized by Chironomidae ($\Phi = 0.612$, $p = 0.018$) (Table S8).

Eutrophic level was characterized by both Brachionidae rotifers ($\Phi = 0.510$, $p = 0.001$) and Cyclopidae crustacean families ($\Phi = 0.409$, $p = 0.002$) in phase I and solely Brachionidae ($\Phi = 0.519$, $p = 0.011$) in phase II, while mesotrophic levels were characterized by the Sididae crustacean family ($\Phi = 0.394$, $p = 0.005$) in phase I and Sciaridae insect family ($\Phi = 0.506$, $p = 0.042$) in phase II (Table S8).

The same observations on the effect of herbicide on the composition of the communities and family preferences were also revealed by the PRCs (Figure S2). HH mesocosms deviated the most from C (baseline). In phase I, the first pulse of herbicide induced greater deviations from controls than the second pulse. In phase II, the herbicide had an effect in all treatments, except mesotrophic-HH mesocosms (Figure S2).

3.4 | Intraspecific Genetic Dynamics

We identified three species of rotifers (*Asplanchna sieboldi*, *Euchalnis dilatata*, *Keratella cochlearis*, and *Polyarthra* sp.; 53 total haplotypes), two of crustaceans (*Chydorus brevilabris* and *Sida crystallina*; five total haplotypes), and four species of insect (*Callibaetis fluctuans*, *Cloeon dipterum*, *Smittia stercoraria*, and *Tanytarsus mendax*; 22 total haplotypes) (Figure 5A). The *Polyarthra* genus was included because only one species of the genus *Polyarthra* was identified through microscopy (Hébert et al. 2021).

Overall, we observed a lower number of haplotypes in the treated mesocosms than in the control mesocosms, regardless of the nutrient level (Figure 5B, Figure S3). For example, *K. cochlearis* haplotype richness ranged between 0 and four in the treated mesocosms but reached eight haplotypes in most controls (MCP, EC, and ECP, Figure S3). Mesotrophic controls showed the highest intraspecific genetic variation and were mostly represented by rotifer species (Figure 5B). For example, *Polyarthra* sp. showed a peak of haplotype richness (16 haplotypes) in the mesotrophic control a week after the first pulse, while in the eutrophic control variation did not show large fluctuations (Figure S3). The lowest number of haplotypes was observed in the mesotrophic MH mesocosm (Figure 5B). The highest haplotype proportion of crustacean was observed in the mesotrophic MH mesocosm, while the highest haplotype proportion of insects was observed in the mesotrophic HH mesocosm (Figure 5B).

Haplotype richness in the rotifers *K. cochlearis* and *Polyarthra* sp. was positively correlated with their abundance estimates obtained through microscopy assessments (*K. cochlearis*: $z = 4.156$, $p = 3.238\text{e-}05$, $\tau = 0.548$; *Polyarthra* sp.: $t = 5.172$, $p = 2.311\text{e-}07$, $\tau = 0.622$). In all treated mesocosms, the number of *Polyarthra* sp. haplotypes decreased from the beginning of the experiment, while the number of *K. cochlearis*

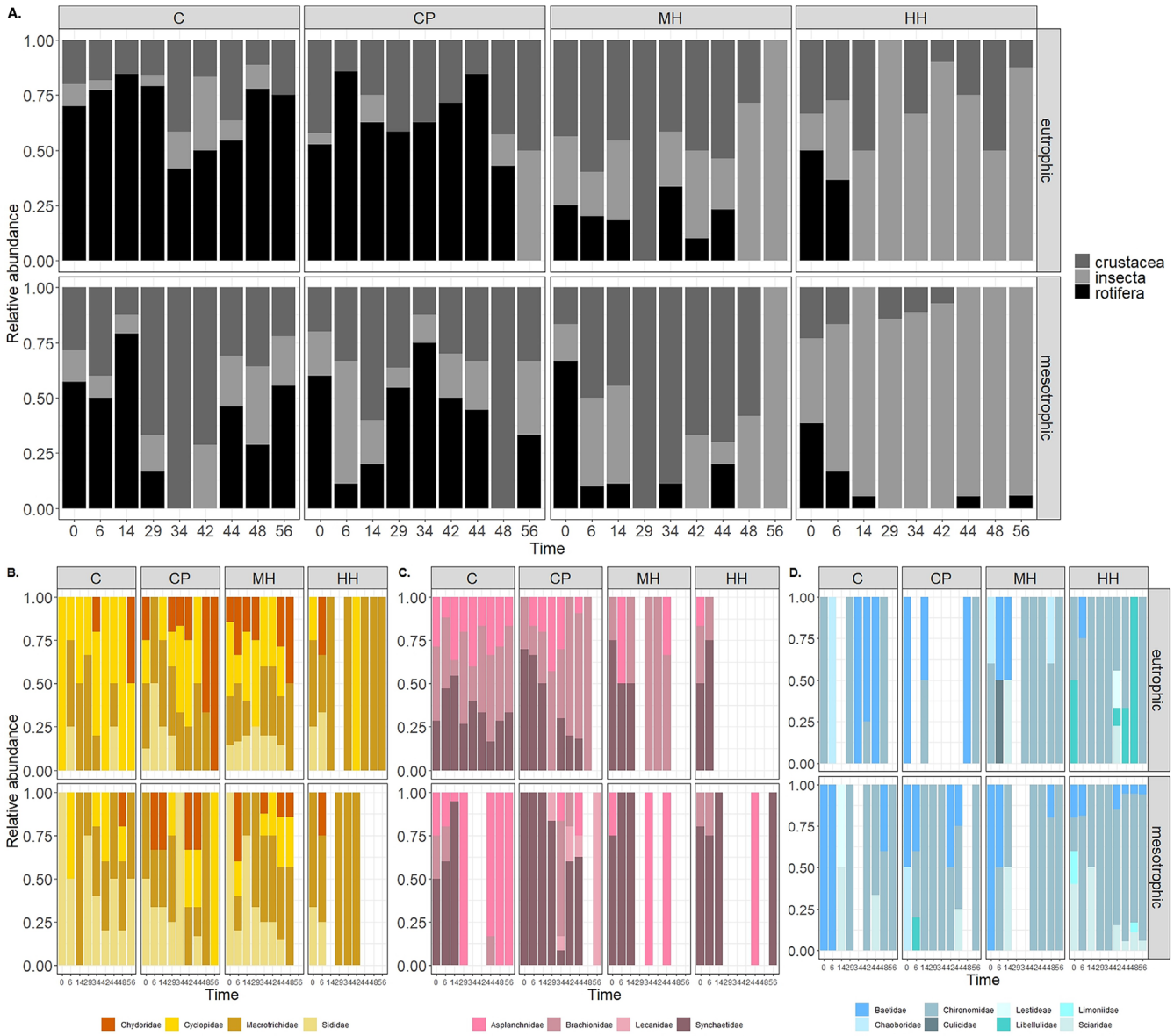

FIGURE 3 | Relative reads abundance in each treatment (herbicide and nutrient level): (A) among the three groups: Rotifers (black), crustaceans (dark gray), and insects (light gray), (B) among crustacean families, (C) among rotifer families, and (D) among insect families. "C" = Control, "CP" = Control with 40 mg/L pulse in phase II, "MH" = 0.3 mg/L herbicide treatment, "HH" = 15 mg/L herbicide treatment. Times 6, 34, and 44 are the sampling days with first, second and third pulse, respectively.

haplotypes increased between days 29 and 34 (EMH) and between day 34 and day 42 (EHH) (Figure S3). During phase I, the haplotype richness of *K. cochlearis* and *Polyarhra* sp. decreased with increasing herbicide concentrations (*K. cochlearis* estimate = −0.132, SE = 0.035, $p = 0.001$; *Polyarthra* estimate = −0.093, SE = 0.023, $p = 0.0004$) (Table S9), and the loss of haplotypes was more severe in eutrophic mesocosms than mesotrophic mesocosms for *K. cochlearis* (estimate = 0.108, SE = 0.053, $p = 0.020$) (Table S9). We also found a significant effect of time on *Polyarthra* sp. haplotype richness, with a significant difference between day 0 and days 29 ($\text{estimate}_{0\text{-}29} = 1.047$, $\text{SE}_{0\text{-}29} = 0.281$, $p\text{-adjust}_{0\text{-}29} = 0.008$), 34 ($\text{estimate}_{0\text{-}34} = 1.117$, $\text{SE}_{0\text{-}34} = 0.281$, $p\text{-adjust}_{0\text{-}34} = 0.004$) and 42 ($\text{estimate}_{0\text{-}42} = 1.117$, $\text{SE}_{0\text{-}42} = 0.281$, $p\text{-adjust}_{0\text{-}42} = 0.004$) (Table S9). Finally, none of the variables influenced *K. cochlearis* haplotype richness in phase II ($p > 0.05$), while *Polyarthra* sp. haplotype richness was influenced by herbicide concentration (estimate = −0.088, SE = 0.029, $p = 0.040$) (Table S9).

4 | Discussion

4.1 | Promises and Limitations of the Metabarcoding Approach

While metabarcoding recovered a broad range of taxa, some zooplankton families that were previously identified with microscopy in the mesocosms by Hébert et al. (2021) were not recovered here (false negative) or were discarded when filtering

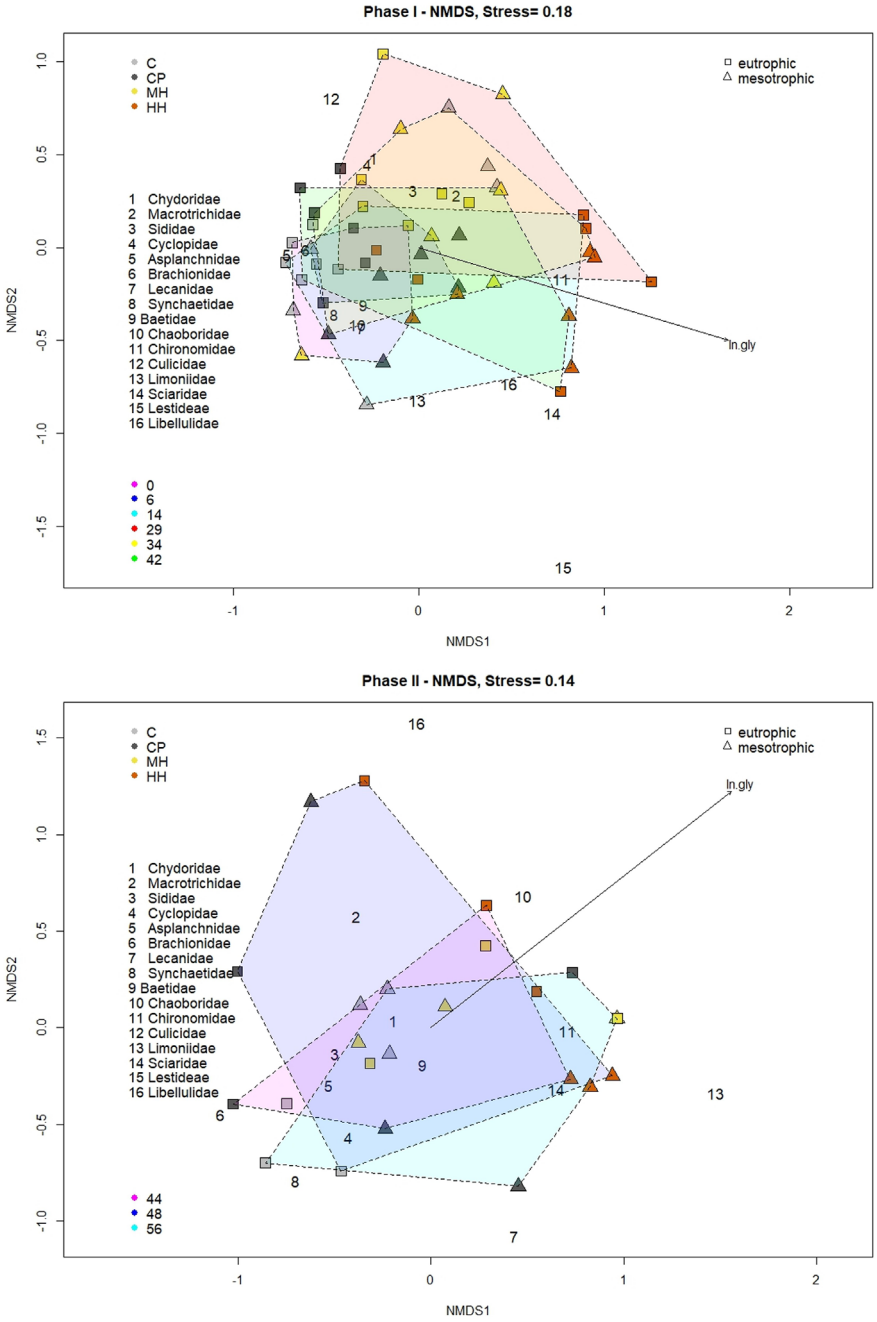

FIGURE 4 | NMDS biplot representation of community composition in Phase I (top) and Phase II (bottom) based on Bray–Curtis dissimilarity (Hellinger transformed data), using metabarcoding. "C" = Control, "CP" = Control with 40 mg/L pulse at day 43, "MH" = 0.3 mg/L herbicide treatment, "HH" = 15 mg/L herbicide treatment. The arrow depicts the glyphosate-fitted environmental variable ("ln.gly" = ln + 1 transformed glyphosate concentration) significantly correlated to ordination ($p < 0.05$). The NMDS axes explained 50% of the variation in glyphosate concentration ($r^2 = 0.502$, $p = 0.001$) in phase I and 29% of the variation in glyphosate concentration ($r^2 = 0.291$, $p = 0.023$) in phase II.

the data (e.g., Daphniidae). The detection inconsistencies could also result from the small filter pore size as the water samples were initially collected for microbial analyses (Barbosa da Costa et al. 2021), or missing species in the reference database. The latter was mitigated by using the gold standard marker for arthropod barcoding (COI) with a relatively good zooplankton coverage in Canada (Tournayre et al. 2024), and by working at the family level with a relaxed identity threshold. Another explanation could be the many degenerate nucleotides (every two bases) in the Leray primer sequences. This high degeneracy enables the recovery of a broad range of species from invertebrates to vertebrates, but it also increases the competition during PCR amplification, thereby increasing the probability of false negatives. While the Leray primers have been designed and mainly used for invertebrate metabarcoding, they have also shown affinity toward vertebrates in water samples (Leray et al. 2013; Tournayre et al. 2024). Using zooplankton-specific primers would likely improve our taxonomic coverage, both in number of taxa and number of reads, by reducing non-target invertebrate and vertebrate amplification (Leese

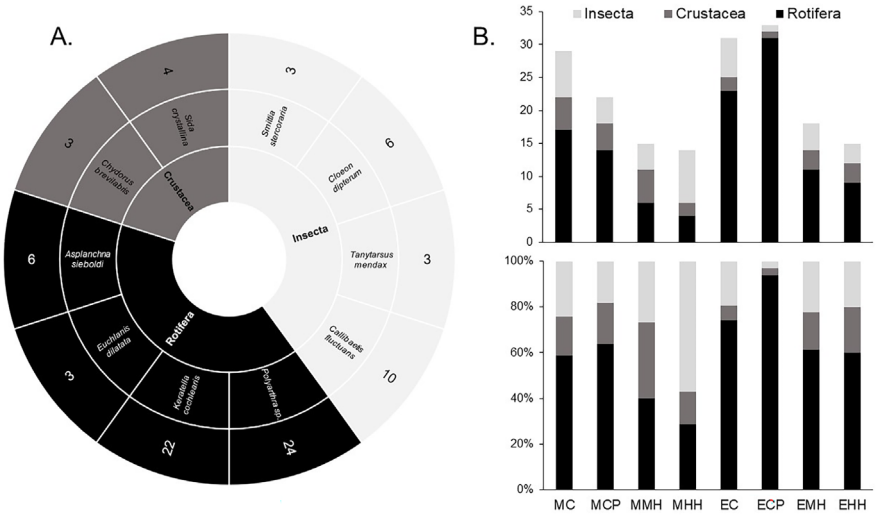

FIGURE 5 | (A) Species that were reliably identified using the Last Common Ancestor Algorithm (BASTA) and their relative number of haplotypes. (B) Total number of haplotypes for rotifers, crustaceans, and insects across ponds (relative estimates at the top right and percentages at the bottom right). Mesocosm ponds are coded as MC (mesotrophic control), MCP (mesotrophic control with 40 mg/L pulse at day 43), MMH (mesotrophic with 0.3 mg/L herbicide pulse), MHH (mesotrophic with 15 mg/L herbicide pulse), EC (eutrophic control), ECP (eutrophic control with 40 mg/L herbicide pulse at day 43), EMH (eutrophic with 0.3 mg/L herbicide pulse), and EHH (eutrophic with 15 mg/L herbicide pulse).

et al. 2021; Collins et al. 2019). Combining primers pairs, especially on different genes, would be another valuable alternative to limit the effect of individual primer bias and therefore improves taxa recovery (Zhang et al. 2018; Duarte et al. 2023; Song and Liang 2023). The presence of false negatives at certain time points in our study could also be associated with low sequencing depth of the target taxa that led to the underestimation of families (Alberdi et al. 2018). However, despite these limitations, metabarcoding recovered taxonomic groups not easily captured or identified by microscopy (Djurhuus et al. 2018; Frontalini et al. 2018; Chen et al. 2023). For example, benthic insect larvae are traditionally sampled with emergence traps (Davies 1984) or benthic sampling techniques (Malison, Benjamin, and Baxter 2010) and are likely missed by water surface sampling which is more appropriate for zooplankton. A good performance of metabarcoding in identifying insects was also observed in most studies (Ekrem, Stur, and Hebert 2010; Silva and Wiedenbrug 2014; Lin, Stur, and Ekrem 2018; Sun et al. 2019), which highlights the added value of using metabarcoding to efficiently characterize communities with reduced sampling effort.

A common concern with eDNA metabarcoding is that it cannot distinguish dead from living organisms (whereas it is relatively easy to exclude decayed individuals with microscopy), and it can pick up signals from organisms no longer present because of the persistence of eDNA in water (Marshall, Vanderploeg, and Chaganti 2021). In this study, the mesocosms were stagnant, enabling dead zooplankton organisms to quickly sink to the bottom, thereby limiting their collection for microscopy assessments (Hébert et al. 2021). However, because of the limited water depth, dead organisms at the bottom were still within <1 m of the water surface. Thus, dead and decaying organismal DNA in such relatively small and closed experimental systems could have been picked up by the metabarcoding approach, potentially increasing false positive detection rates. Moreover, eDNA samples were collected a few days apart during the experiment which may not be a sufficient time for complete degradation of eDNA from previous time points. New approaches such as eRNA metabarcoding, would open new opportunities to provide even more accurate estimate snapshots of aquatic diversity at a fine temporal resolution (Cristescu 2019; Greco et al. 2022; Giroux et al. 2023).

4.2 | Effect of Herbicide and Nutrient Enrichment on Intra- and Inter-Specific Diversity

Despite these concerns, the metabarcoding approach showed partially congruent results with those obtained using the microscopy approach (Hébert et al. 2021), highlighting the ability of metabarcoding to assess rapid community changes. We showed that herbicide applications caused a significant decrease in both crustacean and rotifer family richness, but an increase in insect families (Table S6). Moreover, the communities in the controls were characterized by rotifers in both phases, those in the 0.3 mg/L herbicide mesocosms (MH) by crustaceans (phase I) and insects (phase II, 40 mg/L pulse of herbicide), and those in the 15 mg/L herbicide mesocosms (HH) by insects (Figure 3). The dominance of insect families in high-herbicide treatments and phase II of the experiment could be explained by their lower susceptibility to herbicides than the zooplankton taxa (Folmar, Sanders, and Julin 1979; Relyea 2005). Thus, in accordance with the microscopy study, eDNA metabarcoding suggested a gradient of tolerance in rotifer, crustacean, and insects to this herbicide (Hébert et al. 2021); with both approaches indicating greater sensitivity in rotifers. Additionally, the severe decline of rotifers in high herbicide mesocosms, also observed by Hébert et al. (2021), could be due to the transient loss of algae upon the first pulse, causing a quick collapse of short-lived rotifers.

We did not find any effect of nutrient level (eutrophic vs. mesotrophic) on the number of crustacean, rotifer, and insect families,

but we did observe an effect on community composition and intraspecific diversity. While we can not fully exclude the possibility of false haplotypes due to sequencing errors (e.g., undetected chimera), it is also possible that the full genetic diversity was not captured because of variation in biomass, primer bias, loss of sequence variants with low abundance, and unoptimized PCR conditions for haplotype recovery (Elbrecht et al. 2018; Serrana and Watanabe 2023). Moreover, the short amplicon length used in eDNA studies, typically under ~400 bp, can lack enough variation to distinguish closely related species and resolve intraspecific diversity (Serrana and Watanabe 2023; Thomasdotter et al. 2023). However, as predicted, the rotifer *K. cochlearis* and *Polyarthra* sp. haplotype richness was positively correlated to population size (as the number of individuals assessed with microscopy) and was negatively affected by the herbicide treatment (phase I *K. cochlearis* and phases I and II *Polyarthra* sp). Genetic erosion due to chemical stress represents a factor of concern in risk assessment (review in van Straalen and Timmermans 2002). Toxicants can affect genetic diversity by increasing mutational load, by natural selection on resistance genotypes, by inducing bottleneck events, and by altering migration (van Straalen and Timmermans 2002). For example, a parallel study by Barbosa da Costa et al. (2022) with the same mesocosms, showed that this herbicide strongly cross-selected for antibiotic resistance in natural freshwater bacteria. In our study, nutrient enrichment (eutrophic level) exacerbated the effect of active glyphosate on haplotype richness of the two rotifer species. Rotifers are dominant components of zooplankton communities, channeling basal resources to higher trophic levels while contributing to secondary production and nutrient recycling. The few existing studies on the chronic effects of pesticides on rotifers revealed high sensitivity depending on genotype diversity and experimental conditions (see Moreira et al. 2016). Parallel studies by Fugère et al. (2020) and Hébert et al. (2021) with the same mesocosms, suggested that the herbicide increased algal resources perhaps due to the P content of glyphosate and that this fertilizing bottom-up effect can transfer to higher trophic levels (i.e., zooplankton); an observation also made in previous studies (Pérez et al. 2007; Vera et al. 2012). However, by including even higher trophic levels (insects), our eDNA metabarcoding approach suggested that the advantage of crustacean gained by the loss of highly sensitive competitors and/or predator may have been counteracted by increased predation from insect larvae (top-down regulation; Chang, Sakamoto, and Hanazato 2005; Fussmann and Gonzalez 2013; Kovach-Orr and Fussmann 2013; Yamamichi and Miner 2015; Bell et al. 2019).

Finally, the two pulses of herbicide during phase I and the pulse in phase II showed different effects on zooplankton communities. For example, the community composition of both MH (0.3 mg/L) and HH (15 mg/L) herbicide treatments differed from the controls in phase I, while only the HH treatment differed from the control ("C" herbicide-free) in phase II. Pre-exposure to phase I doses of the herbicide may have represented an advantage for coping with the 40 mg/L pulse, as also observed on phytoplankton by Fugère et al. (2020), and zooplankton by Hébert et al. (2021). Because commercial formulations of the herbicide contain multiple molecules, such as surfactants that can be even more toxic than the active ingredient (review in Mesnage and Antoniou 2018), the observed effects in our study can not be attributed to a single ingredient (i.e., active glyphosate). Further research is required for better risk assessment of commercial chemical mixtures and identification of the actual driver(s) of the observed effects.

5 | Conclusion

Metabarcoding assessed biodiversity response to glyphosate-based herbicide and nutrient enrichment, from the intraspecific genetic diversity up to the community scale and enabled a better understanding of their effects along the trophic chain compared with microscopy by recovering more trophic levels with limited sampling effort. Further validations, including comparison with zooplankton specific primers, are now needed to confirm whether metabarcoding could be used for quantitative assessments of changes in abundance of zooplankton taxa as suggested by the correlations we observed between richness (obtained with metabarcoding) and abundance estimates (obtained with morphological assessments) (Serrana et al. 2019; Song and Liang 2023). While our samples were initially collected and processed for microbial analyses (Barbosa da Costa et al. 2021), we were able to detect the main effects of a glyphosate-based herbicide on the target communities. Metabarcoding is therefore very promising even if DNA collection methods are not optimal, which suggests an opportunity for further exploring freshwater communities with existing samples. Finally, additional research must be conducted to understand the long-term consequences of herbicide on biodiversity, and to what extent abiotic and biotic factors influence the toxic, fertilizing or synergistic effect of glyphosate-based herbicides when combined with other pollutants in the environment.

Author Contributions

Conception and design of the study: A.L., V.F., A.G., R.D.H.B., M.-P.H., B.E.B., M.E.C. Acquisition of the data: A.L., M.-P.H., N.B.C., V.F. Bioinformatics: A.L. Analysis of the data: A.L., M.-P.H., V.F., O.T. Writing of the manuscript: A.L., O.T. with contributions from all authors.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data are archived in the Figshare public repository: 10.6084/m9.figshare.25769157.

References

Adams, C. I., M. Knapp, N. J. Gemmell, et al. 2019. "Beyond Biodiversity: Can Environmental DNA (eDNA) Cut It as a Population Genetics Tool?" *Genes* 10, no. 3: 192.

Alberdi, A., O. Aizpurua, M. T. P. Gilbert, and K. Bohmann. 2018. "Scrutinizing Key Steps for Reliable Metabarcoding of Environmental Samples." *Methods in Ecology and Evolution* 9: 134–147.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. "Basic Local Alignment Search Tool." *Journal of Molecular Biology* 215: 403–410.

Anderson, A., G. Byrtus, J. Thompson, D. Humphries, B. Hill, and M. Bilyk. 2002. "Baseline Pesticide Data For Semi-Permanent Wetlands In The Aspen Parkland of Alberta." Prepared for: Alberta Environment Water Research User Group, Alberta Environment Ecosystem User Group, and Alberta North American Waterfowl Management Plan Partnership. Alberta Environment. Pub No. T/673, ISBN No. 07785-2441-8 (Printed Edition), ISBN No. 07785-2442-6 (On-line Edition). http://www3.gov.ab.ca/env/info/infocentre/publist.cfm.

Anderson, A.-M. 2005. *Overview of Pesticide Data in Alberta's Surface Waters Since 1995*. Edmonton, AB: Alberta Environment, Environmental Monitoring & Evaluation Branch.

Annett, R., H. R. Habibi, and A. Hontela. 2014. "Impact of Glyphosate and Glyphosate-Based Herbicides on the Freshwater Environment." *Journal of Applied Toxicology* 34: 458–479.

Antich, A., C. Palacín, J. Zarcero, O. S. Wangensteen, and X. Turon. 2023. "Metabarcoding Reveals High-Resolution Biogeographical and Metaphylogeographical Patterns Through Marine Barriers." *Journal of Biogeography* 50, no. 3: 515–527.

Barbosa da Costa, N., V. Fugère, M. P. Hébert, et al. 2021. "Resistance, Resilience, and Functional Redundancy of Freshwater Bacterioplankton Communities Facing a Gradient of Agricultural Stressors in a Mesocosm Experiment." *Molecular Ecology* 30, no. 19: 4771–4788.

Barbosa da Costa, N., M. P. Hébert, V. Fugère, et al. 2022. "A Glyphosate-Based Herbicide Cross-Selects for Antibiotic Resistance Genes in Bacterioplankton Communities." *mSystems* 7, no. 2: e0148221.

Barnes, M. A., and C. R. Turner. 2016. "The Ecology of Environmental DNA and Implications for Conservation Genetics." *Conservation Genetics* 17: 1–17.

Bartoń, K. 2024. "MuMIn: Multi-Model Inference." R Package Version 1.48.4. https://CRAN.R-project.org/package=MuMIn.

Bates, D., M. Mächler, B. Bolker, and S. Walker. 2014. "Fitting Linear Mixed-Effects Models Using lme4." *Journal of Statistical Software* 67, no. 1: 48. https://doi.org/10.18637/jss.v067.i01.

Bell, G., V. Fugère, R. Barrett, et al. 2019. "Trophic Structure Modulates Community Rescue Following Acidification." *Proceedings of the Royal Society B: Biological Sciences* 286, no. 1904: 20190856.

Bohan, D. A., C. Vacher, A. Tamaddoni-Nezhad, A. Raybould, A. J. Dumbrell, and G. Woodward. 2017. "Next-Generation Global Biomonitoring: Large-Scale, Automated Reconstruction of Ecological Networks." *Trends in Ecology & Evolution* 32: 477–487.

Brown, E. A., F. J. Chain, T. J. Crease, H. J. MacIsaac, and M. E. Cristescu. 2015. "Divergence Thresholds and Divergent Biodiversity Estimates: Can Metabarcoding Reliably Describe Zooplankton Communities?" *Ecology and Evolution* 5, no. 11: 2234–2251.

Byer, J. D., J. Struger, P. Klawunn, A. Todd, and E. Sverko. 2008. "Low Cost Monitoring of Glyphosate in Surface Waters Using the ELISA Method: An Evaluation." *Environmental Science & Technology* 42: 6052–6057.

Cahill, A. E., J. K. Pearman, A. Borja, et al. 2018. "A Comparative Analysis of Metabarcoding and Morphology-Based Identification of Benthic Communities Across Different Regional Seas." *Ecology and Evolution* 8, no. 17: 8908–8920.

Callahan, B. J., P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. A. Johnson, and S. P. Holmes. 2016. "DADA2: High-Resolution Sample Inference From Illumina Amplicon Data." *Nature Methods* 13: 581–583.

Camacho, C., G. Coulouris, V. Avagyan, et al. 2009. "BLAST+: Architecture and Applications." *BMC Bioinformatics* 10: 421.

Carles, L., H. Gardon, L. Joseph, J. Sanchís, M. Farré, and J. Artigas. 2019. "Meta-Analysis of Glyphosate Contamination in Surface Waters and Dissipation by Biofilms." *Environment International* 124: 284–293.

Carraro, L., H. Hartikainen, J. Jokela, E. Bertuzzo, and A. Rinaldo. 2018. "Estimating Species Distribution and Abundance in River Networks Using Environmental DNA." *Proceedings of the National Academy of Sciences of the United States of America* 115: 11724–11729.

Ceballos, G., P. R. Ehrlich, and R. Dirzo. 2017. "Biological Annihilation via the Ongoing Sixth Mass Extinction Signaled by Vertebrate Population Losses and Declines." *Proceedings of the National Academy of Sciences of the United States of America* 114: E6089–E6096.

Chang, K., M. Sakamoto, and T. Hanazato. 2005. "Impact of Pesticide Application on Zooplankton Communities With Different Densities of Invertebrate Predators: An Experimental Analysis Using Small-Scale Mesocosms." *Aquatic Toxicology* 72: 373–382.

Chen, Y., H. Wang, Y. Gong, et al. 2023. "Benefits of Combined Environmental DNA and Microscopy for Diversity Monitoring in Rotifer Community: A Mesocosm Experiment." *Ecological Indicators* 155: 110930.

Collins, R. A., J. Bakker, O. S. Wangensteen, et al. 2019. "Non-specific Amplification Compromises Environmental DNA Metabarcoding With COI." *Methods in Ecology and Evolution* 10, no. 11: 1985–2001.

Cowart, D. A., M. Pinheiro, O. Mouchel, et al. 2015. "Metabarcoding Is Powerful Yet Still Blind: A Comparative Analysis of Morphological and Molecular Surveys of Seagrass Communities." *PLoS One* 10, no. 2: e0117562.

Cristescu, M. E. 2019. "Can Environmental RNA Revolutionize Biodiversity Science?" *Trends in Ecology & Evolution* 34: 694–697.

Darling, J. A., and A. R. Mahon. 2011. "From Molecules to Management: Adopting DNA-Based Methods for Monitoring Biological Invasions in Aquatic Environments." *Environmental Research* 111: 978–988.

Davies, I. J. 1984. "Sampling Aquatic Insect Emergence." In *A Manual on Methods for the Assessment of Secondary Productivity in Fresh Waters*, edited by J. A. Downing and F. H. Rigler, 2nd ed., IBP Handbook 17, 161–227. Oxford, UK: Blackwell Scientific Publications.

De Cáceres, M., and P. Legendre. 2009. "Associations Between Species and Groups of Sites: Indices and Statistical Inference." *Ecology* 90: 3566–3574. https://doi.org/10.1890/08-1823.1.

Des Roches, S., L. H. Pendleton, B. Shapiro, and E. P. Palkovacs. 2021. "Conserving Intraspecific Variation for Nature's Contributions to People." *Nature Ecology & Evolution* 5, no. 5: 574–582.

Djurhuus, A., K. Pitz, N. A. Sawaya, et al. 2018. "Evaluation of Marine Zooplankton Community Structure Through Environmental DNA Metabarcoding." *Limnology and Oceanography: Methods* 16, no. 4: 209–221.

Duarte, S., P. E. Vieira, B. R. Leite, M. A. Teixeira, J. M. Neto, and F. O. Costa. 2023. "Macrozoobenthos Monitoring in Portuguese Transitional Waters in the Scope of the Water Framework Directive Using Morphology and DNA Metabarcoding." *Estuarine, Coastal and Shelf Science* 281: 108207.

Edgar, R. C. 2016. "UNOISE2: Improved Error-Correction for Illumina 16S and ITS Amplicon Sequencing." *BioRxiv*, 081257.

Ekrem, T., E. Stur, and P. D. Hebert. 2010. "Females Do Count: Documenting Chironomidae (Diptera) Species Diversity Using DNA Barcoding." *Organisms Diversity & Evolution* 10: 397–408.

Elbrecht, V., T. W. Braukmann, N. V. Ivanova, et al. 2019. "Validation of COI Metabarcoding Primers for Terrestrial Arthropods." *PeerJ* 7: e7745.

Elbrecht, V., E. E. Vamos, D. Steinke, and F. Leese. 2018. "Estimating Intraspecific Genetic Diversity From Community DNA Metabarcoding Data." *PeerJ* 6: e4644.

Emmons, S. C., Z. G. Compson, M. C. Malish, et al. 2023. "DNA Metabarcoding Captures Different Macroinvertebrate Biodiversity Than Morphological Identification Approaches Across a Continental Scale." *Environmental DNA* 5, no. 6: 1307–1320.

Folmar, L. C., H. Sanders, and A. Julin. 1979. "Toxicity of the Herbicide Glyphosate and Several of Its Formulations to Fish and Aquatic Invertebrates." *Archives of Environmental Contamination and Toxicology* 8: 269–278.

Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek. 1994. "DNA Primers for Amplification of Mitochondrial Cytochrome c Oxidase Subunit I From Diverse Metazoan Invertebrates." *Molecular Marine Biology and Biotechnology* 3: 294–299.

Fox, J., and S. Weisberg. 2019. *An R Companion to Applied Regression*. 3rd ed. Thousand Oaks, CA: Sage. https://socialsciences.mcmaster.ca/jfox/Books/Companion/index.html.

Frontalini, F., M. Greco, L. Di Bella, et al. 2018. "Assessing the Effect of Mercury Pollution on Cultured Benthic Foraminifera Community Using Morphological and eDNA Metabarcoding Approaches." *Marine Pollution Bulletin* 129, no. 2: 512–524.

Fugère, V., M. P. Hébert, N. B. da Costa, et al. 2020. "Community Rescue in Experimental Phytoplankton Communities Facing Severe Herbicide Pollution." *Nature Ecology & Evolution* 4, no. 4: 578–588.

Fussmann, G. F., and A. Gonzalez. 2013. "Evolutionary Rescue Can Maintain an Oscillating Community Undergoing Environmental Change." *Interface Focus* 3: 20130036.

Gibson, J., S. Shokralla, T. M. Porter, et al. 2014. "Simultaneous Assessment of the Macrobiome and Microbiome in a Bulk Sample of Tropical Arthropods Through DNA Metasystematics." *Proceedings of the National Academy of Sciences of the United States of America* 111, no. 22: 8007–8012.

Gill, J. P. K., N. Sethi, A. Mohan, S. Datta, and M. Girdhar. 2018. "Glyphosate Toxicity for Animals." *Environmental Chemistry Letters* 16: 401–426.

Giroux, M. S., J. R. Reichman, T. Langknecht, R. M. Burgess, and K. T. Ho. 2023. "Using eRNA/eDNA Metabarcoding to Detect Community-Level Impacts of Nanoplastic Exposure to Benthic Estuarine Ecosystems." *Environmental Pollution* 338: 122650.

Greco, M., F. Lejzerowicz, E. Reo, et al. 2022. "Environmental RNA Outperforms eDNA Metabarcoding in Assessing Impact of Marine Pollution: A Chromium-Spiked Mesocosm Test." *Chemosphere* 298: 134239.

Grooten, M., and R. E. A. Almond. 2018. *Living Planet Report – 2018: Aiming Higher*. Gland, Switzerland: WWF.

Hajibabaei, M., T. M. Porter, M. Wright, and J. Rudar. 2019. "COI Metabarcoding Primer Choice Affects Richness and Recovery of Indicator Taxa in Freshwater Systems." *PLoS One* 14: e0220953.

Halstead, N. T., T. A. McMahon, S. A. Johnson, et al. 2014. "Community Ecology Theory Predicts the Effects of Agrochemical Mixtures on Aquatic Biodiversity and Ecosystem Properties." *Ecology Letters* 17, no. 8: 932–941.

Harper, L. R., L. Lawson Handley, C. D. Sayer, et al. 2021. "Assessing the Impact of the Threatened Crucian Carp (*Carassius carassius*) on Pond Invertebrate Diversity: A Comparison of Conventional and Molecular Tools." *Molecular Ecology* 30, no. 13: 3252–3269.

Hébert, M. P., V. Fugère, B. E. Beisner, et al. 2021. "Widespread Agrochemicals Differentially Affect Zooplankton Biomass and Community Structure." *Ecological Applications* 31, no. 7: e02423.

Hébert, M. P., V. Fugère, and A. Gonzalez. 2019. "The Overlooked Impact of Rising Glyphosate Use on Phosphorus Loading in Agricultural Watersheds." *Frontiers in Ecology and the Environment* 17: 48–56.

Hebert, P. D., S. Ratnasingham, and J. R. De Waard. 2003. "Barcoding Animal Life: Cytochrome c Oxidase Subunit 1 Divergences Among Closely Related Species." *Proceedings of the Royal Society of London. Series B: Biological Sciences* 270: S96–S99.

Herve, M. 2023. "RVAideMemoire: Testing and Plotting Procedures for Biostatistics." R package version 0.9-83-7. https://CRAN.R-project.org/package=RVAideMemoire.

Hoffmann, A. A., and Y. Willi. 2008. "Detecting Genetic Responses to Environmental Change." *Nature Reviews Genetics* 9: 421–432.

Holman, L., M. de Bruyn, S. Creer, G. Carvalho, J. Robidart, and M. Rius. 2018. "The Detection of Novel and Resident Marine Non-Indigenous Species Using Environmental DNA Metabarcoding of Seawater and Sediment." *bioRxiv*, 440768.

Hughes, A. R., B. D. Inouye, M. T. Johnson, N. Underwood, and M. Vellend. 2008. "Ecological Consequences of Genetic Diversity." *Ecology Letters* 11: 609–623.

International Union of Conservation of Nature. 2015. "The IUCN Red List of Threatened Species." Version 2015.2. www.iucnredlist.org.

Kahlke, T., and P. J. Ralph. 2019. "BASTA–Taxonomic Classification of Sequences and Sequence Bins Using Last Common Ancestor Estimations." *Methods in Ecology and Evolution* 10: 100–103.

Kolpin, D. W., E. M. Thurman, E. A. Lee, M. T. Meyer, E. T. Furlong, and S. T. Glassmeyer. 2006. "Urban Contributions of Glyphosate and Its Degradate AMPA to Streams in the United States." *Science of the Total Environment* 354, no. 2–3: 191–197.

Kovach-Orr, C., and G. F. Fussmann. 2013. "Evolutionary and Plastic Rescue in Multitrophic Model Communities." *Philosophical Transactions of the Royal Society of London B: Biological Sciences* 368: 20120084.

Kruskal, J. B. 1964. "Multidimensional Scaling by Optimizing Goodness of Fit to a Nonmetric Hypothesis." *Psychometrika* 29: 1–27. https://doi.org/10.1007/BF02289565.

Larigauderie, A., A. H. Prieur-Richard, G. M. Mace, et al. 2012. "Biodiversity and Ecosystem Services Science for a Sustainable Planet: The DIVERSITAS Vision for 2012–20." *Current Opinion in Environmental Sustainability* 4, no. 1: 101–105.

Leasi, F., J. L. Sevigny, E. M. Laflamme, et al. 2018. "Biodiversity Estimates and Ecological Interpretations of Meiofaunal Communities Are Biased by the Taxonomic Approach." *Communications Biology* 1, no. 1: 112.

Leese, F., M. Sander, D. Buchner, V. Elbrecht, P. Haase, and V. M. Zizka. 2021. "Improved Freshwater Macroinvertebrate Detection From Environmental DNA Through Minimized Nontarget Amplification." *Environmental DNA* 3, no. 1: 261–276.

Lenth, R. 2024. "Emmeans: Estimated Marginal Means, Aka Least-Squares Means." R package version 1.10.3. https://CRAN.R-project.org/package=emmeans.

Leray, M., J. Y. Yang, C. P. Meyer, et al. 2013. "A New Versatile Primer Set Targeting a Short Fragment of the Mitochondrial COI Region for Metabarcoding Metazoan Diversity: Application for Characterizing Coral Reef Fish Gut Contents." *Frontiers in Zoology* 10: 1–14.

Lin, X.-L., E. Stur, and T. Ekrem. 2018. "DNA Barcodes and Morphology Reveal Unrecognized Species in Chironomidae (Diptera)." *Insect Systematics & Evolution* 49: 329–398.

Lobo, J., S. Shokralla, M. H. Costa, M. Hajibabaei, and F. O. Costa. 2017. "DNA Metabarcoding for High-Throughput Monitoring of Estuarine Macrobenthic Communities." *Scientific Reports* 7: 1–13.

Lozano, V. L., and H. N. Pizarro. 2024. "Glyphosate Lessons: Is Biodegradation of Pesticides a Harmless Process for Biodiversity?" *Environmental Sciences Europe* 36, no. 1: 55.

Malison, R. L., J. R. Benjamin, and C. V. Baxter. 2010. "Measuring Adult Insect Emergence From Streams: The Influence of Trap Placement and a Comparison With Benthic Sampling." *Journal of the North American Benthological Society* 29: 647–656.

Marques, A., I. S. Martins, T. Kastner, et al. 2019. "Increasing Impacts of Land Use on Biodiversity and Carbon Sequestration Driven by Population and Economic Growth." *Nature Ecology & Evolution* 3: 628–637.

Marshall, N. T., and C. A. Stepien. 2019. "Invasion Genetics From eDNA and Thousands of Larvae: A Targeted Metabarcoding Assay That Distinguishes Species and Population Variation of Zebra and Quagga Mussels." *Ecology and Evolution* 9, no. 6: 3515–3538.

Marshall, N. T., H. A. Vanderploeg, and S. R. Chaganti. 2021. "Environmental (e)RNA Advances the Reliability of eDNA by Predicting Its Age." *Scientific Reports* 11: 2769.

Martin, M. 2011. "Cutadapt Removes Adapter Sequences From High-Throughput Sequencing Reads." *EMBnet Journal* 17: 10–12.

Mesnage, R., and M. N. Antoniou. 2018. "Ignoring Adjuvant Toxicity Falsifies the Safety Profile of Commercial Pesticides." *Frontiers in Public Health* 5: 330483.

Moreira, R. A., A. da Silva Mansano, O. Rocha, and M. A. Daam. 2016. "The Use of Rotifers as Test Species in the Aquatic Effect Assessment of Pesticides in the Tropics." *Hydrobiologia* 773: 1–9.

Myers, J. H., G. Rose, E. Odell, P. Zhang, A. Bui, and V. Pettigrove. 2022. "Household Herbicide Use as a Source of Simazine Contamination in Urban Surface Waters." *Environmental Pollution* 299: 118868.

Myers, J. P., M. N. Antoniou, B. Blumberg, et al. 2016. "Concerns Over Use of Glyphosate-Based Herbicides and Risks Associated With Exposures: A Consensus Statement." *Environmental Health* 15, no. 1: 19.

Oksanen, J., G. Simpson, G. Blanchet, et al. 2022. "Vegan: Community Ecology Package." R Package Version 2.6–4. https://CRAN.R-project.org/package=vegan.

Pérez, G. L., A. Torremorell, H. Mugni, et al. 2007. "Effects of the Herbicide Roundup on Freshwater Microbial Communities: A Mesocosm Study." *Ecological Applications* 17, no. 8: 2310–2322.

Pimm, S. L., C. N. Jenkins, R. Abell, et al. 2014. "The Biodiversity of Species and Their Rates of Extinction, Distribution, and Protection." *Science* 344, no. 6187: 1246752.

Pont, D., M. Rocle, A. Valentini, et al. 2018. "Environmental DNA Reveals Quantitative Patterns of Fish Biodiversity in Large Rivers Despite Its Downstream Transportation." *Scientific Reports* 8, no. 1: 10361.

Qiu, X., L. Wu, H. Huang, et al. 2001. "Evaluation of PCR-Generated Chimeras, Mutations, and Heteroduplexes With 16S rRNA Gene-Based Cloning." *Applied and Environmental Microbiology* 67, no. 2: 880–887.

R Core Team. 2019. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org/.

Relyea, R. A. 2005. "The Impact of Insecticides and Herbicides on the Biodiversity and Productivity of Aquatic Communities." *Ecological Applications* 15: 618–627.

Sales, N. G., O. S. Wangensteen, D. C. Carvalho, et al. 2021. "Space-Time Dynamics in Monitoring Neotropical Fish Communities Using eDNA Metabarcoding." *Science of the Total Environment* 754: 142096.

Serrana, J. M., Y. Miyake, M. Gamboa, and K. Watanabe. 2019. "Comparison of DNA Metabarcoding and Morphological Identification for Stream Macroinvertebrate Biodiversity Assessment and Monitoring." *Ecological Indicators* 101: 963–972.

Serrana, J. M., and K. Watanabe. 2023. "Haplotype-Level Metabarcoding of Freshwater Macroinvertebrate Species: A Prospective Tool for Population Genetic Analysis." *PLoS One* 18, no. 7: e0289056.

Seymour, M., F. K. Edwards, B. J. Cosby, et al. 2021. "Environmental DNA Provides Higher Resolution Assessment of Riverine Biodiversity and Ecosystem Function via Spatio-Temporal Nestedness and Turnover Partitioning." *Communications Biology* 4, no. 1: 512.

Sigsgaard, E. E., I. B. Nielsen, S. S. Bach, et al. 2016. "Population Characteristics of a Large Whale Shark Aggregation Inferred From Seawater Environmental DNA." *Nature Ecology & Evolution* 1, no. 1: 4.

Silva, F., and S. Wiedenbrug. 2014. "Integrating DNA Barcodes and Morphology for Species Delimitation in the *Corynoneura* Group (Diptera: Chironomidae: Orthocladiinae)." *Bulletin of Entomological Research* 104: 65–78.

Song, J., and D. Liang. 2023. "Community Structure of Zooplankton and Its Response to Aquatic Environmental Changes Based on eDNA Metabarcoding." *Journal of Hydrology* 622: 129692.

Struger, J., D. Thompson, B. Staznik, P. Martin, T. McDaniel, and C. Marvin. 2008. "Occurrence of Glyphosate in Surface Waters of Southern Ontario." *Bulletin of Environmental Contamination and Toxicology* 80: 378–384.

Sun, Z., M. Majaneva, E. Sokolova, S. Rauch, S. Meland, and T. Ekrem. 2019. "DNA Metabarcoding Adds Valuable Information for Management of Biodiversity in Roadside Stormwater Ponds." *Ecology and Evolution* 9: 9712–9722.

Thibodeau, G., D. A. Walsh, and B. E. Beisner. 2015. "Rapid Eco-Evolutionary Responses in Perturbed Phytoplankton Communities." *Proceedings of the Royal Society B: Biological Sciences* 282, no. 1814: 20151215.

Thomasdotter, A., P. Shum, F. Mugnai, et al. 2023. "Spineless and Overlooked: DNA Metabarcoding of Autonomous Reef Monitoring Structures Reveals Intra-and Interspecific Genetic Diversity in Mediterranean Invertebrates." *Molecular Ecology Resources* 23, no. 7: 1689–1705.

Thomsen, P. F., and E. Willerslev. 2015. "Environmental DNA–An Emerging Tool in Conservation for Monitoring Past and Present Biodiversity." *Biological Conservation* 183: 4–18.

Tournayre, O., H. Tian, D. R. Lougheed, et al. 2024. "Enhancing Metabarcoding of Freshwater Biotic Communities: A New Online Tool for Primer Selection and Exploring Data From 14 Primer Pairs." *Environmental DNA* 6, no. 4: e590.

Tsui, M. T. K., and L. M. Chu. 2008. "Environmental Fate and Non-target Impact of Glyphosate-Based Herbicide (Roundup®) in a Subtropical Wetland." *Chemosphere* 71, no. 3: 439–446.

Tsuji, S., M. Miya, M. Ushio, H. Sato, T. Minamoto, and H. Yamanaka. 2018. "Evaluating Intraspecific Diversity of a Fish Population Using Environmental DNA: An Approach to Distinguish True Haplotypes From Erroneous Sequences." *bioRxiv*, 429993.

Turon, X., A. Antich, C. Palacín, K. Præbel, and O. S. Wangensteen. 2019. "From Metabarcoding to Metaphylogeography: Separating the Wheat From the Chaff." *Ecological Applications* 30: e02036.

Vacher, C., A. Tamaddoni-Nezhad, S. Kamenova, et al. 2016. "Learning Ecological Networks From Next-Generation Sequencing Data." *Advances in Ecological Research* 54: 1–39.

Valdez-Moreno, M., N. V. Ivanova, M. Elias-Gutierrez, S. L. Pedersen, K. Bessonov, and P. D. Hebert. 2019. "Using eDNA to Biomonitor the Fish Community in a Tropical Oligotrophic Lake." *PLoS One* 14: e0215505.

van Straalen, N. M., and M. J. Timmermans. 2002. "Genetic Variation in Toxicant-Stressed Populations: An Evaluation of the "Genetic Erosion" Hypothesis." *Human and Ecological Risk Assessment* 8: 983–1002.

Vera, M. S., E. Di Fiori, L. Lagomarsino, et al. 2012. "Direct and Indirect Effects of the Glyphosate Formulation Glifosato Atanor® on Freshwater Microbial Communities." *Ecotoxicology* 21: 1805–1816.

Vera, M. S., L. Lagomarsino, M. Sylvester, et al. 2010. "New Evidences of Roundup® (Glyphosate Formulation) Impact on the Periphyton Community and the Water Quality of Freshwater Ecosystems." *Ecotoxicology* 19: 710–721.

Wiens, J. J. 2016. "Climate-Related Local Extinctions Are Already Widespread Among Plant and Animal Species." *PLoS Biology* 14: e2001104.

Wood, D. E., and S. L. Salzberg. 2014. "Kraken: Ultrafast Metagenomic Sequence Classification Using Exact Alignments." *Genome Biology* 15: R46.

Yamamichi, M., and B. E. Miner. 2015. "Indirect Evolutionary Rescue: Prey Adapts, Predator Avoids Extinction." *Evolutionary Applications* 8: 787–795.

Zhang, G. K., F. J. Chain, C. L. Abbott, and M. E. Cristescu. 2018. "Metabarcoding Using Multiplexed Markers Increases Species Detection in Complex Zooplankton Communities." *Evolutionary Applications* 11: 1901–1914.

Zuur, A. F., E. N. Ieno, N. J. Walker, A. A. Saveliev, and G. M. Smith. 2009. *Mixed Effects Models and Extensions in Ecology With R*, 574. New York, NY: Springer.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.