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RNA metabarcoding helps reveal zooplankton community response to environmental stressors☆

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ABSTRACT

DNA metabarcoding can provide a high-throughput and rapid method for characterizing responses of communities to environmental stressors. However, within bulk samples, DNA metabarcoding hardly distinguishes live from the dead organisms. Here, both DNA and RNA metabarcoding were applied and compared in experimental freshwater mesocosms conducted for assessment of ecotoxicological responses of zooplankton communities to remediation treatment until 38 days post oil-spill. Furthermore, a novel indicator of normalized vitality (NV), sequence counts of RNA metabarcoding normalized by that of DNA metabarcoding, was developed for assessment of ecological responses. DNA and RNA metabarcoding detected similar taxa richness and rank of relative abundances. Both DNA and RNA metabarcoding demonstrated slight shifts in measured α-diversities in response to treatments. NV presented relatively greater magnitudes of differential responses of community compositions to treatments compared to DNA or RNA metabarcoding. NV declined from the start of the experiment (3 days pre-spill) to the end (38 days post-spill). NV also differed between Rotifer and Arthropoda, possibly due to differential life histories and sizes of organisms. NV could be a useful indicator for characterizing ecological responses to anthropogenic influence; however, the biology of target organisms and subsequent RNA production need to be considered.

1. Introduction

Biodiversity affects ecosystem functioning for maintaining critical services (Siddig et al., 2016), while living organisms contribute most to the present biological activities driving critical ecosystem processes. Metabarcoding is transforming assessments of ecological and ecotoxicological responses of freshwater ecosystems by providing cost-effective, high-throughput, and high-resolution analyses. Previously, DNA metabarcoding effectively described biodiversity of communities of zooplankton, macroinvertebrates, and fish and described their responses to chemical and physical stressors (Elbrecht and Leese, 2017; Emilson et al., 2017; Miya et al., 2015; Xiong et al., 2019; Yang et al., 2017a; Yang et al., 2017b). DNA extracted directly from community tissue or bulk samples for metabarcoding records both current and recent biodiversity (Thomsen and Willerslev, 2015). Alternatively, because RNA is mostly isolated from living cells or organisms and degrades faster than DNA or is recycled, RNA metabarcoding can be used to characterize biodiversity of living organisms (Baldrian et al., 2012). However, compared to DNA metabarcoding, fewer RNA metabarcoding studies have been conducted. Information about alive organisms can be concealed in DNA metabarcoding.

Since they are global, ecologically important, and typically sensitive to stressors, zooplankton can be used for assessing status and trends of freshwater ecosystems (Lougheed and Chow-Fraser, 2002; Marmorek and Korman, 1993; Sladecek, 1983). Previously, DNA metabarcoding of zooplankton has been used to assess effects of contaminants in water on zooplankton communities (Xiong et al., 2019; Xiong et al., 2017; Yang and Zhang, 2020; Yang et al., 2017a). However, current gaps in the use

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of DNA metabarcoding for biomonitoring and toxicological assessment of stressors on zooplankton include an inability to describe the dynamic, vital community by determining living organisms. This can be due to 7.4–47.6% of biomass of freshwater zooplankton being comprised of carcasses (Tang et al., 2014). RNA metabarcoding of zooplankton communities provides a method for assessing the status of alive zooplankton (Ankley et al., 2021).

By coupling RNA and DNA metabarcoding, a more comprehensive characterization of dynamic community responses to stressors could be obtained (Ankley et al., 2021; Laroche et al., 2018). Since turnover of pools of RNA is associated with growth, RNA fluctuates according to the biology of the target organism (Garcia-Martinez et al., 2016). Results of previous research have suggested that metabarcoding of environmental RNA (eRNA) has greater sensitivity in measuring responses of local biodiversity (α-diversity) to stressors. Alternatively, environmental DNA (eDNA) metabarcoding is more efficient in describing changes in β-diversity, which is defined as the ratio between local and regional diversity (Laroche et al., 2017; Whittaker et al., 2001). eRNA or eDNA is classified as organismal genetic material that are obtained from environmental matrices without apparent signs of the source (Thomsen and Willerslev, 2015). Due to changes in production and recycling of messenger RNA (mRNA), similar measurements using bulk metabarcoding is anticipated. Due to the differential biological characteristics of nucleic acids, the key question remains how to integrate DNA and RNA metabarcoding for a sensitive assessment of ecological response to environmental stressors.

To compare performances of DNA and RNA metabarcoding for characterizing responses of communities to environmental stressors in aquatic environments, DNA and RNA metabarcoding of bulk zooplankton samples were used to characterize biodiversities of dynamic zooplankton communities in response to various methods of remediation of oil-spills. Normalized vitality (NV), which is the ratio of sequence counts of RNA metabarcoding normalized to that of DNA metabarcoding, was tested as a method to detect effects of environmental stressors on the zooplankton community. Traditional RNA:DNA ratios derived from nucleic acid content have been shown to reveal changes in the life history and fitness of zooplankton (Chicharo and Chicharo, 2008; Vrede et al., 2002; Wagner et al., 1998), and have been used as indices for marine ecology (Chicharo and Chicharo, 2008). It was hypothesized that NV could be more descriptive than either DNA or RNA metabarcoding alone for assessing responses of communities to environmental stressors. Objectives of the study were to: (1) Compare abilities of RNA and DNA metabarcoding to capture zooplankton community composition in shoreline enclosures with rock and cobble substrate and describe responses of α-diversity to treatments; (2) Compare DNA and RNA metabarcoding with NV to capture the responses of communities to treatments; (3) Investigate the ability of normalized vitalities to discern the response of the zooplankton community to environmental influence. Zooplankton was sampled from field mesocosm experiments for ecological assessment of remediation practices (enhanced monitored natural recovery and shoreline cleaner application) after a simulated spill of diluted bitumen (dilbit) in a boreal lake. Mitochondrial cytochrome oxidase 1 (COI) was selected as target gene for metabarcoding.

2. Materials and methods

2.1. Experimental design

The experiment was conducted at the IISD Experimental Lakes Area (IISD-ELA), located in northwestern Ontario, Canada, which contains 58 boreal lakes set aside for whole-ecosystem experimentation (49°41′45.0″ N, 93°46′03.4" W) (Kidd et al., 2007; Schindler et al., 1996). In June 2019, seven enclosures (15 × 5 m) were established along the shoreline area with rock and cobble substrata in Lake 260 at the IISD-ELA (See Table S1 for enclosure GPS points). On June 22nd, 2019, after baseline measurements were completed, six randomly selected enclosures were treated with model spills of dilbit applied to the surface of the water approximately 50 cm from the shore (Palace et al., 2021). One enclosure remained untreated to serve as a reference. Details of shoreline enclosures and subsequent exposures have been published previously (Ankley et al., 2021). Enclosures treated with dilbit were then selected to receive one of two remediation practices to determine their effectiveness for promoting longer-term recovery from residual oil contamination. The first method, enhanced monitored natural recovery (eMNR; $n = 3$), included addition of nutrients designed to promote the bacterially-mediated decomposition of remaining oil products. The second method consisted of active cleaning of the shoreline using the oil surface washing agent COREXIT® EC9580A (Nalco, Co, Illinois, USA) (SCA; $n = 3$) (Fig. S1). One shoreline enclosure remained untreated serving as the reference (REF; $n = 1$). Total polycyclic aromatic compounds (PACs) and total 4 ringed PACs measured by using gas chromatography-mass spectrometry (GC-MS) from methods outlined previously (Idowu et al., 2018), was used to establish the relative aqueous exposure of dilbit constituents to the zooplankton and within the experimental enclosures (Fig. S2; Table S2). Water samples used for PAC profiling were collected from the same location and close to the same sampling days for zooplankton, being 1 m from the far-end of the enclosures relative to the shoreline.

2.2. Collection of zooplankton

Triplicate 20-L samples of water were collected consecutively from each experimental enclosure three days before the simulated spill of dilbit, then 11 and 38 days after the spill (Fig. S1). Zooplankton were enriched by two-step filtering by use of a pump with an in-line 53 μm mesh filter and final enrichment with a 5 μm Durapore® PVDF membrane filter (Millipore, Germany). Samples were preserved in LifeGuard Solution (Qiagen, Germany) and stored at −80 °C before extraction of nucleic acid. To avoid and detect cross-contamination, use of filter pumps specified for each treatment, single-use filter-units, changing of gloves at each enclosure, and decontamination of equipment between each replicate was conducted, with field blanks collected from each enclosure. Field blanks consisted of a decontaminated 500 mL Nalgene™ bottle (Thermo Fisher Scientific, USA) containing Nanopure™ water opened throughout the sampling event for each treatment.

2.3. Co-isolation of DNA and RNA, PCR amplification, and next-generation sequencing (NGS)

DNA and RNA were simultaneously isolated by use of AllPrep DNA/RNA Mini Kit (Qiagen, Germany) following the manufacturers specifications, with exception of tissue disruption, which included use of four sizes of beads (one 2.8 mm ceramic bead; two 1.4 mm ceramic beads; 100 μL 0.1 mm glass beads; 100 μL 0.5 mm glass beads) for lysing zooplankton tissue. Contamination of extracted RNA by DNA was removed by digestion with RNase-Free DNase (Qiagen, Germany). Extracted DNA and RNA were measured and checked for quality using Qubit 4 Fluorometer (Thermo Fisher Scientific, USA) and purity by use of NanoDrop Spectrophotometer (Thermo Fisher Scientific, USA), respectively. Concentrations of DNA and RNA from extraction blanks used for quality control (QC) were less than the limit of detection. Complementary DNA (cDNA) was synthesized by use of SuperScript IV Reverse Transcriptase (Invitrogen, CA, USA) along with ezDNase to remove residual DNA.

Amplification by polymerase chain reaction (PCR) was performed on normalized cDNA and DNA (10 ng/μL) by use of unique dual tagged primers targeting a 313bp region of the cytochrome oxidase subunit region 1 (*COI*) by use of primers mICOIintF (5′-GGWACWGGWTGAACWGTWTAYCCYCC-3′) and jgHCO2198R (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) with a "touchdown" cycle program (Leray et al., 2013; Yang et al., 2017b). To minimize potential bias during

amplification, PCR was performed in triplicate using Platinum Taq Hot Start II High-Fidelity DNA Polymerase (Invitrogen, USA), with plate set-up containing multiple PCR blanks for QC. PCR products were checked by use of agarose gel electrophoresis and purified using the QIAquick PCR Purification kit (Qiagen, Germany). Construction of the sequencing library and next-generation sequencing by use of Illumina chemistry on a MiSeq platform (Illumina, USA) were performed as previously described (DeBofsky et al., 2020). Sequencing data can be accessed at https://doi.org/10.20383/102.0332.

2.4. Bioinformatics

Raw reads were demultiplexed based on dual tags of both forward and reverse primers for each sample using fastq-multx (version 1.3.1). Paired-end sequences were then merged using VSEARCH (version 2.14.2), with forward and reverse primers removed and sequences filtered to remove lesser quality (ee > 1.0), chimeras, and shorter length (<300 bp) sequences thereafter (Rognes et al., 2016). Zero-radius operational taxonomic units (ZOTUs) were generated using unoise3 command with a minimum abundance set to 5 (Edgar, 2016), and afterward resulting ZOTU open reading frames (ORFs) were searched *via* NCBI ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/). Resulting pseudogenes and short open reading frames (<300 bp) were removed.

Identifications of taxa were conducted as previously described (Ankley et al., 2021), with target taxa being Phylum Rotifera or select orders in Subphylum Crustacea (Orders Calanoida, Cyclopoida, and Cladocera). ZOTUs that remained unassigned or classified as non-target taxa were removed. Before downstream analyses, replicate samples for each enclosure were merged. Taxa hereafter refers to features assigned to the best attainable level, being either species or genus, with genus-level identification being denoted with *uc.* representative of unclassified species. Singleton taxa and taxa found to occur in only one sample, were removed with samples being rarefied to equal depths of 9182 using QIIME 2 command line feature-table rarefy (Bolyen et al., 2019; Weiss et al., 2017). Further details on Illumina MiSeq sequencing output can be found in supporting information (Appendix S1). Bioinformatics was conducted under command line (Ubuntu version 19.04), QIIME 2 (version 2020.6), and R environment (version 4.0.3) (Team, 2013). A workflow chart for zooplankton metabarcoding and applied bioinformatics can be found in supporting information (Fig. S3).

2.5. Statistics

All statistics were performed in the R environment (version 4.0.3), with use of package ggplot2 (version 3.3.5) for graphics (Wickham, 2016). Rarefied DNA and RNA metabarcoding data were filtered to have equivalent taxa to eliminate potential erroneous annotations and for comparison to NV, with five unique taxa collectively being removed (Fig. S4). Spearman rank correlation was used to compare between DNA and RNA metabarcoding relative abundance and biomass of zooplankton at the genus-level. For assessment of zooplankton α-diversity response to remediation practices eMNR and SCA, responses were partitioned into subacute (Day 11) and chronic (Day 38). Normalized α-diversity indices ($\Delta\Delta a$) relative to reference enclosure were calculated for each treatment enclosure for subacute and chronic response (Eq. (1)). To remove the effect of differential baseline of each enclosure, normalized α-diversity indices were calculated for each treatment enclosure (Eq. (2)) and reference enclosure (Eq. (3)). A two sample t-test was used to test for differences in normalized α-diversity between eMNR and SCA, with assumptions of normal distribution and homogeneity of variance being met.

$$\Delta\Delta a_{E_i,\,D_j} = \Delta a_{E_i,\,D_j} - \Delta a_{E_{REF},\,D_j} \quad (1)$$

$$\Delta a_{E_i,\,D_j} = a_{E_i,\,D_j} - a_{E_i,\,D_{-3}} \quad (2)$$

$$\Delta a_{E_{REF},\,D_j} = a_{E_{REF},\,D_j} - a_{E_{REF},\,D_{-3}} \quad (3)$$

where, E, treatment SCA or eMNR; i, enclosure of treatment groups; j, sampling time point post-spill, 11 or 38 days; REF, reference enclosure.

To enable interpretation of viable and dormant taxa, relative counts inferred from cDNA amplicon sequencing, were divided by relative counts deduced from DNA for each respective taxon referred to as NV. Infinite values were set to "0". Hellinger distance of taxa-level count data between treatment enclosures and reference for each time point sampled was used to compare community response and different methods (i.e., DNA, RNA metabarcoding and NV). Principal Coordinates Analyses (PCoA) were performed to visualize β-diversities of zooplankton communities for all time points sampled using Hellinger distance. Treatment group differences of β-diversities were tested using nested adonis2 (e.g., adonis2 (Distance ~ Treatment, data = metadata, permutations = 9999)) with time set as a block using package vegan (version 2.5–7) (Oksanen et al., 2007). Pairwise multilevel comparisons, using nested adonis2 with Time set as block (e.g., pairwise. adonis2 (Distance ~ Treatment, data = metadata, strata = 'Time', nperm = 9999, p. method = "fdr")), were conducted to test between SCA and eMNR using package pariwiseAdonis (version 0.0.1) adjusting p-values using a false discovery rate (fdr) (Martinez Arbizu, 2017).

ANOVA with Tukey-HSD and Welch's t-test were used to test for differences in number of NV values ≥ 2 between factors of interest, with assumptions of normal distribution and homogeneity of variance being met. NV values ≥ 2 were used to adjust for the variability and biases posed by PCR, with similar interpretation made if using >1 values (Elbrecht and Leese, 2015). Fold-change of 2 is also typically used for analysis of gene expression change. A heatmap was used to visualize NV values over time and between treatments for each taxa with the use of the pheatmap package (version 1.0.12) (Kolde and Kolde, 2015).

3. Results

3.1. Characterizing zooplankton community in rock habitat

Thirty-seven taxa, with 15 taxa in the phylum Arthropoda and 22 taxa in the phylum Rotifera, were detected by both DNA and RNA metabarcoding. DNA metabarcoding top abundant taxa included *Cyclops uc.* (29.9 ± 5.24%; average ± standard error of the mean (SEM)), *Keratella uc.* (21.9 ± 4.93%), *Leptodiaptomus minutus* (14.2 ± 2.69%), *Epischura lacustris* (13.3 ± 2.64%), *Keratella cochlearis* (12.7 ± 2.45%), and *Asplanchna uc.* (2.47 ± 0.743%) (Fig. 1A). RNA metabarcoding top abundant taxa included *Keratella uc.* (36.6 ± 6.86%; average ± SEM), *Leptodiaptomus minutus* (16.62 ± 3.33%), *Cyclops uc.* (14.36 ± 3.21%), *Epischura lacustris* (12.3 ± 2.46%), *Keratella cochlearis* (10.1 ± 1.77%), and *Asplanchna uc.* (3.10 ± 0.304%) (Fig. 1A). Taxa with greatest overall NV values included *Synchaeta uc.* (149.1), *Brachionus uc.* (143.1), *Asplanchna uc.* (81.2), *Trichocerca uc.* (72.6), *Ploesoma uc.* (71.5), and *Lecane uc.* (65.3) (Fig. 1B). NV values of top 15 taxa were from the phylum Rotifera, indicating increase in RNA sequence counts relative to DNA. Taxa with lowest overall NV values included *Alonella uc.* (0.000), *Holopedium glacicialis* (0.154), *Sinergasilus uc.* (0.282), *Collotheca campanulata* (0.818), *Macrothrix uc.* (1.18), and *Diaphanosoma uc.* (1.29), five of which were from the phylum Arthropoda. Genetic variability also differed between phyla, with a greater overall feature (i.e., ZOTUs) count assigned to phylum Rotifera (n = 112) relative to phylum Arthropoda (n = 21) (Fig. S5).

Overall, RNA metabarcoding resulted in lesser Shannon index compared to DNA metabarcoding at 38 days post-spill (t-value = 2.61, p = 0.0229), with similar observed taxa richness (Fig. S6; Appendix S2). Sequence counts inferred from both DNA and RNA metabarcoding served as an acceptable indicator of zooplankton biomass at the genus level (Fig. 2). For Arthropoda genera, both DNA and RNA metabarcoding based relative abundances ($\log_e$-transformed) were moderately

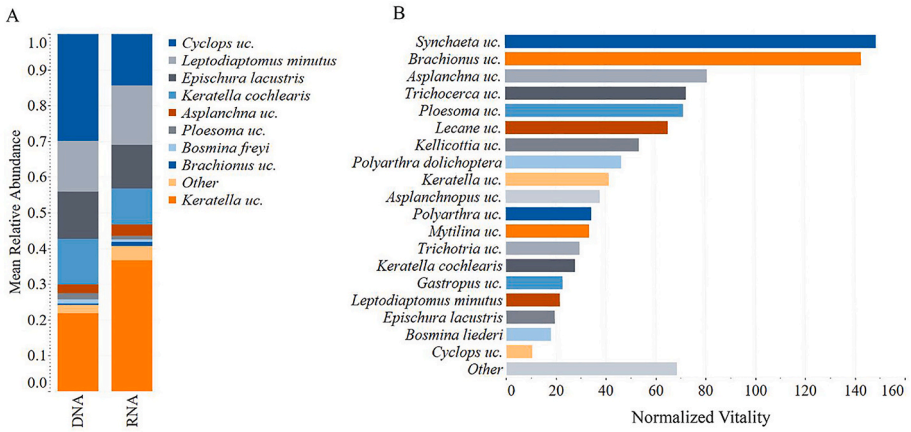

Fig. 1. (A) Detected taxa with ≥1% mean relative abundance for DNA and RNA metabarcoding. Taxa with <1% mean relative abundance were clustered into "Other". (B) Normalized vitality values of taxa, with taxa and associated values less than 10 clustered into "Other".

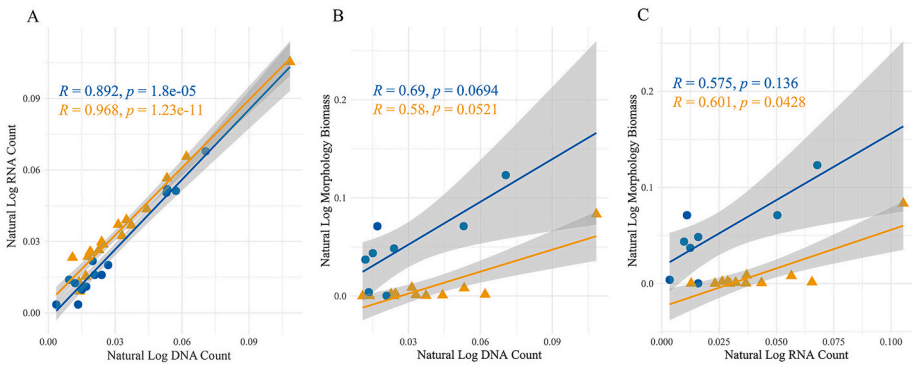

Fig. 2. Correlations of shared genera of loge-transformed metabarcoding rarefied count data and loge-transformed morphology biomass using Spearman rank correlation. Correlation relationship was assessed between (A) RNA and DNA metabarcoding, (B) biomass and DNA metabarcoding, and (C) biomass and RNA metabarcoding. Blue indicates genera in the phylum Arthropoda and orange indicates genera in the phylum Rotifera. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

correlated with $\log_e$-transformed biomass (Fig. 2B, R = 0.69, $p = 0.0694$; Fig. 2C, R = 0.575, $p = 0.136$). Rotifera genera DNA and RNA based relative abundances also correlated with biomass (Fig. 2B, R = 0.58, $p = 0.0521$; Fig. 2C, R = 0.601, $p = 0.0428$). DNA and RNA metabarcoding inferred relative abundances were significantly correlated for both Arthropoda and Rotifera genera (Fig. 2A, R ≥ 0.892, $p \leq 1.8e\text{-}05$).

3.2. Subacute and chronic impacts of dilbit remediation practices

eMNR had similar magnitude of subacute effects on α-diversities (taxa richness and Shannon index) of zooplankton as SCA (Fig. 3A–D). Slightly less DNA metabarcoding-based normalized taxa richness resulted from SCA, relative to that of eMNR (Fig. 3A), while no difference in RNA metabarcoding-based normalized α-diversities was observed between eMNR and SCA (Fig. 3B, D). Both DNA and RNA metabarcoding-based normalized Shannon index increased at day 11 post-spill for SCA and eMNR. For chronic effects, an increase in normalized taxa richness was observed for both eMNR and SCA, with relatively greater magnitude for RNA metabarcoding-based response compared to DNA metabarcoding (Fig. 3E and F). SCA had differing chronic effects on normalized Shannon index of zooplankton compared to subacute. A lesser DNA metabarcoding-based normalized Shannon index was caused by SCA relative to that of eMNR (Fig. 3G), while only a slight difference of RNA metabarcoding-based normalized Shannon index between eMNR and SCA was observed (Fig. 3H).

eMNR had less of an impact on normalized taxa richness at subacute compared to chronic exposure, whereas had greater impact for normalized Shannon index (Fig. 3). SCA had greater effects on normalized taxa richness at subacute relative to chronic exposure, and for DNA-based normalized Shannon index, an inverse impact was observed for subacute versus chronic (Fig. 3C, G). Total PACs were greatest for SCA at subacute (6 days post-spill) having mean concentration of 1.1×10^4 ng/L (Table S2), with eMNR and REF having lesser concentrations of 3.8×10^3 and 1.2×10^3 ng/L, respectively, at subacute. Total PACs at chronic (20 days post-spill) for both eMNR and SCA was similar with average concentrations of 2.3×10^3 and 2.5×10^3 ng/L, respectively.

A distinct pattern of beta-diversities, derived as the Hellinger distance relative to the reference, was revealed by DNA, RNA metabarcoding and NV. Treatments SCA and eMNR both showed similar changes in distance to reference for both methods of identification (Fig. 3I and J). NV revealed the greatest distance at each time point measured relative to DNA or RNA metabarcoding (Fig. 3I and J). At day 38, Hellinger distance, based on RNA metabarcoding, was significantly less than the distance determined at day 11 (Fig. 3J, Tukey HSD, $p = 0.0111$). NV revealed stronger clustering of treatment groups compared to either DNA or RNA metabarcoding alone (PCoA, Fig. S7). β-diversities, based on NV, were not significantly different between treatments (Table S3, Nested adonis2, $F_{2,18} = 1.06$, $p = 0.0752$), although it had a greater magnitude of difference relative to either DNA or RNA metabarcoding, alone (Table S3, $F_{2,18} \leq 0.440$, $p \geq 0.372$). Differences in β-diversities, derived from either DNA or RNA metabarcoding alone, between eMNR and SCA, were not statistically significant (Table S3, Nested pairwise adonis2, $F_{1,16} \leq 0.475$, $p \geq 0.320$), but NV did detect significant ($F_{1,16} = 1.42$, $p = 0.023$) differences between the two treatments (Table S3).

3.3. Normalized vitality

Differences in NV of zooplankton taxa were detected through time

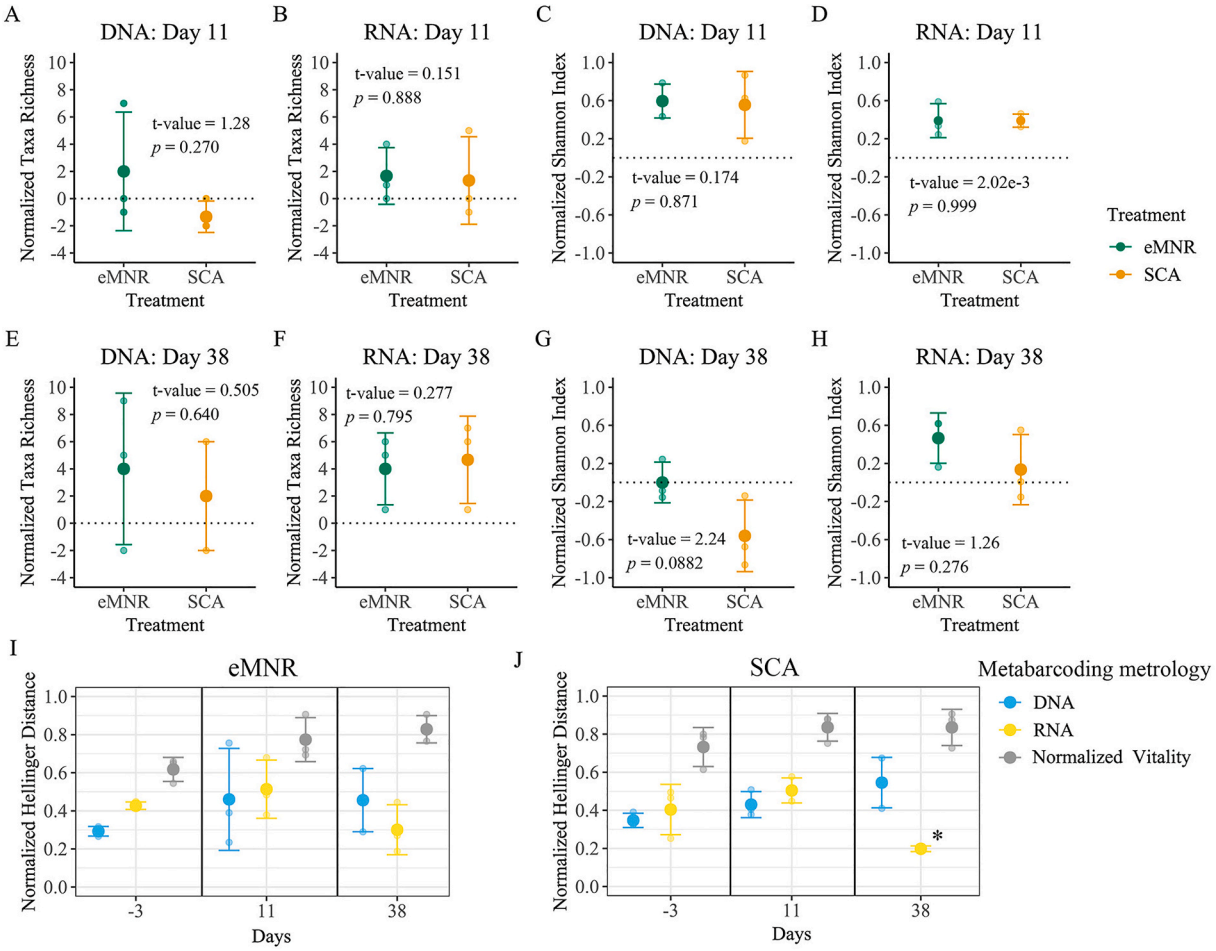

Fig. 3. Normalized alpha (α) diversity indices of (A–D) subacute and (E–H) chronic response for DNA and RNA metabarcoding with black dotted line indicating no difference relative to reference and day −3. Hellinger distance to reference enclosure of treatments (I) eMNR and (J) SCA for DNA, RNA metabarcoding, and normalized vitality. Plotted is average and standard deviation. Treatment groups consisted of enhanced monitored natural recovery (eMNR; $n = 3$) and shoreline cleaner application (SCA; $n = 3$). DNA refers to DNA metabarcoding and RNA refers to RNA metabarcoding. Time points consisted of 3 days pre-spill and 11- and 38-days post-spill. Asterisk (*) indicates significant difference ($p < 0.05$) between day 38 and 11 using Tukey HSD.

and among phyla. Time or taxonomic group was an indicator of NV, with lesser NV at day 38 (July 30th) relative to days −3 (June 19th) or 11 (July 3rd) (Fig. 4A). Zooplankton taxa in either phylum, Rotifera or Arthropoda (subphylum Crustacea), differed in patterns of NV (Fig. 4B). No significant differences in numbers of normalized vitalities ≥ 2 between treatments were observed when considering only days 11 and 38 post-spill (Fig. S8). NV visualized by use of heatmap indicated greatest abundance at day −3 and least abundance 38 days post-spill (Fig. 4C). *Cyclops uc.* had a consistently smaller, NV whereas several Rotifera taxa, such as *Keratella uc.* and *Asplanchna uc.*, exhibited relatively greater vitality throughout. Several Rotifera taxa with greater normalized vitalities at day −3 included *Asplanchna uc., Brachionus uc., Kellicottia uc., Synchaeta uc.,* and *Trichocera uc.* Shannon diversity index seemed to better represent trends in NV of the zooplankton community, with RNA metabarcoding having significantly greater Shannon diversity relative to DNA metabarcoding at days −3 and 11, but was less pronounced at day 38 (Fig. S6; Appendix S2).

4. Discussion

Integrated DNA and RNA metabarcoding served as a useful approach for characterizing the zooplankton community and its response to environmental perturbation. DNA and RNA metabarcoding were comparable in detecting taxa and associated rank of relative abundance. However, a discrepancy between RNA and DNA metabarcoding detecting Rotifera taxa was observed. RNA metabarcoding indicated greater relative abundance compared to taxa in the phylum Arthropoda, but DNA metabarcoding indicated the opposite. DNA and RNA metabarcoding detected comparable responses to treatments, with DNA metabarcoding detecting moderately greater responses for both α- and β-diversity indices. RNA metabarcoding normalized by DNA, or NV, was able to better discern the response of the zooplankton community to remediation practices, measured *via* β-diversity. NV was additionally able to discern temporal changes in the community and detect differential dynamics of taxa between phyla Rotifera and Arthropoda.

DNA and RNA metabarcoding revealed similar composition to historical morphological identification data from IISD-ELA (Patalas, 1971; Schindler and Novén, 1971). Crustaceans found in the lakes are typically dominated by three species, with one cyclopoid (e.g., *Cyclops*), one diaptomid (e.g., *Leptodiaptomus minutus*), and one cladoceran (e.g., *Bosmina*) being the most common structure (Patalas, 1971). Rotifer communities in the IISD-ELA are dominated by *Keratella* spp., which numerically are the most prevalent (Kidd et al., 2014; Schindler and Novén, 1971). Based on previous research, issues still remain with classification of metabarcoding to the species level for zooplankton in the IISD-ELA region and when inferring absolute biomass (Ankley et al., 2021), however, updates to molecular taxonomic databases and use of multiple gene regions can improve resolution (Yang et al., 2017c; Zhang et al., 2018).

Overall responses of zooplankton communities to oil-spill

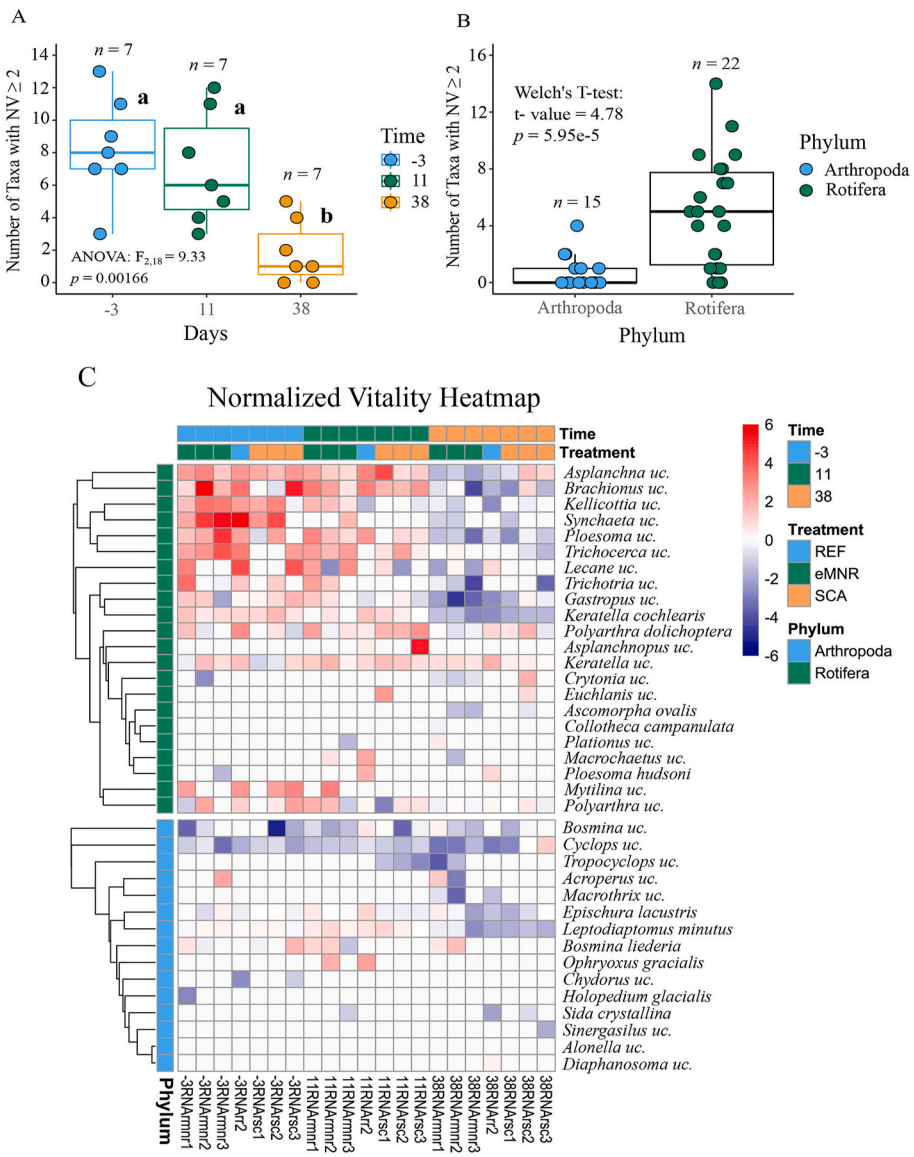

Fig. 4. (A) Number of taxa with normalized vitality (NV) values ≥ 2 per sampling day, with sampling days consisting of 3 days pre-spill and 11- and 38-days post-spill. ANOVA was used to test for differences between time points with Tukey HSD as post-hoc test. Letters denote significant differences inferred from Tukey HSD; (B) Number of taxa with NV values ≥ 2 per taxa for phyla Rotifera or Arthropoda (subphylum Crustacea). Significant differences were inferred by use of Welch's two sample t-test; (C) Heatmap of normalized vitality values of zooplankton taxa. Log base 2 transformations was applied to the normalized vitality values. Rows were clustered according to unweighted pair-group method with arithmetic mean (UPGMA), separately, for each phylum (i.e., Arthropoda and Rotifera).

remediation practices were relatively minor. Compared with metabarcoding based on RNA, that based on DNA revealed that SCA likely had the greatest effects on zooplankton α-diversity during subacute and chronic exposures. Chemistry data collected 6 days post-spill, indicated experimental treatments both received PAC exposure, with lesser exposure in eMNR versus SCA enclosures (Fig. S2; Table S2). Petroleum constituents from the shoreline were expectedly washed into the water column by the application of the shoreline washing agent (i.e., COREXIT® EC9580A). Results of previous studies have shown that COREXIT® 9580A can be acutely toxic to zooplankton and macroinvertebrates, separately and coupled with dilbit (Ankley et al., 2021; Black et al., 2020; Hansen et al., 2014). eMNR indicated relative increases in α-diversity for both subacute and chronic response, which could be due to the greater initial influx of nutrients (Azevedo et al., 2015), although slight increases in TDP and TDN were observed for treatment eMNR at day 11 post-spill (Table S4). Overall, DNA metabarcoding indicated a greater magnitude of differences in α-diversity response, which was unexpected (Pawlowski et al., 2014). DNA metabarcoding also demonstrated a greater magnitude of difference for β-diversity response when compared to RNA metabarcoding, which was consistent with results of previous studies and theoretical expectations (Laroche et al., 2017).

By combination of metabarcoding based on DNA and RNA, NV could assist in detailing zooplankton community of dynamic ecosystems. NV could reflect the changing aspects of the community in response to chemical stress, with β-diversity analyses revealing a larger magnitude of difference between treatment groups for NV compared to DNA and RNA metabarcoding, individually. Metabarcoding of zooplankton based on NV could reflect the active zooplankton community response to environmental influence, which is the case for microbes inferred from the same techniques (Blazewicz et al., 2013). DNA extracted directly from community tissue and bulk sample record current and recent biodiversity (Thomsen and Willerslev, 2015), while RNA derived from bulk zooplankton samples are typically from living organisms (Baldrian et al., 2012; Cristescu, 2019; Hui et al., 2014). For zooplankton metabarcoding, mitochondrial *COI* is a protein-coding gene, with the abundance of DNA relative sequence counts being comparatively reflective of the biomass of target organisms (Ankley et al., 2021; Elbrecht and Leese, 2015; Yang et al., 2017b). Mitochondrial RNA could be reflective of cell proliferation or vitality and growth of the organisms (Pochon et al., 2017). It could also reflect the reproduction cycles of organisms, with more energy, or RNA in this case, being passed to offspring (Bamstedt, 1983). In microbial studies, the ratio of 16s rRNA:rDNA amplicons was suggested to indicate metabolic activity or potential synthesis activity of

microbial communities (Blazewicz et al., 2013; Zhou et al., 2021). However, the method in this study was developed and tested for macro- (>500 μm) and meso-organisms (200–500 μm). Due to primer biases, there are limitations of the use of *COI* for inferring abundance (Deagle et al., 2014; Elbrecht and Leese, 2015), but methods are continuously being improved (Andujar et al., 2018).

NV could describe changes of zooplankton community to temporal influence and associated fluxes in nutrients. At 3 days pre-spill (June 22nd), temperatures in Lake 260 were increasing, with the cycling of nutrients having occurred in previous weeks due to lake turnover (Planas and Hecky, 1984; Wetzel, 2001). Ratios of amounts of crustacean RNA:DNA have previously been shown to be influenced by nutrients, with the copepod *Calanus finmarchicus* having lesser RNA:DNA ratios when fed growth-limiting amounts of phytoplankton, and the cladoceran *Daphnia galeata* having an enhanced RNA:DNA ratios with increasing food P:C ratio (Table S4, S5) (Vrede et al., 2002; Wagner et al., 1998). For Rotifera species *Brachionus calyciflorus*, a diet composed of lesser C:N and C:P ratios, resulted in the greatest RNA: Protein ratios (Wojewodzic et al., 2011). Temperature might also influence RNA:DNA ratios, with greater concentrations of RNA seen for *Daphnia pulex* adapted to lower temperatures (Van Geest et al., 2010); However, this has been debated as a major driver, as in the case of calanoid copepods collected from various pelagic zones (Table S6) (Ikeda et al., 2007).

Application of NV might help reveal differences in life history and body size/composition of separate phyla. Inferred from NV values, taxa in the phylum Rotifera had greater numbers of active taxa than Crustacean taxa (Phylum Arthropoda). Taxa in phylum Rotifera and subphylum Crustacea can differ widely in life-history traits, which could lead to differences in NV values (Allan, 1976). Copepods (e.g., *Cyclops spp.*) typically have longer life cycles and fewer generations relative to more rapid rates of growth and reproduction and shorter life cycles of Rotifera and Cladocerans, but there is variability between these categories (Allan, 1976). Rotifera can have life cycles of days in duration, whereas copepods are upwards of weeks and months long. Taxa in subphylum Crustacea typically had greater DNA sequence counts relative to RNA, with exoskeletons of these organisms potentially enhancing total DNA sequence counts. Rotifera taxa lack an exoskeleton and are composed instead of a transparent external cuticle. Amounts of RNA can vary according to the dry masses of zooplankton, with RNA content inversely proportional to body mass (Bamstedt, 1983). At day 38, for the phylum Rotifera, fluctuations of feature richness indicated relatively greater feature counts based on DNA than RNA metabarcoding (Fig. S9). This could be due to species complexes, dormant haplotypes, lab and bioinformatic variation, or mitochondrial pseudogenes (Declerck and Papakostas, 2017; Elbrecht et al., 2018; Song et al., 2008). DNA and RNA content of target organisms and its variability should be taken into consideration when using RNA:DNA ratios derived from amplicon sequence count data for ecological community assessment, including the use of the mitochondrial *COI* gene when inferring relative vitality and abundance (Louca et al., 2018).

Due to several limitations of the resulting metabarcoding data, benchmarking of NV is required. NV included setting infinite values to "0" which has been shown to capture similar patterns as other normalization methods, including the addition of pseudo "1" to every feature in both DNA and RNA datasets (Bowsher et al., 2019). Classification of the living community *via* RNA:DNA ratios, termed NV here, can identify more active taxa, however, false negatives can occur with active taxa sometimes being misclassified as dormant (Steven et al., 2017). Currently, RNA:DNA ratios inferred from metabarcoding would be more useful and accurate as a biodiversity measurement compared to a measurement of growth, due to uncertainty for individual levels in RNA amounts compared to DNA between different taxa. Future steps would be to assess changes in RNA and DNA amounts from representative taxa in a controlled environment with a greater frequency, to distinguish dynamics to simulated stressors using metabarcoding techniques.

5. Conclusions

Here the value of utilizing NV to reveal the dynamics of the zooplankton community to temporal and anthropogenic change was demonstrated. This method is still in development and further development and benchmarking will be required to gain a greater understanding of the physiological and community vitality and the corresponding amplicon sequence output. The index of the RNA:DNA ratio has been used historically in an ecological context, however, the use of these indices inferred from metabarcoding is still an emerging area of research. Overall, we have shown that NV could serve as a sensitive method for measuring the ecological response of communities to environmental change.

Credit author statement

Phillip J. Ankley: Conceptualization, Investigation, Formal analysis, Methodology, Data curation, Writing – original draft. **Yuwei Xie**: Conceptualization, Methodology, Writing – review & editing. **Sonya Havens**: Resources, Data curation. **Lisa Peters**: Resources, Data curation. **Lauren Timlick**: Resources. **Jose Luis Rodriguez-Gil**: Resources. **John P. Giesy**: Conceptualization, Project administration, Funding acquisition, Writing – review & editing. **Vince P. Palace**: Conceptualization, Funding acquisition, Resources, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.118446.

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Supporting Information

RNA metabarcoding reveals zooplankton community response to environmental stressors

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Summary of the number of figures and tables: Figures: 9; Tables: 6

CONTENT

Tables

Appendix S1. Metabarcoding consisted of a total of 2400775 sequence reads after demultiplexing from the two MiSeq runs, with run 1 having 612977 reads and run 2 having 1787798 reads for target samples. Technical replicates had sequence reads of 14290 ± 956.82 (average ± standard error o the mean (SEM)). Samples for day 11 post-spill were resequnced on run 2 and were merged after checking for differences due to batch effect via Procrustes analysis. After denoising and merging technical replicates, and removing features that only occurred in one sample, a total of 1,284,906 sequence reads were assigned to target taxa (e.g., Phylum Rotifera and Order's Calanoida, Cyclopoida, and Cladocera) to at least the family level, with merged technical replicates (n = 3) having sequence reads of 51494 ± 4990.1 (average ± SEM) prior to rarefaction to equal depth of 9182.

Appendix S2. Alpha (α) diversity indices were tested for differences between metabarcoding methods while controlling for time effects using a random intercept model. Method effect on α-diversity indices were not significant via random intercept models when blocking time (e.g., lmer (Diversity Index ~ Treatment + (1|Time), data = α); Figure S4, $F_{1,38} \leq 0.0653$, $p \geq 0.800$)). This was conducted using the lme4 and lmerTest packages in R (version 4.0.3) (Bates et al., 2014; Kuznetsova et al., 2017; Team, 2013). Two-sample t-tests were used to denote difference at each time point for Shannon diversity index due to visual evidence of differences, with significance difference observed at -3 (t-value = 3.32, p = 0.00607), 11 (t-value = 3.49, p = 0.00444), and 38-days post-spill (t-value = 2.61, p = 0.0229).

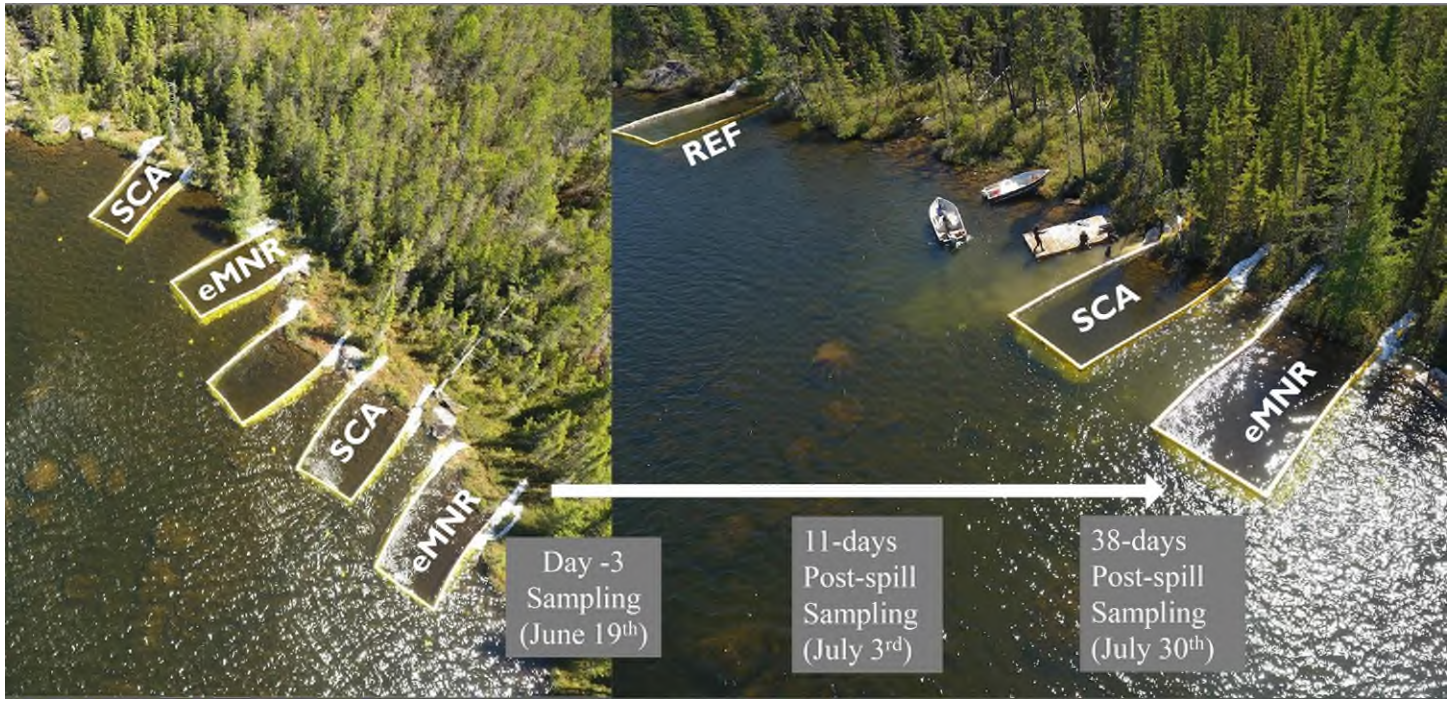
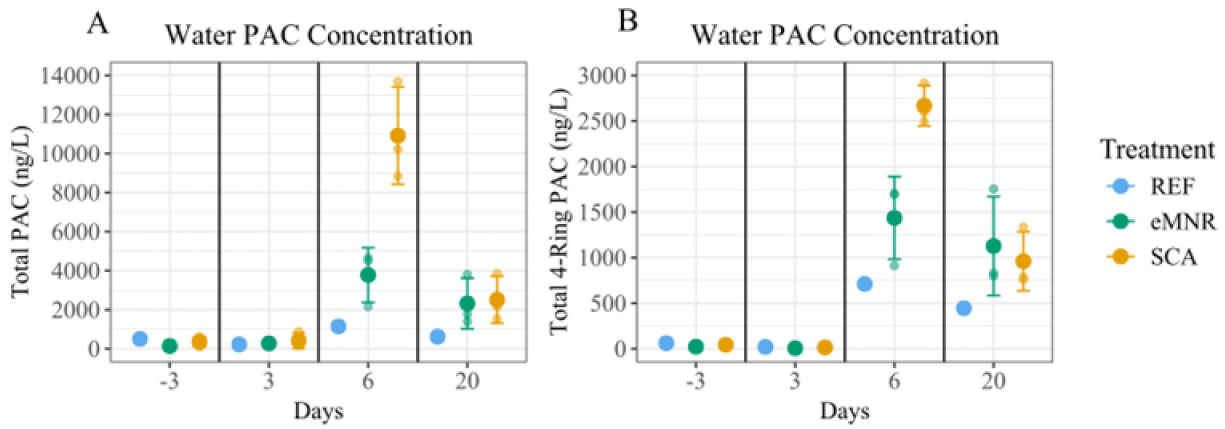
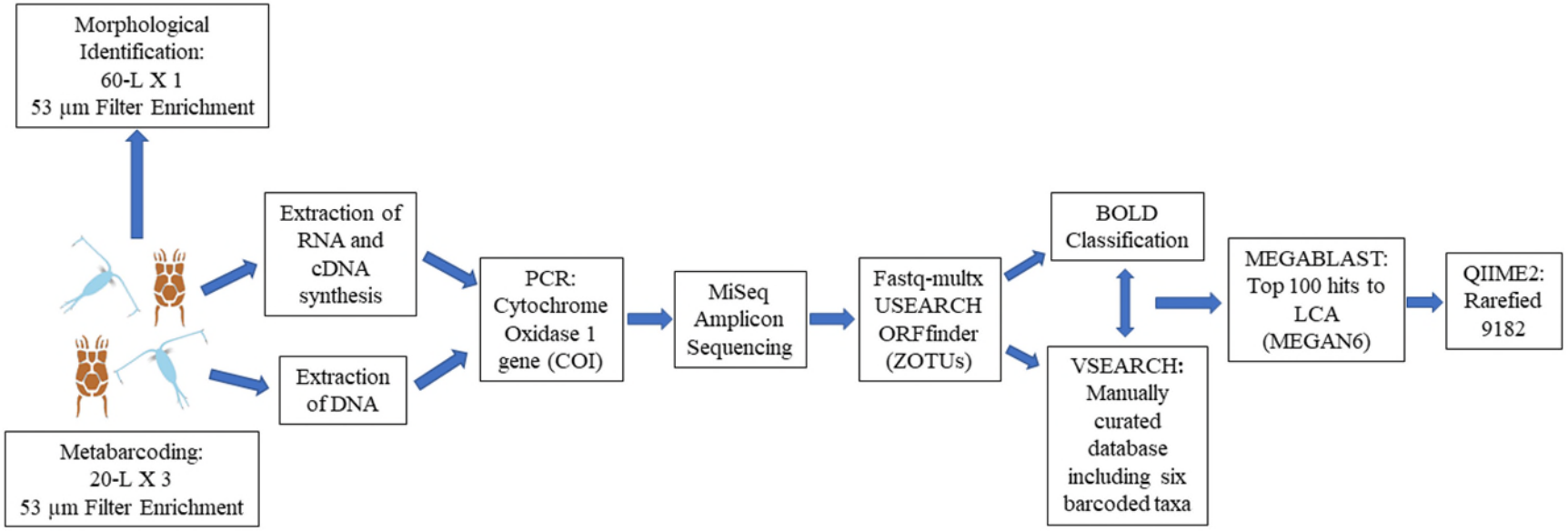


Figure S1. Aerial photo of the enclosures used in DNA and RNA metabarcoding studies of zooplankton in assessing the ecotoxicological effects of two oil spill remediation practices – enhanced monitored natural recovery (eMNR) using the addition of nitrogen and phosphorous, and a shoreline cleaner, COREXIT® EC9580A (SCA) – relative to a reference enclosure (REF). Diluted bitumen was applied to enclosures on June 22nd, with the selected remediation practices being applied on June 26th. Unlabeled enclosures are not part of this select experiment. White arrow represents the elapse of time, with gray boxes indicating zooplankton sampling days and respective dates. The two side-by-side photos are used to depict all the shoreline enclosures used in the experiment with rock and cobble substrates.



84

85 **Figure S2.** (A) Total Polycyclic Aromatic Compounds (PAC) and (B) total 4-ring PAC aqueous
86 concentration in experimental enclosures over time. Treatment groups consisted of reference
87 (REF; *n* =1), enhanced monitored natural recovery (eMNR; *n* = 3), and shoreline cleaner
88 application (SCA; *n* = 3). Time points consisted of 4 days pre-spill and 3-, 6-, and 20-days post-
89 spill. Measured concentrations of total PAC and total 4-ring PAC are in ng/L. Plotted is average
90 and standard deviation.



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92 Figure S3: Workflow chart for zooplankton metabarcoding and bioinformatics applied.

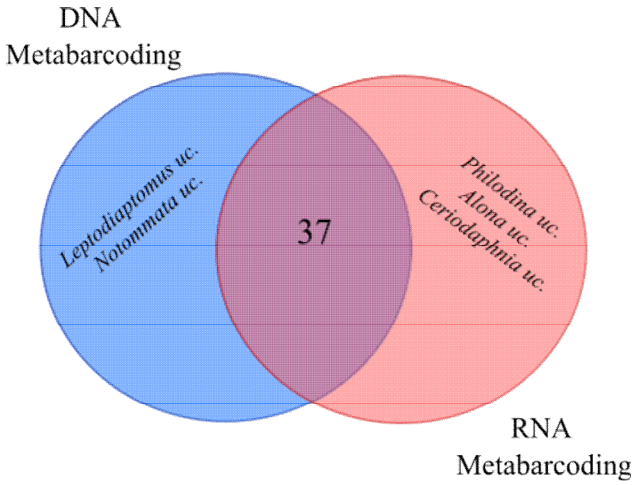


Figure S4. Venn diagram of taxa detected using DNA and RNA metabarcoding. DNA metabarcoding is shown in blue, with two unique taxa, and RNA metabarcoding is shown in red with three unique taxa. 37 taxa were shared between the two methods.

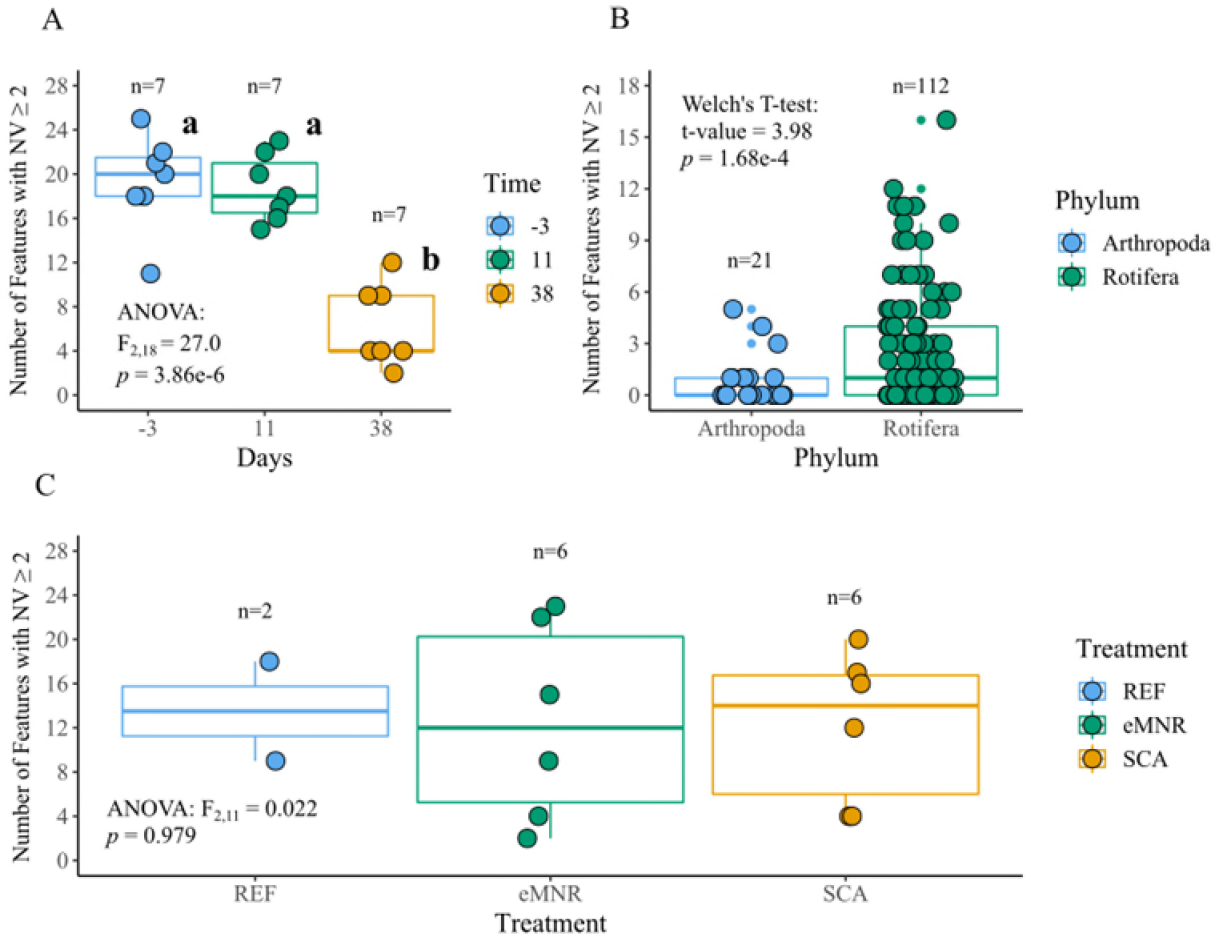
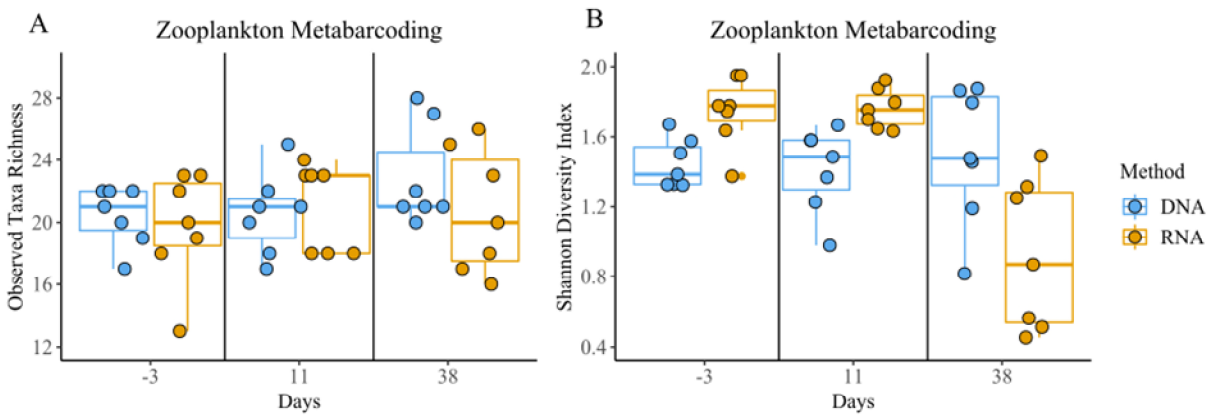


Figure S5. (A) Number of features (e.g., ZOTUs) with normalized vitality (NV) values ≥ 2 per sampling day, with sampling days consisting of 3 days pre-spill and 11- and 38-days post-spill. ANOVA was used to test for differences between time points. Letters denote significant differences inferred from Tukey HSD; (B) Number of features with NV values ≥ 2 per feature for phylum's Rotifera or Arthropoda. Significant differences were inferred by use of Welch's Two Sample T-test; (C) Number of features with NV values ≥ 2 for treatments including 11- and 38-days post-spill. Treatment groups consisted of reference (REF; *n* =1), enhanced monitored natural recovery (eMNR; *n* = 3), and shoreline cleaner application (SCA; *n* = 3). ANOVA was used to test for differences between treatments.



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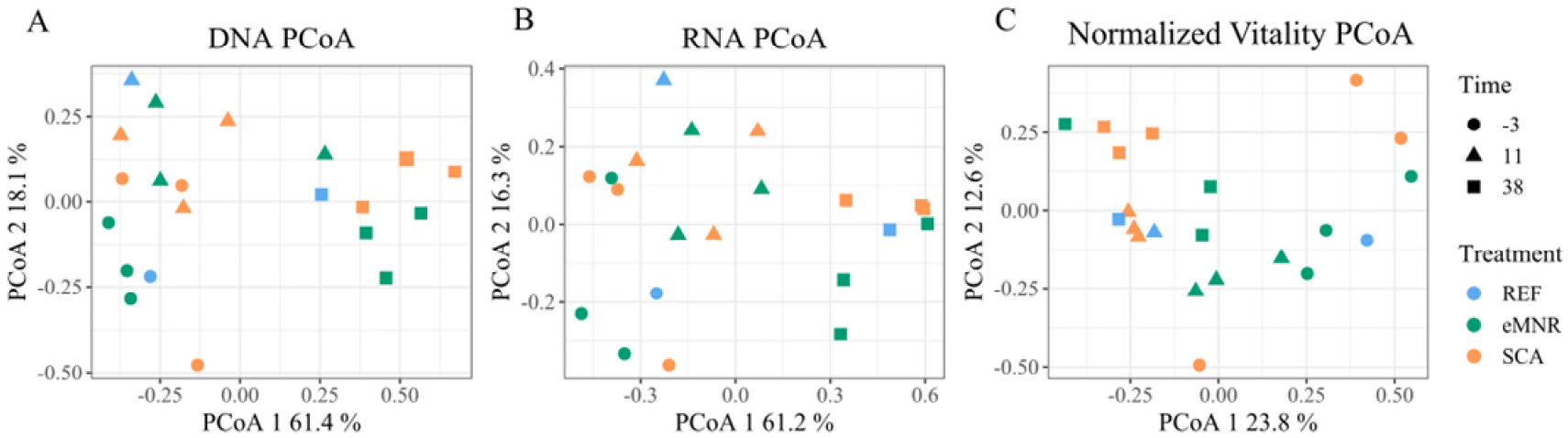
108 **Figure S6.** Boxplots of α-diversity indices (A) observed taxa richness and (B) Shannon diversity

109 index for the two metabarcoding methods, DNA and RNA metabarcoding. See Appendix 2 for

110 details on significant differences between treatments for each respective α-diversity index.



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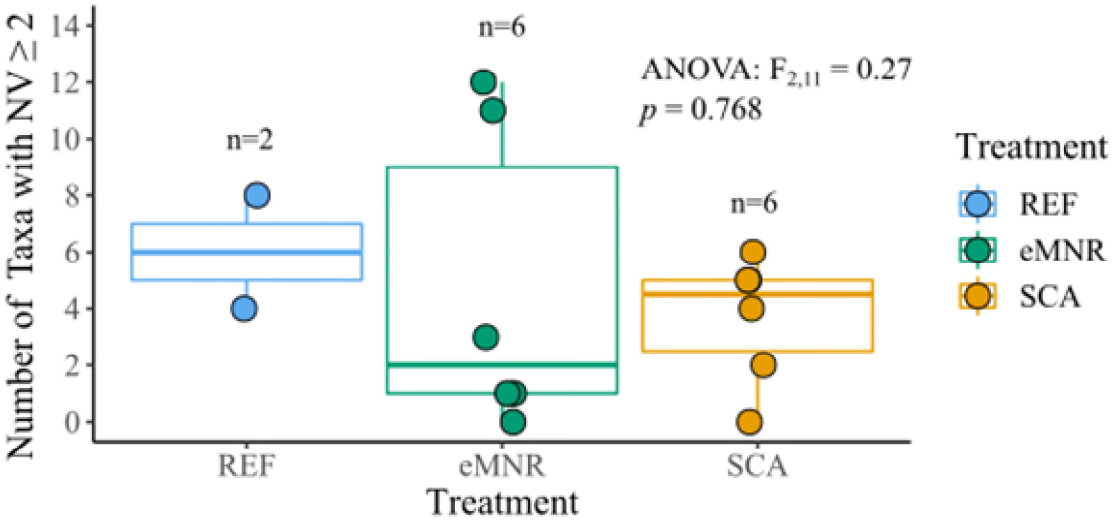
112 **Figure S7.** PCoA of Hellinger distance for (A) DNA metabarcoding, (B) RNA metabarcoding and (C) normalized vitality. Treatment

113 groups consisted of enhanced monitored natural recovery (eMNR; *n* = 3), shoreline cleaner application (SCA; *n* = 3), and reference

114 (REF; *n* =1). Time points consisted of 3 days pre-spill and 11- and 38-days post-spill.



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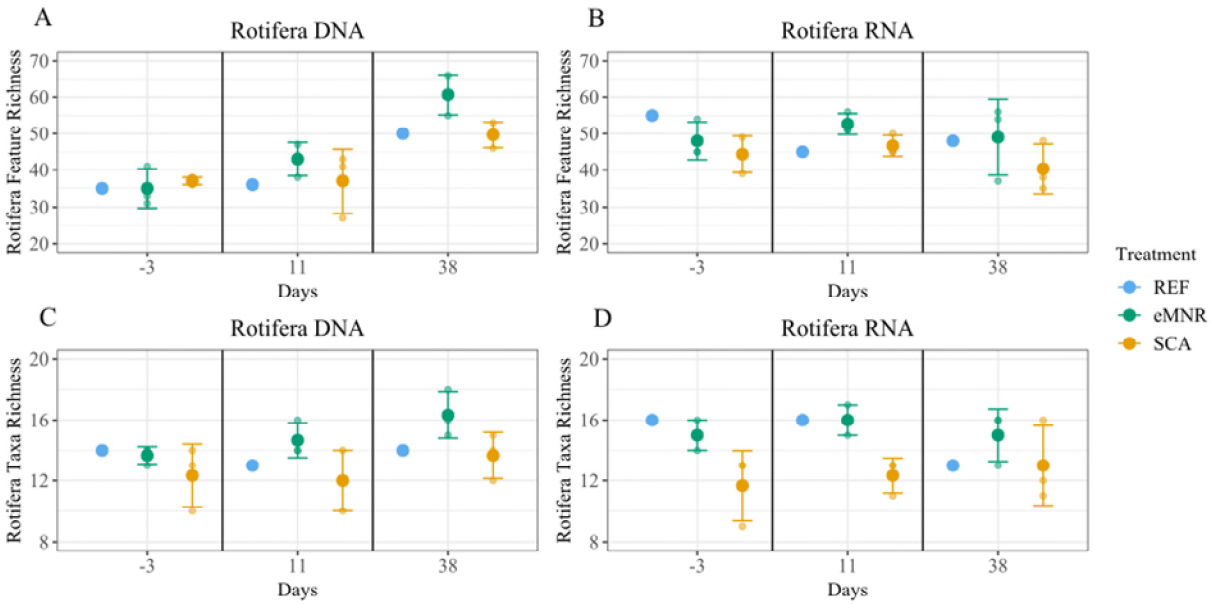
116 **Figure S8.** Number of taxa with normalized vitality (NV) values ≥ 2 for treatments including 11-

117 and 38-days post-spill. Treatment groups consisted of reference (REF; *n* =1), enhanced

118 monitored natural recovery (eMNR; *n* = 3), and shoreline cleaner application (SCA; *n* = 3). No

119 significant difference was determined between the factors by use of ANOVA.



120

121 **Figure S9.** Observed richness for (A, B) features and (C, D) taxa assigned to phyla Rotifera for

122 DNA and RNA metabarcoding. Treatment groups consisted of reference (REF; *n* =1), enhanced

123 monitored natural recovery (eMNR; *n* = 3), and shoreline cleaner application (SCA; *n* = 3). Time

124 points consisted of 3 days pre-spill and 11- and 38-days post-spill. Plotted is average and

125 standard deviation.

Table S1: Experimental shoreline enclosure locations within Lake 260.

Enclosure	Treatment	Latitude	GPS Latitude	Longitude	GPS Longitude
rEMNR1	eMNR	N	49.69256	W	93.76707
rEMNR2	eMNR	N	49.69556	W	93.76495
rEMNR3	eMNR	N	49.69579	W	93.76488
rR2	Reference	N	49.69571	W	93.76295
rSC1	SCA	N	49.69262	W	93.76698
rSC2	SCA	N	49.69563	W	93.76493
rSC3	SCA	N	49.69598	W	93.76492

Table S2. Total Polycyclic Aromatic Compounds (PAC) and total 4-ring PAC aqueous concentrations (ng/L) presented as average and standard error of the mean (SEM) per treatment. Treatment groups consist of enhanced monitored natural recovery (eMNR; $n = 3$), shoreline cleaner application (SCA; $n = 3$), and reference (REF; $n = 1$). Samples for PAC analysis were taken at 4 days pre-spill (e.g., -4), 3-, 6- and 20-days post-spill.

Total PAC Concentration (ng/L)				
Treatment	06-18-2019 (-4 Days)	06-25-2019 (3 Days)	06-28-2019 (6 Days)	07-12-2019 (20 Days)
REF	507.8	224.38	1147.0	619.9
eMNR	141.1 ± 55.12	276.5 ± 84.48	3778.1 ± 807.1	2321.6 ± 748.2
SCA	359.4 ± 153.1	419.9 ± 230.9	10916.5 ± 1438.8	2512.5 ± 694.5
Total 4-Ring PAC Concentration (ng/L)				
Treatment	06-18-2019 (-4 Days)	06-25-2019 (3 Days)	06-28-2019 (6 Days)	07-12-2019 (20 Days)
REF	62.25	20.54	711.4	444.9
eMNR	23.17 ± 4.41	6.78 ± 1.36	1436.5 ± 262.2	1127.4 ± 313.2
SCA	44.43 ± 15.45	14.59 ± 8.67	2667.3 ± 127.8	960.8 ± 187.1

Table S3. Nested adonis2 statistic and nested pairwise adonis2 statistic for treatment effects and eMNR vs SCA, respectively, while blocking the effects of time. Methods consisted of DNA metabarcoding, RNA metabarcoding, and normalized vitality.

	Treatment (Time as Block)					
Method	**DF**	**R^2**	**Pseudo-F**	**Pr(>F)**	**eMNR vs SCA (Pseudo-F)**	**eMNR vs SCA (Pr(>F))**
DNA	2,18	0.0466	0.440	0.372	0.463	0.323
RNA	2,18	0.0358	0.334	0.622	0.475	0.320
Normalized Vitality	2,18	0.106	1.06	0.0752	1.42	0.023

138 **Table S4.** Nutrient and water quality data presented as average per treatment and standard error of the mean (SEM). Samples for
139 nutrient and water quality were taken at 3 days pre-spill (e.g., -3), and 11- and 38 days post-spill. Sample collection took place prior to
140 afternoon (e.g., 8:00 AM – 12:00 PM). Treatment groups consist of enhanced monitored natural recovery (eMNR; $n = 3$), shoreline
141 cleaner application (SCA; $n = 3$), and reference (REF; $n = 1$).

Treatment	Time	Chl*a* (µg/L)	DIC (µM)	pH (SU)	TDN (µg/L)	TDP (µg/L)	TDP:DIC (µg/L)/(µM)	TDN:TDP
REF		2.13	174.8	6.79	258.1	4.2	0.0240	61.5
eMNR	06-19-2019 (-3 Days)	2.56 ± 0.377	182.4 ± 17.5	6.70 ± 0.0538	264.1 ± 4.99	4.6 ± 0.23	0.0174 ± 0.0009	57.7 ± 2.78
SCA		2.34 ± 0.131	175.8 ± 11.6	6.83 ± 0.0686	272.2 ± 7.09	5.10 ± 0.067	0.0291 ± 0.002	53.8 ± 2.08
REF		2.14	166.7	6.70	286.3	4.7	0.0282	60.9
eMNR	07-03-2019 (11 Days)	1.85 ± 0.258	164.6 ± 5.06	6.81 ± 0.0509	292.4 ± 3.54	5.1 ± 0.25	0.0310 ± 0.0008	57.6 ± 2.93
SCA		2.24 ± 0.198	162.3 ± 4.19	6.80 ± 0.0137	288.2 ± 6.84	4.4 ± 0.60	0.0270 ± 0.003	67.4 ± 6.92
REF		2.06	168.1	6.40	294	4.1	0.0244	71.7
eMNR	07-30-2019 (38 Days)	2.10 ± 0.541	182.2 ± 24.6	6.37 ± 0.0843	284.5 ± 12.1	3.6 ± 0.17	0.0203 ± 0.0027	79.9 ± 2.68
SCA		1.85 ± 0.0726	164.0 ± 3.71	6.41 ± 0.177	284.3 ± 14.6	3.7 ± 0.48	0.0227 ± 0.0025	78.1 ± 8.04

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Table S5. Nutrient data averaged over time with standard error of the mean (SEM). Samples for nutrient and water quality were taken at 3 days pre-spill (e.g., -3), and 11- and 38 days post-spill. Sample collection took place prior to afternoon (e.g., 8:00 AM – 12:00 PM).

	06-19-2019 (-3 days)	**07-03-2019 (11 days)**	**07-30-2019 (38 days)**
Chlorophyll *a* (µg/L)	2.41 ± 0.163	2.06 ± 0.145	1.99 ± 0.212
TDP:DIC (µg/L)/(µM)	0.0270 ± 0.00174	0.0288 ± 0.00139	0.0219 ± 0.00152
TDN:TDP	56.6 ± 1.71	62.3 ± 3.39	78.0 ± 3.38

Table S6. Temperature (°C) for treatments (average ± standard error of the mean (SEM)) over time. Treatment groups consist of enhanced monitored natural recovery (eMNR; $n = 3$), shoreline cleaner application (SCA; $n = 3$), and reference (REF; $n = 1$). Samples for temperature were taken at 4 days pre-spill (e.g., -4), and 13- and 38 days post-spill.

Treatment	06-18-2019 (-4 days)	07-05-2019 (13 days)	07-31-2019 (38 days)
REF	18.0	22.8	20.2
eMNR	18.5 ± 0.252	23.1 ± 0.115	21.0 ± 0.120
SCA	18.7 ± 0.296	22.9 ± 0.146	20.9 ± 0.203

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