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Environmental RNA-Based Metatranscriptomics as a Novel Biomonitoring Tool: A Case Study of Glyphosate-Based Herbicide Effects on Freshwater Eukaryotic Communities

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ABSTRACT

Traditional morphology- and molecular-based biodiversity surveys provide essential information on species composition and diversity, but they rarely provide information about the physiological states of organisms, which are key indicators of ecosystem health. Environmental RNA (eRNA) has the potential to significantly enhance biomonitoring by providing insights beyond species detection. Recent studies suggest that extra-organismal RNA released into the environment could help identify differentially expressed genes of single species. However, the feasibility of eRNA-based metatranscriptomics on complex environmental samples, containing both extra-organismal and organismal eukaryotic RNA, remains untested due to numerous experimental and analytical challenges. In this study, we explored the potential of eRNA-based metatranscriptomics, enriched for eukaryotes, as a tool to monitor environmental stress. We used outdoor mesocosms to examine the acute effects of a glyphosate-based herbicide (GBH) on gene transcription across diverse freshwater eukaryotic taxa. Our metatranscriptomics data revealed diverse eukaryotic taxa spanning multiple trophic levels, including phytoplankton, zooplankton, ciliates, and aquatic insects. GBH treatment significantly altered the relative transcript abundances of most eukaryotic classes, with longer-lived taxa demonstrating greater tolerance compared to shorter-lived taxa. Differential expression analysis showed more gene downregulation than upregulation in response to GBH, likely due to its acute toxicity. Many differentially expressed genes were involved in molecular pathways associated with responses to GBH exposure, such as oxidative stress response and detoxification. Our results demonstrate that eRNA-based metatranscriptomics captures transcriptional signals from diverse aquatic eukaryotic taxa, providing insights into functional gene expression. As such, its application to support environmental monitoring of aquatic ecosystems warrants further exploration.

1 | Introduction

Molecular methods of species identification using DNA collected either directly from organisms (organismal DNA) or indirectly from their environment (environmental DNA) have revolutionised biodiversity surveys, offering high-throughput, cost- and time-efficient approaches that provide valuable biological data for environmental biomonitoring (Cristescu and Hebert 2018; Ficetola et al. 2008; Hebert et al. 2003; Pawlowski et al. 2014). Recent advances in environmental RNA (eRNA) may significantly enhance environmental assessments by providing additional information beyond species detection (Cristescu 2019). Similar to environmental DNA (eDNA), eRNA metabarcoding can be used to detect species and characterise community composition (Littlefair et al. 2022; Miyata et al. 2022). As RNA degrades faster than DNA, it has been suggested that eRNA may better reflect the real-time status of species presence and community composition (Kagzi et al. 2022; Yates et al. 2021), thereby improving spatio-temporal resolution, particularly in heterogenous environments with rapid species turnover. Most importantly, eRNA has the potential to measure expression levels of functional genes, providing additional ecological and physiological information (Cristescu 2019).

Gene expression profiling using organismal RNA (oRNA) has been employed to assess the health status and stress levels of organisms (Akbarzadeh et al. 2018; Logan and Somero 2011; Ramaswamy et al. 2001), as the regulation of gene expression is a fundamental strategy for organisms to adapt to environmental changes and cope with stress (Schulte 2004). Therefore, analyzing gene expression using eRNA may also provide insights into the physiological status of organisms in complex communities. Recent transcriptomic analyses based on extra-organismal RNA have been used to detect the responses to heat stress in the water flea *Daphnia pulex* (Hechler et al. 2025) and to pyrene exposure in the Japanese rice fish *Oryzias latipes* (Hiki et al. 2023). Although extra-organismal RNA covered only a small portion of genes when compared to the full repertoire of genes derived from oRNA, these single-species studies found that extra-organismal RNA detected differentially expressed genes in response to stress. In the fish and *Daphnia* studies, 14.0% and 53.1% of the genes identified as differentially expressed in the eRNA data were also found to be differentially expressed in the oRNA data, respectively. Although these pioneering studies are promising, it remains to be tested whether eRNA can be used to detect transcriptomic responses of complex eukaryotic communities to environmental stress.

Traditionally, environmental metatranscriptomics studies have focused on bulk samples of prokaryotic organisms or specific eukaryotic taxonomic groups (e.g., Carradec et al. 2018; Knapik et al. 2020; Mojib et al. 2017), with the extracted RNA originating mainly from living organisms. However, when focusing on complex eukaryotic communities containing both macro- and micro-eukaryotes, a typical water sample includes extra-organismal RNA as well as oRNA. Thus, in this study, we define eRNA as the RNA extracted from environmental samples, consisting of a mixture of extra-organismal RNA primarily derived from macro-eukaryotes and oRNA mainly derived from whole micro-eukaryotes (adapted from Hechler et al. 2025). Recovering taxonomic and functional information of eukaryotes based on environmental metatranscriptomics presents inherent challenges. The eukaryotic community is highly diverse both taxonomically and ecologically, and capturing such diversity requires a substantial number of sequences per sample. Moreover, most eukaryotes lack a reference genome, and public databases have insufficient nucleotide and amino acid sequences for eukaryotic genes (Lewin et al. 2018; Shakya et al. 2019). This shortage impacts the accuracy of taxonomic and functional annotation of RNA-Seq reads. There are also concerns related to the quantity and quality of eRNA (particularly of extra-organismal origin) when it comes to library preparation (Hiki et al. 2023). Lastly, differential expression analysis is more challenging than single-species studies because it is difficult to determine if changes in expression levels are due to gene expression regulation or differences in species composition among sample groups (Klingenberg and Meinicke 2017; Zhang et al. 2021). Despite these challenges, the potential advantages of eRNA-based metatranscriptomics include non-invasive sampling and the ability to capture a broader range of eukaryotic groups than bulk samples. In addition, the continual growth of reference genomes makes eRNA-based metatranscriptomics an attractive choice for monitoring environmental changes and stress.

Here we investigate how metatranscriptomics based on eRNA can be applied beyond species detection to assess the physiological status of complex eukaryotic communities under environmental stress. As a case study, we examined the acute effects of a glyphosate-based herbicide (GBH) on gene transcription in diverse freshwater eukaryotic taxa using outdoor mesocosms. The widespread use of GBHs in agriculture has led to contamination of aquatic environments, directly causing mortality in many species (Gonçalves et al. 2019; Rico-Martínez et al. 2012) and reducing species richness in plankton communities (Fugère et al. 2020; Hébert et al. 2021). Using an eRNA-based metatranscriptomics approach enriched for eukaryotes, we exposed complex freshwater communities to a GBH for 24 h and assessed changes in gene transcription of major eukaryotic groups. Given that GBH exposure may alter the transcriptional activity of sensitive taxa, our first objective was to compare the relative transcript abundance of eukaryotic taxa before and after treatment. The second objective was to analyze the effects of GBH on gene transcription in major eukaryotic taxonomic groups. We predicted that GBH would trigger gene transcription responses, reflecting the toxic impacts of GBH and the responses of organisms to mitigate the damage. Given that GBH has been reported to cause oxidative stress via reactive oxygen species production in various organisms, including algae, invertebrates, and vertebrates (Klátyik et al. 2024; Modesto and Martinez 2010), we predicted that many differentially expressed genes would be involved in oxidative stress response pathways.

2 | Materials and Methods

2.1 | Experimental Design and Sample Collection

The GBH treatment experiment was conducted at the Large Experimental Array of Ponds (LEAP) facility, located within a 1000-ha protected forest at Gault Nature Reserve (Mont-Saint-Hilaire, Quebec, Canada). Four pond mesocosms (approximately

1000 L each) were set up and filled with water from a reservoir connected to Lac Hertel (45°32′N, 73°09′W) between June 13 and 17, 2022. Lac Hertel is considered free of herbicide pollution due to its location within a protected forested watershed with no history of agricultural activity (Fugère et al. 2020). The mesocosms were left undisturbed for approximately six weeks to allow the communities to stabilize before the GBH treatment. Control samples were collected from the four ponds on July 26, 2022. Subsequently, 57.2 mL of Roundup Super Concentrate Grass and Weed Control (reg. no. 22759; Bayer) was added to each pond to target a total glyphosate concentration of 15 mg/L, assuming a pond volume of 1000 L. We selected a targeted concentration of 15 mg/L for our acute effect experiment based on previous studies that found strong responses of phytoplankton and zooplankton to this dose of GBH at the same facility (Fugère et al. 2020; Hébert et al. 2021). This concentration falls between the Canadian freshwater quality guidelines for long-term (0.8 mg/L) and short-term (27 mg/L) exposure limits (CCME 2012). Treatment samples were collected approximately 24 h after the GBH exposure from the same four ponds on July 27, 2022. However, we cannot attribute these effects solely to glyphosate, as the commercial GBH formulation used contains other toxic ingredients in addition to glyphosate (Mesnage and Antoniou 2017).

Water samples were collected from the upper 35 cm at multiple locations within each mesocosm using dedicated PVC tubes (one tube per pond). Two water samples (500 mL each) were filtered through 0.7 μm glass microfiber filters (Millipore) for each pond on each sampling day. Filters were placed in 1.5 mL microcentrifuge tubes containing 375 μL of RLT buffer (Qiagen) and β-mercaptoethanol (Sigma-Aldrich), mixed at a volume ratio of 100:1. To prevent eRNA degradation, water filtration was completed on site within 30 min from collection. Samples were immediately placed on dry ice and then stored at −80°C until RNA extraction. A filtration blank was included on each sampling day by filtering 500 mL of distilled water using the same method as for the eRNA samples. In total, 16 eRNA samples and two filtration blanks were collected. Samples were identified using the format Pond_Treatment&ReplicateID. For example, sample "A_C1" refers to Replicate 1 from the Control group in Pond A.

2.2 | RNA Extraction and RNA-Seq Library Preparation

RNA extraction, DNase treatment, and library preparation were conducted in a clean laboratory dedicated to environmental nucleic acid research. Environmental RNA was extracted from the filters using the RNeasy Mini Kit (Qiagen) and ZR BashingBead Lysis Tubes (0.1 and 0.5 mm) (Zymo Research). Samples were thawed on ice. Each ZR BashingBead lysis tube was wetted with 200 μL of RLT buffer (with β-mercaptoethanol). Each filter and its preservation buffer were then transferred into one lysis tube, followed by the addition of another 175 μL of RLT buffer (with β-mercaptoethanol). Samples were homogenised using a TissueLyser (Qiagen), then centrifuged at 13,000 rpm for 3 min. The liquid from each sample was transferred to a new 1.5 mL microcentrifuge tube and centrifuged again at 13,000 rpm for 3 min. The supernatants were carefully transferred to new 1.5 mL tubes. Subsequent steps followed the Qiagen RNeasy Mini Kit Handbook. One RNA extraction blank was included for each batch of RNA extraction. In total, RNA was extracted from 20 samples: 16 eRNA samples, two filtration blanks, and two extraction blanks.

RNA was quantified using a Qubit RNA High Sensitivity kit, and RNA quality was checked using an Agilent RNA 6000 Pico Kit (Agilent). There was no detectable RNA in the filtration and extraction blanks, as indicated by the Qubit results and the absence of bands in the gel image results from the BioAnalyzer. Therefore, RNA extracted from filtration and extraction blanks was not used for library preparation. Total RNA was subject to DNase digestion for 30 min using a Turbo DNA-free Kit (Thermo Fisher) prior to library preparation. Sixteen RNA-Seq libraries were prepared from eRNA samples, and one library preparation negative control was prepared using molecular-grade water, with the Illumina Stranded mRNA Prep kits and IDT for Illumina RNA UD Indexes Set B (Illumina). During library preparation, mRNA with poly(A) tails was enriched using oligo(dT) magnetic beads according to the Illumina protocol. Libraries were sent to the McGill Genome Centre for quality checking, quantification, and sequencing using the Illumina NovaSeq 6000 S4 platform with paired-end 150 bp reads. One eRNA sample from the treatment group failed during library preparation.

2.3 | De Novo Transcriptome Assembly and Annotation

The data analysis workflow involved bioinformatic analysis and post-bioinformatic analysis, each step of which is summarised in Figure 1. Raw sequences were processed using Trimmomatic 0.36 to remove low-quality reads and Illumina adapters (Bolger et al. 2014). Ribosomal RNA (rRNA) sequences were discarded using SortMeRNA v4.3.6 (Kopylova et al. 2012), which aligned our sequences with several rRNA genes (i.e., 5S rRNA, 5.8S rRNA, 16S rRNA, 18S rRNA, 23S rRNA, and 28S rRNA) from the SILVA and Rfam databases (Griffiths-Jones et al. 2003; Quast et al. 2013) included in the SortMeRNA v4 default database. rRNA-depleted reads were assembled into contigs using MEGAHIT (Li et al. 2016). Then, rRNA-depleted reads were mapped to the assembled contigs using Bowtie2 (Langmead and Salzberg 2012), and abundances for each contig in each sample were estimated using SAMtools (Li et al. 2009). A count matrix for each contig in each sample was generated using the get_count_table.py (https://github.com/metajinomics/mapping_tools).

Taxonomy of the contigs was annotated following the DIAMOND+MEGAN pipeline for long reads (Bağcı et al. 2021). The contig sequences were split into three files using SeqKit (Shen et al. 2016) and subsequently aligned against the NCBI nr database (database downloaded on January 25, 2024) (Benson et al. 2005) using DIAMOND v2.1.7 with the sensitive mode, frameshift-aware alignment mode, and range culling reporting mode (Buchfink et al. 2021). The output DAA files were processed using the *daa-meganizer* tool of MEGAN v6.25.9 in long-read mode, applying the interval-union lowest common ancestor algorithm for taxonomic analysis of contigs (setting for *daa-meganizer*: --longReads -me 0.00001 -mpi 40) (Huson

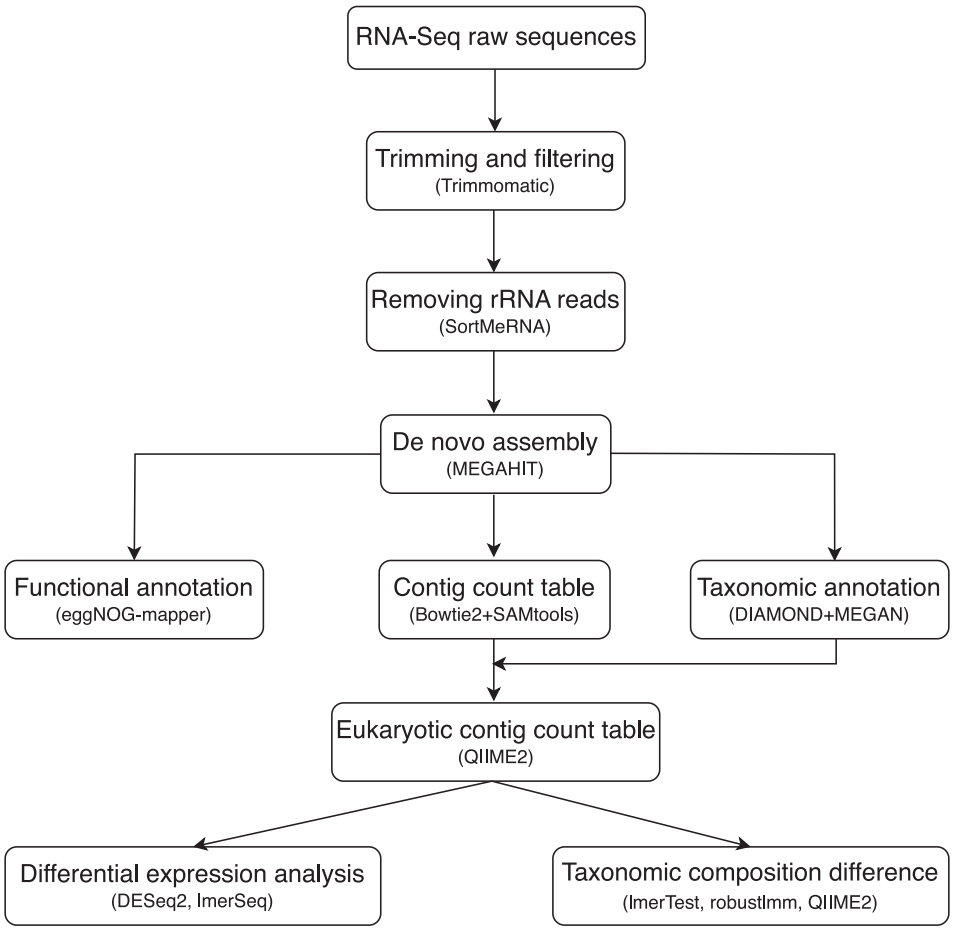

FIGURE 1 | Flowchart illustrating the general bioinformatic and post-bioinformatic analysis workflow used in this study.

et al. 2016, 2018). The meganized DAA files were imported into the graphical user interface of MEGAN to export the taxonomic assignment results of the contigs. Based on the taxonomy assignment results at the domain level, contigs that were not assigned to Eukaryota were discarded and a count table for eukaryotic contigs was generated using the *filter-table* plugin of QIIME2 (Bolyen et al. 2019). Functional annotation of the contigs was performed using the eggNOG-mapper v2.1.12 with the eggNOG v5.0.2 database (Cantalapiedra et al. 2021; Huerta-Cepas et al. 2019).

2.4 | Analyses of Class-Level Transcript Profiles

The eukaryotic contig count table was collapsed at the class level based on taxonomic assignment results from MEGAN. Contigs that were assigned to Eukaryota and could not be assigned at the class level were categorised as "Unassigned_Eukaryota" and retained in the table. To confirm that the major taxonomic classes we detected were indeed present in the ecosystem, we employed two approaches: cross-referencing classes reported in previous studies that used freshwater communities derived from the same source lake (Fugère et al. 2020; Hébert et al. 2021; Loria et al. 2025) and confirming the widespread occurrence of these classes in freshwater ecosystems (Table S1). The class count table, which contained the number of reads assigned to each class in each sample, was rarified to 600,000 reads per sample using QIIME2 (Bolyen et al. 2019). Bray-Curtis dissimilarities were estimated using the rarified table, and Principal Coordinate Analysis (PCoA) was performed on the resulting dissimilarity matrix.

The relative transcript abundance of each eukaryotic class in each sample was calculated based on the number of reads assigned to each class relative to the total number of reads assigned to Eukaryota in that sample. These values reflect relative transcriptional contributions rather than organismal abundances. We compared the relative transcript abundances between control and treatment for nine classes with the greatest number of reads across the samples: three classes of zooplankton (Branchiopoda, Eurotatoria, and Hexanauplia), two of phytoplankton (Dinophyceae and Cryptophyceae), two of ciliates (Oligohymenophorea and Spirotrichea), and two additional classes (Insecta and Magnoliopsida). For the nine major classes, differences in the relative transcript abundances between treatment and control were initially analysed using linear mixed effects models (LMM) with the *lmerTest* package (Kuznetsova et al. 2017): the herbicide treatment (before vs. after GBH application) was set as a fixed effect and pond was set as a random effect for each model. Q-Q plots indicated that the residuals of the models for Branchiopoda, Dinophyceae, and Oligohymenophorea followed a normal distribution, while the residuals for the other six classes did not. Consequently, we reanalysed the differences in relative transcript abundances between treatment and control for these six classes using robust linear mixed effects models (rLMM) with the *robustlmm* R

package (Koller 2016), as rLMM are less sensitive to deviations from normality. In the rLMM analyses, 95% confidence intervals (CIs) for the effects of the treatment were estimated using the *confintROB* R package (Mason et al. 2024), and *p* values to determine the significance of the fixed factor effects were calculated following the method outlined in Geniole et al. (2019).

2.5 | Differentially Expressed Gene Analysis

Based on the taxonomy assignment results of the contigs at the class level, a contig count table for each of the nine major classes was generated by extracting the relevant data from the eukaryotic contig count table. We followed the recommendation of Klingenberg and Meinicke (2017) to normalise gene expression data using DESeq2 (Love et al. 2014) for each class separately and recombine the normalised data into a count matrix to analyse differentially expressed genes. Low-count genes were pre-filtered, and only contigs with at least 10 normalised counts in at least four samples were included in the differential gene expression analysis. For statistical analyses, a variance-stabilising transformation was performed on the counts of each contig using DESeq2 (Love et al. 2014), followed by LMM analyses on the transformed counts with lmerSeq (Vestal et al. 2022). In the LMM analyses, treatment was set as a fixed effect and pond was set as a random effect. *p* values were adjusted using the Benjamini-Hochberg (BH) method (Benjamini and Hochberg 1995). KEGG Orthology (KO) enrichment analysis for differentially expressed genes were performed using the *enricher* function of the *clusterProfiler* R package (Yu et al. 2012). The contigs that passed the filtration criteria for differential expression analysis were used as background.

3 | Results

3.1 | Summary of Sequencing and Taxonomy Composition

In total, 3.4 billion reads (i.e., 1.7 billion paired-end reads) were generated for the 16 libraries (15 eRNA samples and one library preparation negative control) (Table S2). After denoising and removing rRNA reads, the reads were assembled into 1,726,846 contigs with 2.5 billion reads mapped to those contigs. Among them, 1.6 billion (64.0%) reads were taxonomically assigned to a domain, while 0.9 billion (36.0%) were not. The average percentage of reads assigned to Eukaryota, Archaea, and Bacteria across the eRNA samples was 94.9% (105,617,564 reads), 1.5% (1,918,501 reads), and 3.6% (2,370,320 reads), respectively, with similar patterns in each sample (Figure S1). In contrast, the corresponding percentages in the library negative control were 13.2% (2039 reads), 0.01% (2 reads), and 86.8% (13,410 reads), respectively. Given that the number of eukaryotic reads in the library negative control sample was 2039, which is very small compared to the average of 105.6 million eukaryotic reads in the eRNA samples (Table S2), this sample was removed from downstream analysis.

Of the eukaryotic reads, the average percentage assigned at the class level across the 15 eRNA samples was 74.4% (Figure 2A). The most transcriptionally represented class was Dinophyceae, with an average percentage of 20.1%. The second and third most represented classes were Branchiopoda and Insecta, with average percentages of 14.2% and 13.1%, respectively. Spirotrichea and Eurotatoria also had substantial representation, averaging 6.3% and 6.2%, respectively. The predominantly terrestrial groups, Magnoliopsida and Mammalia, accounted for average proportions of 1.2% and 0.2%, respectively, and both were considered unexpected taxa. Magnoliopsida reads may originate from airborne plant material, such as pollen, while Mammalia reads could be attributed to the presence of mammals living around the area.

In general, replicates collected from each pond at the same sampling time showed similar class level transcript profiles (Figure 2A). Statistical analyses showed that eight of the nine analysed classes had significantly different relative transcript abundances between treatment and control, with Branchiopoda and Insecta having higher relative transcript abundances in the treatment, and the other six classes having higher relative transcript abundances in the control (Figure 2B and Table S3). The relative transcript abundance of Magnoliopsida was not significantly different between treatment and control. PCoA revealed that GBH treatment influenced the transcriptional profiles of eukaryotic communities. Specifically, PCo1 captures the impact of the treatment, whereas PCo2 captures pond-specific differences (Figure 3A).

3.2 | Differential Expression Analysis

The sample distance heatmap based on all gene expression data revealed two major clusters: one for control samples and the other for treatment samples, with replicates from each tank clustering together within their respective groups (Figure 3B). In total, LMM analyses showed there were 61,957 contigs that exhibited significantly different expression between treatment and control: expression of 59,554 contigs showed downregulation in response to the GBH treatment, and 2403 contigs showed upregulation. Taxonomic annotation of these contigs allowed us to identify how many were differentially expressed within each class, and the numbers revealed a pattern of more downregulation than upregulation in every analysed class (Table 1). Those differentially expressed contigs encode genes involved in diverse molecular pathways (Figure 4 and Figure S2). KEGG ortholog enrichment analysis identified 48 enriched terms (Table S4), representing key biological pathways. For instance, the enriched KO term K08914 (light-harvesting complex II chlorophyll a/b binding protein 3) is associated with photosynthesis, while the enriched KO term K03661 (V-type H^+—transporting ATPase 21 kDa proteolipid subunit) is associated with oxidative phosphorylation.

To interpret the biological significance of differential gene expression, we examined differentially expressed contigs annotated to KEGG pathways potentially affected by GBH treatment and visualised their expression patterns using heatmaps (Figure 5 and Figure S3). To investigate oxidative stress responses, we identified 463 differentially expressed contigs annotated to glutathione metabolism (ko00480), 1513 to oxidative phosphorylation (ko00190), and 610 to peroxisome (ko04146). For detoxification, 263 differentially expressed contigs were

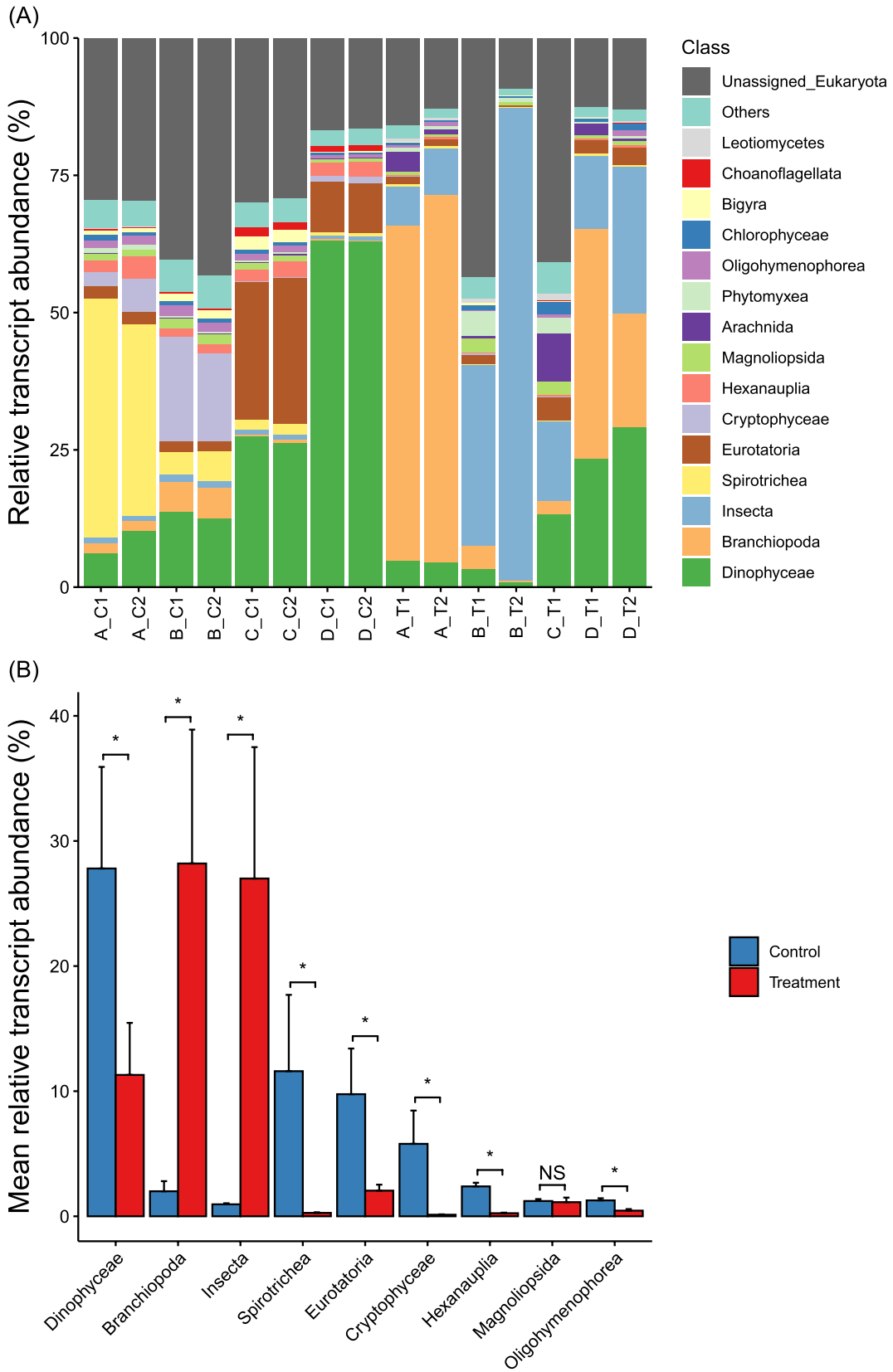

FIGURE 2 | Effect of GBH treatment on relative transcript contributions of freshwater eukaryotic classes. (A) Relative transcript abundances of classes in each sample. "Others" represents the sum of eukaryotic classes with <1% relative transcript abundance in every sample. "Unassigned_Eukaryota" represents the reads assigned to Eukaryota but not to the class level. (B) Comparison of relative transcript abundances between treatment and control for nine major classes. Asterisks ('*') indicate significant differences revealed by either LMM or rLMM, while "NS" denotes non-significant difference.

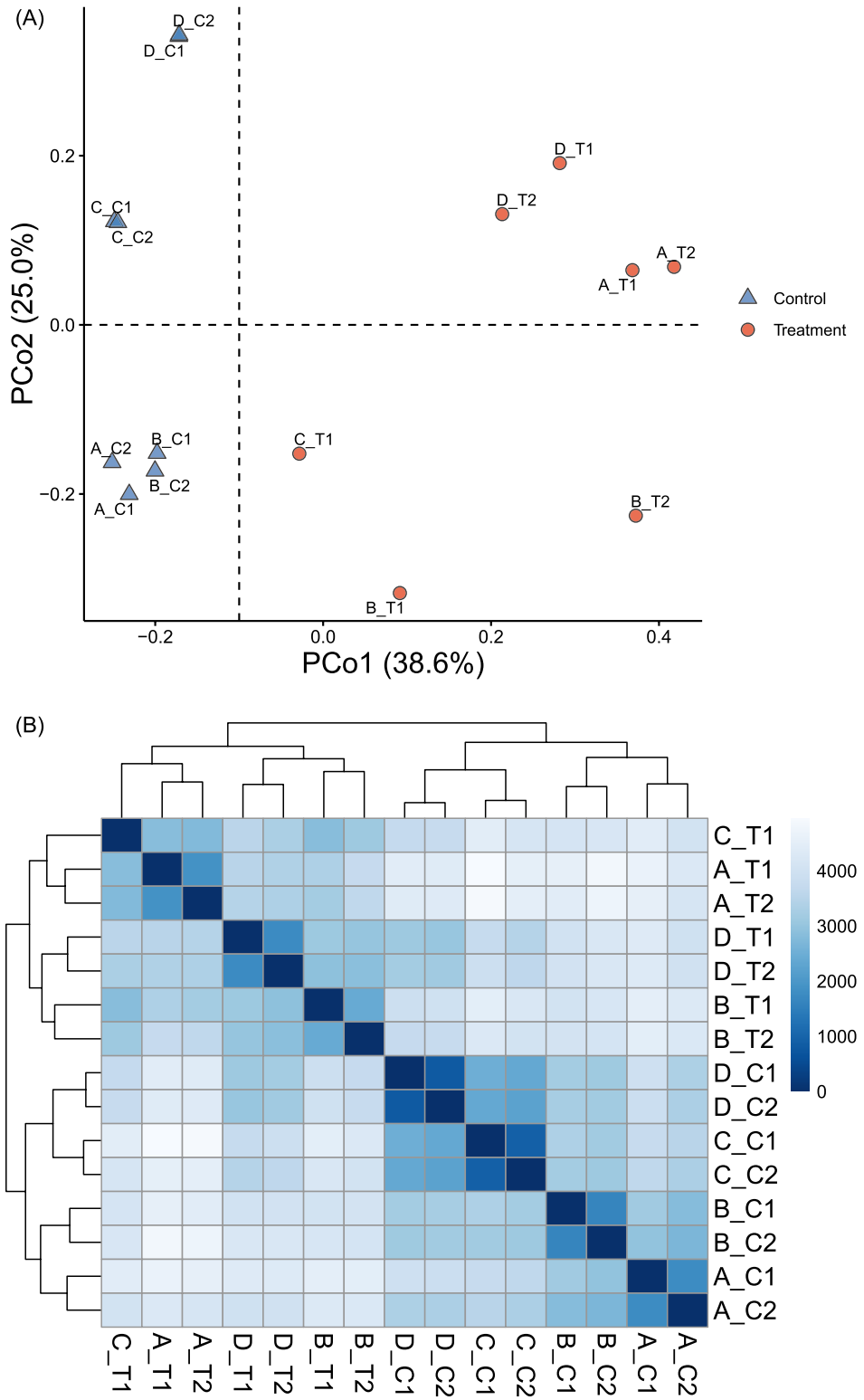

FIGURE 3 | Comparison of samples based on transcript-derived taxonomic composition and overall gene expression. (A) Principal coordinate analysis (PCoA) of Bray–Curtis dissimilarity based on transcript read counts assigned to eukaryotic classes (rarified to 600,000 reads per sample). (B) Heatmap of sample-to-sample distances based on gene expression data. Distances were calculated using eukaryotic contigs with at least 10 reads in at least four samples, and hierarchical clustering was performed on the sample distances.

annotated to the metabolism of xenobiotics by cytochrome P450 (ko00980) molecular pathway. To examine potential impacts on photosynthesis, we identified 649 differentially expressed contigs annotated to the photosynthesis (ko00195) pathway and 228 to the photosynthesis-antenna proteins (ko00196) pathway in the two phytoplankton classes (Cryptophyceae and Dinophyceae). The heatmap of downregulated contigs associated with oxidative stress response (Figure 5A) reveals that treatment samples clustered together on the right and then grouped with control samples on the left, suggesting a consistent transcriptional

TABLE 1 | Numbers of reads, contigs and differentially expressed (DE) contigs in the major classes.

Category	Class	No. of reads	No. of contigs		DE contigs				
			Total	For LMM[a]	Total DE	Up (no.)	Up (%)	Down (no.)	Down (%)
Phytoplankton	Dinophyceae	348,099,868	206,568	76,242	23,732	710	0.93	23,022	30.20
	Cryptophyceae	55,057,730	10,466	4033	3713	16	0.40	3697	91.67
Zooplankton	Branchiopoda	108,145,935	39,377	8474	1202	273	3.22	929	10.96
	Eurotatoria	114,837,167	46,833	14,615	11,341	356	2.44	10,985	75.16
	Hexanauplia	25,975,365	21,396	6388	5261	15	0.23	5246	82.12
Insect	Insecta	310,927,755	48,060	6579	2678	648	9.85	2030	30.86
Ciliate	Oligohymenophorea	13,964,387	19,689	2523	1718	186	7.37	1532	60.72
	Spirotrichea	107,843,917	34,675	11,383	10,611	27	0.24	10,584	92.98
Plant	Magnoliopsida	16,630,772	6305	2025	1701	172	8.49	1529	75.51

[a]Only contigs with at least 10 counts in at least four samples after normalization were included in the differential expression analysis. A contig count table consisting of data from the nine classes was used for the analysis, after which the numbers of differentially expressed contigs in each class were summarized based on the taxonomic annotation of the contigs.

suppression in response to GBH exposure across treatment samples. In contrast, the heatmap of upregulated contigs associated with oxidative stress response (Figure 5B) shows that control samples clustered in the center, with treatment samples on both sides, indicating more variable upregulation among treatment samples. The heatmaps of detoxification-related contigs (Figure 5C,D) exhibited patterns similar to those observed for oxidative stress response contigs in Figure 5A,B. In comparison, the heatmap of contigs associated with photosynthesis-related pathways (Figure S3) displayed less consistent clustering patterns between treatment and control groups, suggesting more heterogeneous transcriptional responses across samples.

To further investigate the genes associated with these KEGG pathways, we examined patterns at the KO level within the three pathway categories: oxidative stress response, detoxification, and photosynthesis. For each KO, we compiled its functional description along with the number of downregulated and upregulated contigs assigned to it across eukaryotic taxonomic classes (Tables S5–S7). Consistent with the overall differential expression patterns (Table 1), most KOs had more downregulated than upregulated contigs. For KOs associated with oxidative stress responses, Dinophyceae had the highest number of downregulated contigs following GBH treatment, while Insecta had the highest number of upregulated contigs (Table S5). For KOs associated with detoxification, Dinophyceae had the highest number of downregulated contigs, whereas Eurotatoria had the highest number of upregulated contigs (Table S6). For KOs associated with photosynthesis, Dinophyceae had more regulated contigs than Cryptophyceae, and no upregulated contigs were detected in Cryptophyceae (Table S7).

4 | Discussion

Biomonitoring based on eRNA has the potential to complement surveys that primarily focus on community composition (e.g., morphotaxonomy and eDNA metabarcoding) and provide a more integrative assessment of environmental changes and ecosystem health (Cristescu 2019; Yates et al. 2021). In this study, we used metatranscriptomics based on eRNA to investigate how a common glyphosate-based herbicide affected gene transcription across multiple eukaryotic taxa in a complex freshwater community. We found that metatranscriptomics based on eRNA can capture gene transcription signals across diverse aquatic eukaryotic taxa and detect changes in relative transcript abundance and functional gene expression in response to environmental stress.

4.1 | Challenges in Metatranscriptomics Based on eRNA

Metatranscriptomics based on eRNA presents several challenges, particularly regarding RNA quality, as no standardized method exists to assess eRNA integrity as is done for single-species RNA. A key concern in previous research was whether mRNA derived from eRNA samples retained poly(A) tails. Due to this uncertainty, two studies comparing extra-organismal and organismal RNA for detecting transcriptomic responses of single species to environmental stress opted for rRNA depletion

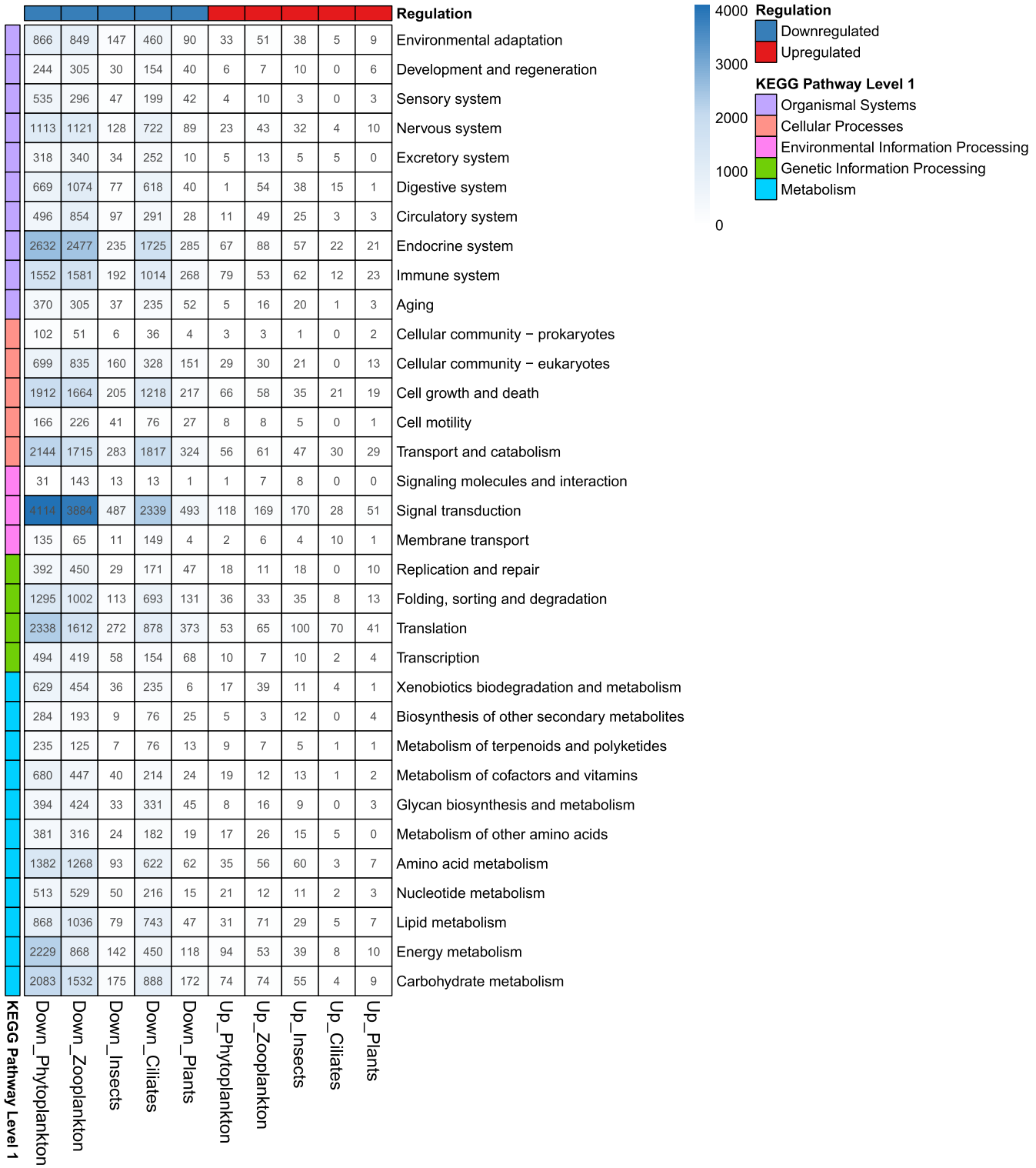

FIGURE 4 | Heatmap showing the number of differentially expressed contigs assigned to KEGG Level 2 pathways across five taxonomic categories. Each row represents one of the KEGG Level 2 pathways, and each column represents the number of contigs that were either downregulated (left five columns) or upregulated (right five columns) in response to the GBH treatment.

during library preparation (Hechler et al. 2025; Hiki et al. 2023). However, this approach led to only 0.5% and 0.6% of reads in eRNA samples mapping to target species genomes, as rRNA remained overwhelmingly abundant. In contrast, our study successfully applied poly(A) selection, achieving an average of 94.9% of reads assigned to eukaryotes among those that could be assigned at the domain level, confirming that eRNA quality was sufficient for library preparation.

Data analysis presents additional challenges. Public databases lack well-annotated genomes and reference sequences for many freshwater plankton species, limiting the accuracy of taxonomic

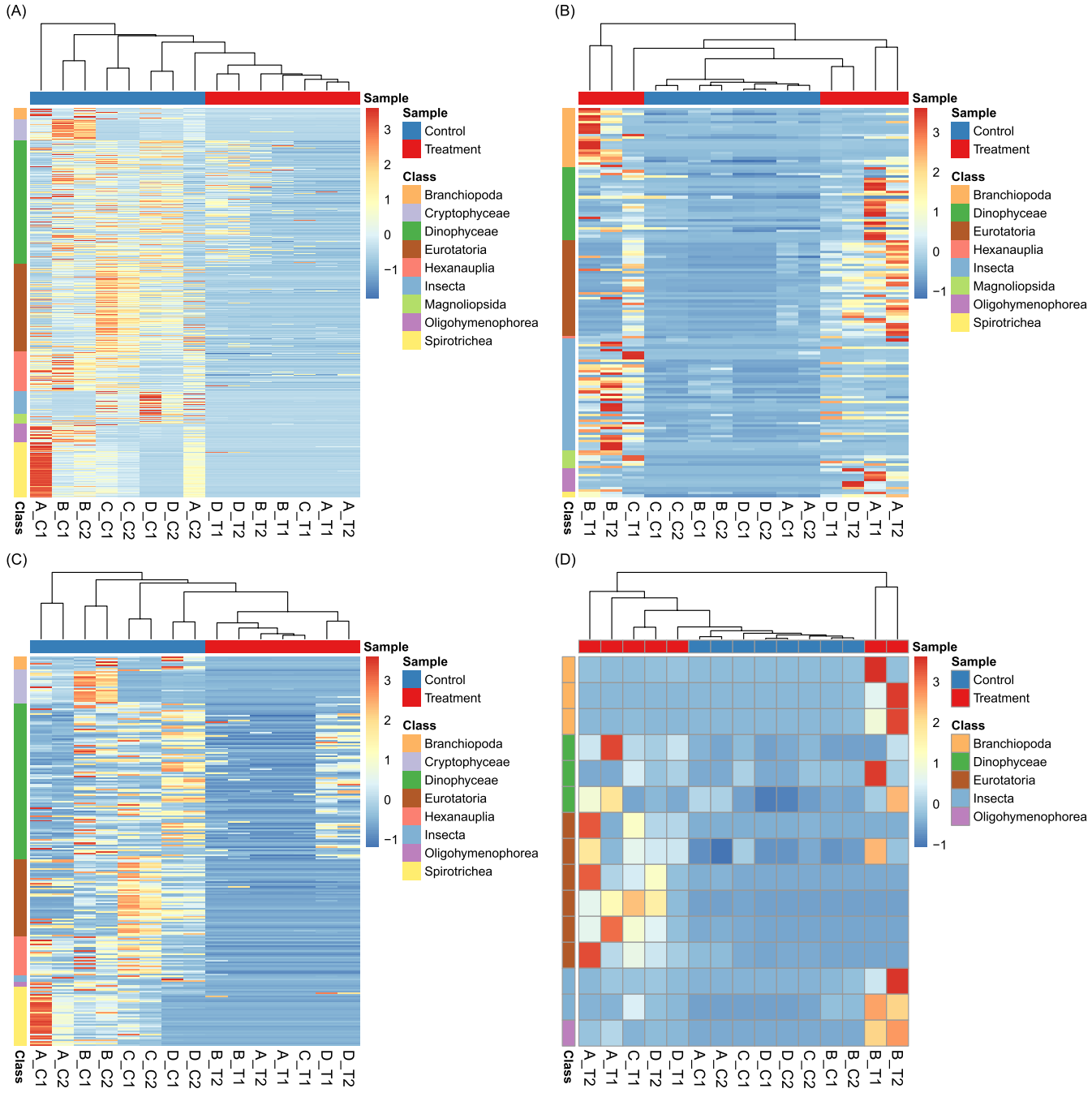

FIGURE 5 | Heatmaps showing expression patterns of differentially expressed contigs associated with oxidative stress response and xenobiotic metabolism pathways across nine major taxonomic classes. *Z*-scores were calculated for each contig for clustering. (A) Downregulated contigs associated with three KEGG pathways related to oxidative stress response. (B) Upregulated contigs associated with three KEGG pathways related to oxidative stress response. (C) Downregulated contigs associated with the "Metabolism of xenobiotics by cytochrome P450" pathway. (D) Upregulated contigs associated with the "Metabolism of xenobiotics by cytochrome P450" pathway.

assignment. To mitigate this limitation, we focused on taxonomic assignment at the class level, which does not require high sequence similarity to reference databases (Levy Karin et al. 2020). Differential gene expression analysis also presents challenges, as observed changes in gene expression may result from variations in taxonomic composition and organismal abundance rather than regulatory processes. To mitigate this, we normalized read counts separately for each major class and combined them for statistical analysis, following Klingenberg and Meinicke (2017). However, new statistical methods, such as those incorporating species or DNA abundance as covariates (Zhang et al. 2021), are less feasible for eukaryotes than for prokaryotes, due to incomplete reference databases and the complexity of eukaryotic genomes.

Interpreting metatranscriptomics results involves further complexities. For example, gene expression can persist post-mortem, as transcriptional shutdown is a gradual process (Bonadio et al. 2021; Pozhitkov et al. 2017). Thus, eRNA likely reflects both gene expression at the time of cellular release and residual

activity during cell death, offering both challenges and opportunities. Additionally, eRNA may not fully capture functional gene expression, as single-species studies show it detects only a subset of genes compared to tissue or whole-organism RNA (Hechler et al. 2025; Hiki et al. 2023). Environmental stressors can potentially accelerate RNA degradation, further complicating comparisons between control and treatment conditions. Despite these challenges, our results demonstrated that eRNA-based metatranscriptomics effectively captured community and gene expression responses to GBH exposure. Continued improvements in reference databases, analytical methods, and statistical models will help overcome current challenges.

Extra-organismal RNA degrades rapidly, with an approximately 10-h half-life in laboratory conditions (Marshall et al. 2021) and even faster decay in natural environments due to biotic (e.g., extracellular enzymes) and abiotic (e.g., UV, chemical) factors (Barnes and Turner 2016). In this study, we used outdoor mesocosms filled with natural lake water, where these degradation factors likely accelerated the breakdown of RNA. Additionally, we selectively captured eukaryotic mRNA through poly(A) tail selection during library preparation, a method more sensitive to RNA integrity compared to qRT-PCR and digital PCR. Together, these factors allowed us to make meaningful comparisons of the relative transcriptional contributions of major taxonomic classes among samples and between treatments.

4.2 | Attribution of GBH Effects

Although we did not include untreated control mesocosms that were sampled at both time points to assess natural temporal variation over 24 h, two previous studies conducted at the same facility using planktonic communities from the same source lake reported no significant weekly changes in community composition in control ponds through morphological and eDNA analyses (Hébert et al. 2021; Loria et al. 2025). Given that the treatment samples were collected 24 h after the control samples in this study, the observed differences between control and treatment samples are unlikely to be due to natural temporal changes and are instead attributable to the GBH treatment.

4.3 | Effects of GBH on Different Eukaryotic Classes

The extensive use of GBHs in agriculture for weed control has led to their widespread presence in aquatic ecosystems (Klátyik et al. 2024). Although GBHs can promote the growth of certain phytoplankton species by acting as a nutrient (Wang et al. 2016), we observed a decrease in the relative transcript abundances of two abundant phytoplankton classes after 24 h of GBH exposure. While GBHs have adverse effects on zooplankton, studies revealed that their impact varies among different zooplankton groups (Hébert et al. 2021; Polla et al. 2022). Among the three zooplankton classes analysed in this study, we found that the relative transcript abundance of cladocerans (Branchiopoda) increased, while those of copepods (Hexanauplia) and rotifers (Eurotatoria) decreased after 24 h of GBH exposure. This pattern may reflect differences in taxon-specific sensitivity or physiological response. These results are consistent with a previous study on the same freshwater community, which found that copepod and rotifer biomass declined 1 day after GBH exposure, whereas cladocerans showed no apparent decrease during the initial 24-h period (Hébert et al. 2021). Changes in relative transcript abundance between control and treatment could also be attributed to the direct effects of GBH on organisms, indirect effects from altered species interactions following the disturbance, or the release of environmental nucleic acids immediately after death. Moreover, the increased relative transcript abundances of cladocerans and insects after GBH exposure may also be attributed to their longer lifespans as metazoan taxa, since the classes that declined in relative transcript abundances are known to be shorter-lived taxa. Despite the short 24-h duration of our GBH treatment, these findings are consistent with studies showing that GBH differently affects taxa and can alter community composition (Hébert et al. 2021; Polla et al. 2022; Wang et al. 2016).

4.4 | Natural Pond Variation

In addition to GBH treatment effects, we observed substantial variation in the relative transcriptional contributions of different taxonomic groups among ponds, despite all being filled with water from the same source lake. This variability likely reflects natural differences in the initial community composition at the time of filling, such as random sampling variation affecting taxa presence. Moreover, the 6-week period before the experiment allowed for further divergence due to microenvironmental differences, species interactions, and ecological drift. Our PCoA analysis (Figure 3A) and class-level taxonomic summaries (Figure 2A) highlight these differences, demonstrating that eRNA-based metatranscriptomics can effectively capture taxonomic variation as reflected in relative transcriptional contributions in environmental samples.

4.5 | Effects of GBH on Gene Expression

The numbers of differentially expressed genes in most functional categories at KEGG Level 2 were similar between phytoplankton and zooplankton (Figure 4 and Figure S2). This similarity may be because GBHs were originally developed to target terrestrial weeds, whereas phytoplankton and zooplankton are non-target organisms. The shared pattern could arise from GBHs inducing detrimental effects through the same underlying mechanism (i.e., oxidative stress), as summarised in Klátyik et al. (2024), which then leads to comparable gene expression changes in both phytoplankton and zooplankton groups.

In terms of functional gene expression changes, we found that the GBH treatment resulted in more downregulation (45.0% of analysed genes) than upregulation (1.8% of analysed genes). This is likely due to the toxic effects of GBH, which can cause cellular and DNA damage (Hao et al. 2019), potentially impairing normal gene transcription and resulting in widespread gene downregulation. A similar trend was reported in the alga *Fucus virsoides*, where RNA-Seq revealed more gene downregulation than upregulation following GBH exposure (Gerdol et al. 2020). It is also possible that for some genes, both organismal and extra-organismal RNA contributed to the control samples, while

only extra-organismal RNA may have remained detectable in the treatment samples if GBH exposure led to organism mortality. This could result in the presence of these genes in both control and treatment samples but lead to downregulation observed in the gene expression analysis. These findings highlight the need for caution when interpreting downregulated transcripts, as they may represent cytotoxic effects rather than just specific metabolic responses. Interestingly, in the two classes that showed an increase in relative transcript abundances after treatment (Branchiopoda and Insecta), 14.2% and 40.7% of the analysed genes, respectively, responded to the GBH treatment. These percentages are lower than those observed in the other seven analysed classes, except for Dinophyceae (Table 1). These lower percentages may reflect greater tolerance to GBH in these classes or slower degradation of RNA due to longer organismal persistence in the environment after exposure.

Although most KO groups showed predominantly downregulated contigs following GBH exposure, some KOs included both upregulated and downregulated contigs (Tables S5–S7). This pattern is expected because each KO represents a functional category that may include homologous genes from multiple taxa and multiple genes from the same species (Kanehisa et al. 2016). Several qPCR studies on single animal species have reported variable transcriptional responses to GBH exposure, with response direction often depending on gene target, GBH formulation and concentration, exposure duration, species, developmental stage, and sex (de Melo et al. 2019; Kronberg et al. 2018; Le et al. 2010). For example, a 24-h glyphosate exposure in *Daphnia magna* decreased the expression of aryl hydrocarbon receptor nuclear translocator (*arnt*) and cytochrome P450 4 (*cyp4*), but had no effect on vitellogenin (*vtg*) or cytochrome P450 314 (*cyp314*) (Le et al. 2010). Similarly, a 7-day GBH exposure in males of the freshwater prawn *Macrobrachium potiuna* increased the expression of ecdysteroid receptor (*ecr*) and moult-inhibiting hormone (*mih*) and decreased the expression of *vtg*, with no changes observed in females under the same conditions (de Melo et al. 2019). Thus, the presence of both upregulated and downregulated contigs within the same KOs likely reflects the complex and dynamic transcriptional responses to GBH exposure.

Among the differentially expressed genes in response to the GBH treatment, the upregulated genes may encode proteins involved in GBH degradation or reflect metabolic responses to GBH exposure. These genes could serve as valuable candidate genes for developing eRNA-based biomarkers for assessing GBH-induced stress or pollution. Although elucidating the mechanisms of toxicity and species recovery following GBH exposure is beyond the scope of this study, future work should incorporate multiple post-exposure timepoints to distinguish eRNA signals reflecting active transcriptional responses from those released by organisms damaged or killed due to GBH toxicity. Complementary data on organismal abundance (e.g., via microscopy or eDNA) would help determine the extent to which shifts in relative transcript abundance reflect changes in organismal abundance. Nonetheless, our results demonstrate that eRNA-based metatranscriptomics can reveal biologically meaningful, taxon-specific transcriptional patterns associated with pollutant exposure, even from a single timepoint survey. This supports the sensitivity of eRNA-based metatranscriptomics for detecting molecular-level stress responses in freshwater ecosystems and underscores its potential as a tool for environmental biomonitoring.

5 | Conclusion

We evaluated eRNA-based metatranscriptomics for assessing gene transcriptional responses of freshwater eukaryotic communities under environmental stress, using GBH exposure as a case study. Our results demonstrate that this non-invasive method can efficiently capture the relative transcriptional contributions and functional gene expression responses of diverse freshwater eukaryotic taxa. We identified numerous differentially expressed genes associated with molecular pathways known to be impacted by GBH. Despite challenges such as data analysis complexities, reference database limitations, and the need for broader testing across systems and stressors, our mesocosm study highlights the significant potential of eRNA-based metatranscriptomics for environmental biomonitoring.

Author Contributions

X.H. and M.E.C. conceived this study with contributions from co-authors. X.H. performed sample collection, molecular lab work, data analysis, and manuscript writing. All authors contributed to manuscript editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The raw sequence data have been deposited in the NCBI SRA database under the BioProject Accession PRJNA1100463. The bioinformatics commands used in this study are available online (https://github.com/XHe20/bioinformatics-codes-for-eRNA-metatranscriptomics).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Relative transcript abundances of (A) domains and (B) phyla in each sample. In panel (B), "Others" represents the sum of eukaryotic phyla with < 1% relative transcript abundance in every sample, while "Unassigned_Eukaryota" includes reads assigned to Eukaryota but not classified at the phylum level. Some sequences were assigned to the class level but not to a specific phylum due to the structure of the NCBI taxonomy database and are therefore included in "Unassigned_Eukaryota". For example, Dinophyceae, classified within the superphylum Alveolata, lacks a defined phylum-level designation, so its reads fall under "Unassigned_Eukaryota" in panel (B). **Figure S2:** Summary of differentially expressed contigs for each KEGG Level 2 pathway. Contigs with significant expression differences between treatment and control were annotated using eggNOG-mapper and summarised by pathway. The bar names on the y-axis represent the KEGG Level 2 pathways, with bars belonging to each KEGG Level 1 pathway separated by dashed lines. **Figure S3:** Expression patterns of differentially expressed contigs associated with two photosynthesis-related molecular pathways in the two phytoplankton classes (Cryptophyceae and Dinophyceae). (A) Downregulated contigs in response to GBH treatment. (B) Upregulated contigs in response to GBH treatment; no upregulated contigs were detected in Cryptophyceae. **Table S1:** Validation of major classes identified in eRNA data based on previous studies and known freshwater distributions. **Table S2:** Number of reads processed at each step. **Table S3:** Statistical analysis of relative transcript abundance differences between treatment and control samples for nine major classes using LMMs and rLMMs. **Table S4:** Results of KEGG Orthology (KO) enrichment analysis. **Table S5:** Number of upregulated and downregulated contigs assigned to KEGG Orthologs (KOs) within three oxidative stress-related pathways (ko04146, ko00480, and ko00190). **Table S6:** Number of upregulated and downregulated contigs assigned to KEGG Orthologs (KOs) within the detoxification-related pathway (ko00980). **Table S7:** Number of upregulated and downregulated contigs assigned to KEGG Orthologs (KOs) within two photosynthesis-related pathways (ko00195 and ko00196) in Cryptophyceae and Dinophyceae.