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SPECIAL ISSUE ARTICLE

Assessing the degradation of environmental DNA and RNA based on genomic origin in a metabarcoding context

Kaushar Kagzi[1,2] | Katie L. Millette[1] | Joanne E. Littlefair[1,3] | Xavier Pochon[4,5] | Susanna A. Wood[4] | Gregor F. Fussmann[1] | Melania E. Cristescu[1]

[1]Department of Biology, McGill University, Québec, Montréal, Canada

[2]Institute of Environmental Sciences (CML), Leiden University, Leiden, The Netherlands

[3]Queen Mary University of London, London, UK

[4]Cawthron Institute, Nelson, New Zealand

[5]Institute of Marine Science, University of Auckland, Warkworth, New Zealand

Correspondence
Kaushar Kagzi, Institute of Environmental Sciences (CML), Leiden University, Leiden, The Netherlands.
Email: k.kagzi@cml.leidenuniv.nl

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Abstract

Molecular tools of species identification based on eNAs (environmental nucleic acids; environmental DNA [eDNA] and environmental RNA [eRNA]) have the potential to greatly transform biodiversity science. However, the ability of eNAs to obtain "real-time" biodiversity estimates may be complicated by the differential persistence and degradation dynamics of the molecular template (eDNA or eRNA) and the barcode marker used. Here, we collected water samples over a 28-day period to comparatively assess species detection using eDNA and eRNA metabarcoding of two distinct barcode markers—a mitochondrial mRNA marker (COI) and a nuclear rRNA marker (18S)—following complete removal of *Arthropoda* taxa in a semi-natural freshwater system. Our findings demonstrate that *Arthropoda* community composition was largely influenced by marker choice, rather than molecular template, individual microcosm, or sampling time point. Furthermore, although eRNA may capture similar species diversity as the established eDNA method, this finding may be marker-dependent. Although we found little to no difference in decay rates observed among sample groups (COI eDNA, COI eRNA, 18S eDNA, 18S eRNA), this result is likely due to limitations in the ability of eNA-based metabarcoding to provide a strong correlation between true eNA copy numbers present in the environment and final read counts obtained (following the metabarcoding workflow). Collectively, our findings provide further support for the use of multi-marker assessments in metabarcoding surveys to unravel the broadest taxonomic diversity possible, highlight the limitations of eNA metabarcoding methods in providing accurate decay rate estimates, as well as establish the need for further comparative studies using both metabarcoding and single-species detection methods to assess the persistence and degradation dynamics of eNAs for a diverse range of taxa.

KEYWORDS
aquatic ecosystems, biodiversity, degradation, environmental nucleic acids (eNAs), metabarcoding, molecular markers

1 | INTRODUCTION

Over the past decade, environmental DNA (eDNA) metabarcoding has emerged as a powerful tool for characterizing biological communities in aquatic ecosystems (Hajibabaei, 2012; Taberlet et al., 2012), with eDNA-based surveys often outperforming traditional methods in biodiversity assessment (Czeglédi et al., 2021; Fediajevaite et al., 2021; Hallam et al., 2021). However, despite several advantages of using eDNA for biomonitoring applications (e.g., reduced cost, non-invasiveness, increased efficiency in detecting rare and/or invasive species, etc.; Evans et al., 2017; Fukumoto et al., 2015; Harper et al., 2019), its potential to persist in the environment long term (Barnes et al., 2014; Dejean et al., 2011; Kagzi et al., 2022) as well as undergo transport downstream and in the water column (Deiner & Altermatt, 2014; Shogren et al., 2017) may result in reduced spatio-temporal acuity in biodiversity estimates. Recently, environmental RNA (eRNA) has been proposed as an alternative biomonitoring tool as it is thought to exhibit rapid decay (Cristescu, 2019; Jo et al., 2022; Kagzi et al., 2022)—owing in part to the single-stranded structure of the RNA molecule (Eigner et al., 1961), susceptibility to spontaneous cleavage of its phosphodiester bond (Fabre et al., 2014; Li & Breaker, 1999; Oivanen et al., 1998), and ubiquity of exogenous and endogenous RNases (Tan & Yiap, 2009). Environmental RNA has thus the potential to enhance discrimination between species detections originating from contemporary (locally present and living/recently alive organisms) versus residual (dead, non-local, or transient organisms) sources (Feist & Lance, 2021).

In recent years, a growing interest in the ability of eNAs (environmental nucleic acids; eDNA and eRNA) to complement—or even replace—traditional biomonitoring approaches has resulted in several studies comparatively investigating the efficacy of each survey methodology. For example, a few studies have demonstrated that eDNA/eRNA metabarcoding performed equally well (Keeley et al., 2018; Lejzerowicz et al., 2015) or better than traditional biomonitoring approaches (Laroche et al., 2018) when monitoring biological impacts associated with fish farm activities and offshore oil and gas production and drilling operations, respectively. When comparing eDNA with eRNA metabarcoding, a few studies indicated that the detection sensitivity of eRNA is equivalent to that of eDNA (Adamo et al., 2020; Littlefair et al., 2022; Miyata et al., 2021), with the "true" positive predictivity of eRNA superior to that of eDNA (Littlefair et al., 2022; Miyata et al., 2021). Recent studies have also suggested that in some contexts, eRNA may even outperform eDNA metabarcoding results. For example, Greco et al. (2022) showed that eRNA-based metabarcoding was better able to assess the impact of heavy metal pollution on foraminiferal diversity. In addition, a growing number of studies have advocated for the use of both eDNA and eRNA in parallel (i.e., using "shared" OTUs between datasets) to control for potential sequence artifacts (Greco et al., 2022), eliminate spurious taxa and better reflect true OTU abundance in different communities (Adamo et al., 2020), as well as better distinguish living biotic assemblages from "legacy" signals originating from dead/absent organisms (Pochon et al., 2017). However, prior to the widespread adoption of eRNA metabarcoding as an accurate, reliable quantitative tool for inferring biodiversity patterns with high spatio-temporal acuity in an aquatic system, it is necessary to comparatively assess its persistence and degradation dynamics relative to eDNA metabarcoding.

Currently, degradation estimates for eDNA and eRNA in aquatic systems vary considerably. For example, the first study by Wood et al. (2020) to comparatively investigate the degradation of eDNA and eRNA (collected from marine invertebrates *Sabella spallanzanii* [Gmelin, 1791] and *Styela clava* [Herdman, 1881]) found no significant differences between estimated eDNA/eRNA decay rates. In contrast, Marshall et al. (2021), who explored variation in decay rates of eDNA and eRNA (collected from non-indigenous dreissenid mussels *Dreissena polymorpha* [Pallas, 1771] and *D. rostriformis bugensis* [Andrusov, 1897]) based on genomic origin found evidence of measurable differences in the decay rates of eDNA and eRNA over three investigated gene regions (mitochondrial 16S and COI, and nuclear 18S). Similarly, both Kagzi et al. (2022) and Jo et al. (2022) found that eRNA decayed more rapidly than eDNA (collected from zooplankton *Daphnia pulex* [Leydig, 1860] and zebrafish *Danio rerio* [Hamilton, 1822], respectively) across a broad range of pH conditions. In comparison, Qian et al. (2022) investigated the decay of eDNA and eRNA (collected from the Chinese prawn *Fenneropenaeus chinensis* [Osbeck, 1765]) in response to changes in water temperature and found that while the degradation of eDNA increased with higher temperatures, the degradation of eRNA changed only slightly; thus, the authors postulated that as water temperature rises, the advantage of using eRNA will diminish as its degradation rate becomes similar to that of eDNA. It is important to note that the aforementioned studies all used single-species quantification methods (i.e., quantitative PCR [qPCR] or droplet digital PCR [ddPCR]) to estimate decay rate patterns for various individual taxa; in contrast, studies that comparatively investigate degradation rates of eDNA and eRNA using metabarcoding methodologies in complex organismal communities remain scarce.

The importance of selecting an appropriate marker for eDNA and eRNA surveys further affects the efficacy of metabarcoding as a reliable survey approach. Indeed, marker selection has been shown to be important in determining ecological community composition (Farrell et al., 2021; Tang et al., 2012); for example, for eDNA, genes of mitochondrial origin (e.g., the *cytochrome c oxidase* subunit I [COI] gene) are often used for targeted species detection as they are expected to possess higher stability (due to their circular structure) and higher density within the cell/environment in comparison with genes of nuclear origin (Bylemans et al., 2018; Foran, 2006). However, some evidence suggests that repetitive ribosomal nuclear genes (e.g., the small subunit 18S) may be present in higher abundance than genes of mitochondrial origin (Marshall et al., 2021; Moushomi et al., 2019), suggesting that nuclear DNA may, in some instances, promote the detectability of target taxa. Furthermore, within eRNA, differences in RNA types (ribosomal [r]RNA or messenger [m]RNA) may also act to influence degradation and detection success (Marshall et al., 2021). For example,

rRNA is thought to be more stable than mRNA due to a combination of factors including molecular length, secondary structure and folding, protein association, copy number (with rRNA comprising >80% to 90% of a total RNA sample), and location within a cell or organelle (Deutscher, 2006; Fontaine & Guillot, 2003; O'Neil et al., 2013). Indeed, knowledge of the degradation of eDNA and eRNA in the context of genomic origin and RNA types is crucial for accurate interpretation of environmental genetic signals detected in a metabarcoding context.

To verify the utility and efficacy of eRNA metabarcoding in characterizing contemporary, complex communities with strong spatio-temporal acuity, it is necessary to comparatively assess eNA metabarcoding results; specifically, the persistence and degradation dynamics of eDNA and eRNA. Here, we use a comparative metabarcoding approach based on two distinct barcode markers—a mitochondrial mRNA marker (COI) and a nuclear rRNA marker (18S) targeting both eDNA and eRNA—to assess species detection following the complete removal of *Arthropoda* taxa over several intervals across a 28-day period. We selected freshwater *Arthropoda* as our target phylum as it encompasses a broad range of trophic/functional groups (e.g., herbivores, filterers, detritivores, etc.) that play an important role in the cycling of energy and nutrients, with their populations often serving as indicators for the health and status of freshwater ecosystems (Thorp & Rogers, 2011). We predict that (1) eDNA and eRNA species detections will differ based on genomic origin and molecular template, (2) eDNA species detections will persist longer than eRNA over time, and (3) within eRNA, species detections with the rRNA-based marker (18S) will outlast that of the mRNA-based marker (COI).

2 | METHODS

2.1 | Experimental design and sample collection

The experiment was conducted at McGill University's Large Experimental Array of Ponds (LEAP) located at the Gault Nature Reserve in Mont St-Hilaire, Québec (Canada). LEAP is comprised of outdoor circular mesocosms (~1000 L Rubbermaid® tanks), each filled with water from a common reservoir fed by the upstream mesotrophic Lake Hertel; thus, all mesocosms contain natural and complex microbial, phytoplankton, and zooplankton communities (Bell et al., 2019). Three mesocosms were filled on May 23–24, 2017, and subsequently received a nutrient spike in the same molar ratio of the source lake (40 mg of phosphorus [KH_2PO_4 and K_2HPO_4] and 600 mg of nitrogen [KNO_3]) on May 31, 2017, to facilitate primary production. The mesocosms were monitored and maintained uncovered for ~15 consecutive weeks.

On September 11, 2017, a 7-L water sample was collected from each mesocosm and subsequently siphoned through a 50-μm Nitex mesh into three sterile carboys (~10 L each) to remove all freshwater *Arthropoda* present in the sample and effectively cease eNA production/input from these organisms. Carboys were stored (covered; with day/night light cycling conditions) in an indoor laboratory space at room temperature (~24°C) throughout the duration of the experiment. Samples of eDNA and eRNA were carefully collected via siphoning from the center of each carboy (henceforth referred to as microcosms) at seven distinct time points: < 1, 2, 24, 48, 96 h (4 days), 168 h (7 days), and 672 h (28 days) after siphoning. For both eDNA and eRNA, 400 mL of sample water was filtered twice (total 800 mL per microcosm) through 0.7 μm glass micro-fiber filters (47 mm diameter) over ceramic Büchner funnels on a stainless-steel vacuum filtration manifold. A sample volume of 400 mL was selected to optimize the trade-off between maximizing filtration volume and minimizing filtration time (affected by heavy fouling of mesocosms, thus resulting in the rapid clogging of filters). Each filter was cut in half using sterile scissors, folded with sterile forceps, and preserved in a 1.7-mL Eppendorf tube containing either 375 μL of ATL buffer (for eDNA extraction) or 375 μL of RLT buffer with 1% β-mercaptoethanol (for eRNA extraction) (Qiagen) (Kagzi et al., 2022). Samples were frozen immediately at −80°C until extraction.

Filtration equipment was sterilized between samples via soaking of all instruments in a 10% bleach solution (~20 min), followed by a thorough soak and rinse using distilled water. Prior to each filtration, ~100 mL of sample water was passed through the filtration apparatus (without filter) to rinse and prime the equipment for the respective sample. Negative controls consisting of 400 mL of Milli-Q water were filtered at each sampling time point. All filtrations were conducted in a dedicated wet laboratory space, separate from molecular protocols.

2.2 | Extraction of environmental DNA and RNA

All eDNA and eRNA extractions were conducted using DNEasy and RNeasy kits (Qiagen) based on the manufacturer's protocol with the following modifications: eDNA samples containing filter halves were thawed and 20 μL of proteinase-K was added to each sample tube; samples were then incubated at 56°C for 24 h and vortexed thoroughly (3×) during incubation. Environmental RNA samples containing filter halves were thawed and vortexed thoroughly upon buffer liquefication. Both eDNA and eRNA samples were then centrifuged at 3000*g* for 3 min at room temperature prior to transfer into new 1.7-mL sterile Eppendorf tubes for extraction. Samples were eluted from spin columns via two washes of 30 μL each (60 μL total). Negative controls were included in each round of extractions.

Digestion of DNA present in eRNA samples was conducted using the DNA-Eraser Genomic DNA Removal kit (iNtRON Biotechnology) according to manufacturer's instructions using the following sample and reagent volumes: 8 μL of eRNA, 2 μL Reaction Buffer, and 2 μL of Reaction Stopper followed by a 10-min incubation period at room temperature. Complete DNA digestion was verified with failed PCR amplification (using the same primers used for sample amplification) of the post-digestion eRNA product, as RNA will not amplify prior to reverse transcription. Reverse transcription to complementary DNA (cDNA) was conducted with the High-Capacity

cDNA Reverse Transcription Kit (Applied Biosystems). The reaction contained 2.0 μL RT buffer, 0.8 μL dNTP mix, 2.0 μL random primers, 1.0 μL MultiScribe Reverse Transcriptase, 4.2 μL nuclease-free water, and 10 μL eRNA. Cycling conditions were as follows: 10 min at 25°C, 120 min at 37°C, and 5 min at 85°C. Negative controls for DNA digestion and reverse transcription were included on each plate. To prevent contamination, molecular workspaces and equipment were cleaned thoroughly with a 10% bleach solution and RNase WiPER™ (iNtRON Biotechnology) prior to use, and sterile filter pipette tips were used for all pre-amplification steps.

2.3 | Metabarcoding library preparation

Environmental DNA and RNA metabarcoding was performed using two common barcode markers for zooplankton identification: the *cytochrome c oxidase* subunit I fragment, hereafter COI (mlCOIintF and HCO2198; Folmer et al., 1994; Leray et al., 2013), and the V4 region of the nuclear 18S rRNA gene, hereafter 18S (Uni18S and Uni18SR; Zhan et al., 2013). Environmental DNA and cDNA (from eRNA) samples were PCR amplified at the COI and 18S barcode markers with Illumina-adapted primers using separate optimized PCR protocols, as eDNA and cDNA did not amplify with the same PCR reactions. The eDNA reactions for both COI and 18S amplification contained the following reagent and sample volumes: 8.0 μL nuclease-free water (Qiagen), 1.25 μL 10× Taq Buffer (GenScript), 0.5 μL 25 mM $MgCl_2$ (Thermo Fisher Scientific.), 0.125 μL 10 mM dNTP Mix (GeneDireX, Inc.), 0.2 μL each of forward and reverse primers (10 mM), 0.05 μL BSA (Thermo Fisher Scientific), 1 unit of Taq DNA Polymerase (GenScript), and 2 μL of template DNA, for a total of 12.6 μL. The eRNA reactions for both COI and 18S contained the following reagent and sample volumes: 6.25 μL MyFi master mix (BioLine), 3.25 μL nuclease-free water, 0.5 μL of each of the forward and reverse primers (10 mM), and 2 μL of cDNA, for a total of 12.5 μL. The thermocycler regime for 18S amplification of eDNA and cDNA consisted of an initial denaturing step at 95°C for 5 min, followed by 25 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 90 s, and an elongation step at 72°C for 10 min (Zhan et al., 2013). The thermocycler regime for COI amplification of both eDNA and cDNA consisted of touchdown PCR (to reduce the probability of non-specific amplifications) with 16 initial cycles of denaturation at 95°C for 10 s, annealing at 62°C for 30 s (−1°C per cycle) and extension at 72°C for 60 s, followed by 25 cycles with a 46°C annealing temperature (Leray et al., 2013). Fragment size and amplification were verified on a 1% agarose gel. Negative controls consisting of Milli-Q water in lieu of template DNA/RNA were included on each plate.

Amplified products of technical replicates were pooled and cleaned with a 0.875 ratio of AMPure XP beads (Beckman Coulter) to PCR product. The cleaned product was suspended in 30 μL of DNAse-free ultra-sterile water and used as template in the 25 μL indexing reaction. Samples were indexed using 12.5 μL of 2× KAPA HiFi HotStart ReadyMix, 0.1 μM of each Nextera XT index primer (Illumina), and 2.5 μL of template. The indexing thermal cycling regime consisted of denaturation at 95°C for 3 min, followed by 8 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 30 s, and a final extension at 72°C for 5 mins. Indexed PCR product was purified using AMPure XP beads as previously described.

Amplicon libraries were normalized and assembled by pooling equal volumes (22 μL) of uniquely-indexed samples from the three experimental microcosms and seven time points for each marker/molecular template combination (i.e., COI eDNA, 18S eDNA; COI eRNA, 18S eRNA; 84 libraries). Environmental DNA and eRNA samples from negative controls were included for each marker (4 libraries). The concentration, size, and quality of the 88 libraries were verified prior to sequencing. Paired-end (300 bp) sequencing was performed in a single run on the Illumina MiSeq platform by Génome Québec (Montréal).

2.4 | Bioinformatic analyses

Raw paired-end reads were processed separately for COI and 18S using the YAAP metabarcoding pipeline available at http://www.github.com/CristescuLab/YAAP. Briefly, adapters were removed using paired-end trimming in cutadapt v1.9.1 (Martin, 2011) with a quality threshold of 25 and minimum read length of 130 bases. Trimmed sequences were merged in PEAR v0.9.11 (Zhang et al., 2014) using the assembly score method. Due to the minimal overlap between 18S reads, forward and reverse sequences were analyzed separately. Any remaining gene-specific primers were removed using SeqKit v0.10.0 (Shen et al., 2016). Resulting base quality scores were examined with FastQC v0.11.0 (Andrews, 2015) and filtered by sequence length ± 20 bp around the target amplicon size for the COI fragment (293–333 bp) and the 18S fragment (380–420 bp). Sequences <200 bp in length were removed. All sequences were dereplicated using vsearch v2.11.0 (Rognes et al., 2016). We used a denoised zero-radius Operational Taxonomic Unit (ZOTU, sometimes referred to as Amplicon Sequencing Variants [ASV]) approach to analyze sequences, as analyses based on ZOTUs remove arbitrary thresholds in species delimitation which can be a problem with other clustering approaches. In addition, denoising corrects for errors resulting from next-generation sequencing platforms. Separate ZOTU databases for COI and 18S sequences were generated using the unoise3 command in USEARCH v10.0.240 (Edgar, 2016) after removing all ZOTUs which had fewer than four copies in the entire database. The final resulting eDNA and eRNA ZOTU-by-sample matrices for COI and 18S were used for downstream analyses.

Sequence taxonomy was assigned using BLASTn (Camacho et al., 2009) on the NCBI nucleotide database (downloaded on August 12, 2018), retaining the top 20 hits with >90% identity, and *e*-value <0.001. Hits with unknown taxonomy (e.g., "environmental" or "uncultured" sample) were removed. Sequences were assigned to last common ancestor using the algorithm implemented in BASTA (Kahlke & Ralph, 2019) with 95% identity, *e*-value $<1e^{-10}$, >90% sequence alignment query coverage, and minimum number of sequences set to 1.

2.5 | Statistical analyses

All statistical analyses were conducted in the R version 4.0.1 statistical computing environment (R Core Team, 2020). Henceforth, all analyses will refer to either total (i.e., taxonomically assigned; richness estimate) or *Arthropoda* (classified at the family level) ZOTUs. *Arthropoda* were analyzed separately from the full dataset, as they were the target taxa filtered out of mesocosms for the degradation experiment. For all *Arthropoda* analyses, any taxa that did not possess at least one aquatic life stage were removed (for COI, *Aphididae*, *Cecidomyiidae*, *Leiodidae*, *Noctuidae*, *Sciaridae*; for 18S, *Linyphiidae*), along with any taxa occurring in non-freshwater environments (for COI, *Chthamalidae*).

To determine whether there was a significant interaction between molecular template (eDNA and eRNA) and sampling time point on the number of ZOTUs detected (i.e., richness; performed separately for total versus *Arthropoda*-only ZOTUs) within microcosms for both barcode markers (COI and 18S), a two-way repeated measures ANOVA was performed using the R package "Rstatix" (Kassambara, 2021). Furthermore, to test which groups differed significantly, pairwise comparisons between molecular template at each time point (unpaired *t*-test), as well as pairwise comparisons between sampling time points for each molecular template (paired *t*-test) were performed. For each pairwise comparison, *p*-values were adjusted using the Benjamini-Hochberg multiple testing correction method.

The degradation rate of eDNA and eRNA was estimated for each *Arthropoda* family detected in microcosms for each barcode marker and molecular template (i.e., COI eDNA, COI eRNA, 18S eDNA, 18S eRNA) using the exponential decay model $N(t)=N_0 e^{-rt}$, where $N(t)$ is the copy number of eDNA or eRNA at time t, N_0 is the initial copy number of eDNA or eRNA (taken immediately after organismal removal), and r is the decay rate constant. Here, ZOTU read counts (i.e., copy numbers) were summed on a per microcosm basis for each *Arthropoda* family and sample group (COI eDNA, COI eRNA, 18S eDNA, 18S eRNA) for model input. When calculating decay rate constants, latter time points ($t=168$ h and/or $t=672$ h) were omitted in instances where unusually high read numbers were detected (possibly due to disturbance of biofilm at the bottom of microcosms during sampling, and resuspension of eDNA and eRNA; see Table S1), along with any *Arthropoda* families exhibiting low detection (i.e., total of ≤30 reads detected in a given microcosm within the first two time points) or lack of decay in read numbers throughout the duration of the experiment, thus indicating failure of removal via the filtration mesh. Lack of decay in read numbers was designated based on expected decay patterns previously reported in the literature (Kagzi et al., 2022; Marshall et al., 2021; Qian et al., 2022; Wood et al., 2020). Exponential decay models with optimized starting values were fit to raw count data using R packages "easynls" and "minpack.lm" (Arnhold, 2017; Elzhov et al., 2016; Kaps & Lamberson, 2017). Mean model-derived decay rate constants generated for *Arthropoda* eDNA or eRNA at the COI or 18S barcode marker were used to estimate the time taken (in hours) for eDNA or eRNA to degrade below detection limits (limit of detection = 0.1 copies remaining) (Strickler et al., 2015; Wood et al., 2020). Differences in decay rate constants between sample groups (COI eDNA, 18S eDNA, 18S eRNA) were estimated using a linear mixed-effects model generated using the "lme4" package implemented in R (Bates et al., 2015); the "COI eRNA" group was excluded from the model due to insufficient sample size ($n=1$ decay curve). The model included $\log_e$-transformed copies of eDNA and eRNA per *Arthropoda* family in each microcosm as the response variable, sample group (COI eDNA, 18S eDNA, or 18S eRNA), sampling time (hours) and the two-way interaction between sample group and sampling time as fixed effects, and *Arthropoda* families nested within replicate microcosms as random effects. Differences in decay rate constants between genes were determined via a post hoc Tukey test using the R package "emmeans" (Lenth et al., 2022) (Appendix S1).

To visualize changes in *Arthropoda* community composition detected by COI and 18S at each sampling time point and microcosm, ZOTU incidence (i.e., total number of ZOTUs detected), read count, and taxonomic classification were determined for all *Arthropoda* taxa at the family level. A principal coordinates analysis (PCoA) of Jaccard dissimilarity distances was conducted to assess differences in community composition based on (a) molecular template (eDNA or eRNA), (b) microcosm, (c) sampling time point, and (d) barcode marker (COI or 18S) using presence/absence data of *Arthropoda* families detected throughout the experiment. A dissimilarity matrix was generated using the function "vegdist" in the R package "vegan" (Oksanen et al., 2022), and eigenvalues for the PCoA analysis were computed using the function "pco" in the R package "ecodist" (Goslee & Urban, 2007). An Analysis of Similarities (ANOSIM) test using Jaccard dissimilarity distances and 9999 permutations was conducted to estimate differences between *Arthropoda* communities for each PCoA analysis using the function "anosim" in the R package "vegan" (Oksanen et al., 2022).

3 | RESULTS

3.1 | Sequencing

The Illumina MiSeq run resulted in 16,968,080 reads from the 88 eDNA- and eRNA-based libraries submitted. The 42 experimental eDNA-based libraries, representing three microcosms sampled at seven time points with two markers, contained 9,274,384 reads (5,263,014 reads from COI and 4,011,370 reads from 18S). The 42 experimental eRNA-based libraries contained 7,210,787 reads (3,763,376 reads from COI and 3,447,411 reads from 18S).

3.2 | Negative controls

Amplification was not detected by gel electrophoresis in the filtration, extraction, reverse transcription, or PCR blanks at any experimental time point. All blanks were pooled with molecular

template and marker, and sequenced; results indicated refer exclusively to taxonomically assigned ZOTUs. The pooled COI blanks resulted in 419 reads from eDNA and 56 reads from eRNA, while the pooled 18S blanks resulted in 3208 reads from eDNA and 39,369 reads from eRNA after bioinformatic processing. In the 18S eRNA pooled blanks, reads were primarily assigned (at the phylum level) as unknown reads (35.4%), followed by *Ciliophora* (26.6%), *Cercozoa* (10.3%), *Cnidaria* (8.2%), *Rotifera* (5.5%), *Chlorophyta* (4.1%), *Haptista* (2.5%), *Streptophyta* (2.0%), and *Arthropoda* (1.3%). Since complete removal of *Arthropoda* ZOTUs detected in blanks from the sample dataset would result in significant data loss, we elected to retain them for subsequent analyses.

3.3 | Total and *Arthropoda*-only zero-radius OTUs (ZOTUs)

The mean (± standard deviation) number of total taxonomically assigned ZOTUs detected by eDNA and eRNA using the 18S barcode marker at the first sampling time point ($t = <1$ h following organismal removal) were 303 ± 16.5 and 313 ± 26.6, respectively, while the COI marker detected 39 ± 6.56 and 22.3 ± 7.1 total ZOTUs for eDNA and eRNA, respectively. Of these, *Arthropoda* taxa accounted for 5 ± 4.36 ZOTUs for both eDNA and eRNA using the 18S barcode marker; using the COI marker, *Arthropoda* taxa accounted for 13.7 ± 2.52 and 2.33 ± 0.58 ZOTUs by eDNA and eRNA, respectively (Figure 1).

The two-way repeated measures ANOVA showed significant two-way interactions between molecular template (eDNA or eRNA) and sampling time point for total ZOTUs detected using the 18S barcode marker ($p = 0.01$), but not for total ZOTUs detected using COI (Figure 1). For total ZOTUs detected using the 18S barcode marker, the number of ZOTUs detected using eDNA and eRNA did not differ significantly at any time points except at $t = 48$ h ($p = 0.045$), where eRNA detected more ZOTUs than eDNA; further, when comparing the number of ZOTUs detected by eDNA and eRNA across time points, no significant differences were observed (Figure 1a, left panel). For total ZOTUs detected using the COI barcode marker, the number of ZOTUs detected by eDNA and eRNA differed significantly at the first five time points where eDNA detected more ZOTUs than eRNA ($p = 0.040$, 0.004, 0.017, 0.043, and 0.033, respectively), but not at $t = 168$ h or $t = 672$ h; further, the total number of ZOTUs detected by eDNA at $t = <1$ h first declined significantly at $t = 168$ h ($p = 0.04$), while the total number of ZOTUs detected using eRNA did not show significant differences across time points (Figure 1a, right panel).

Furthermore, significant two-way interactions were observed between molecular template (eDNA or eRNA) and sampling time point for *Arthropoda* ZOTUs detected using the COI barcode marker ($p = 0.005$), but not for *Arthropoda* ZOTUs detected using 18S (Figure 1). For *Arthropoda* detected using the 18S barcode marker, the number of ZOTUs detected by eDNA and eRNA at each time point did not differ significantly except at $t = 168$ h, where eDNA detected more ZOTUs than eRNA ($p = 0.026$); further, when comparing the number of ZOTUs detected by either eDNA or eRNA across time points, no significant differences were observed (Figure 1b, left panel). For *Arthropoda* detected using the COI barcode marker, eDNA detected more ZOTUs than eRNA at all time points, except for at $t = 48$ h (no significant difference observed) and $t = 672$ h (eRNA detects more ZOTUs than eDNA) (Figure 1b, right panel).

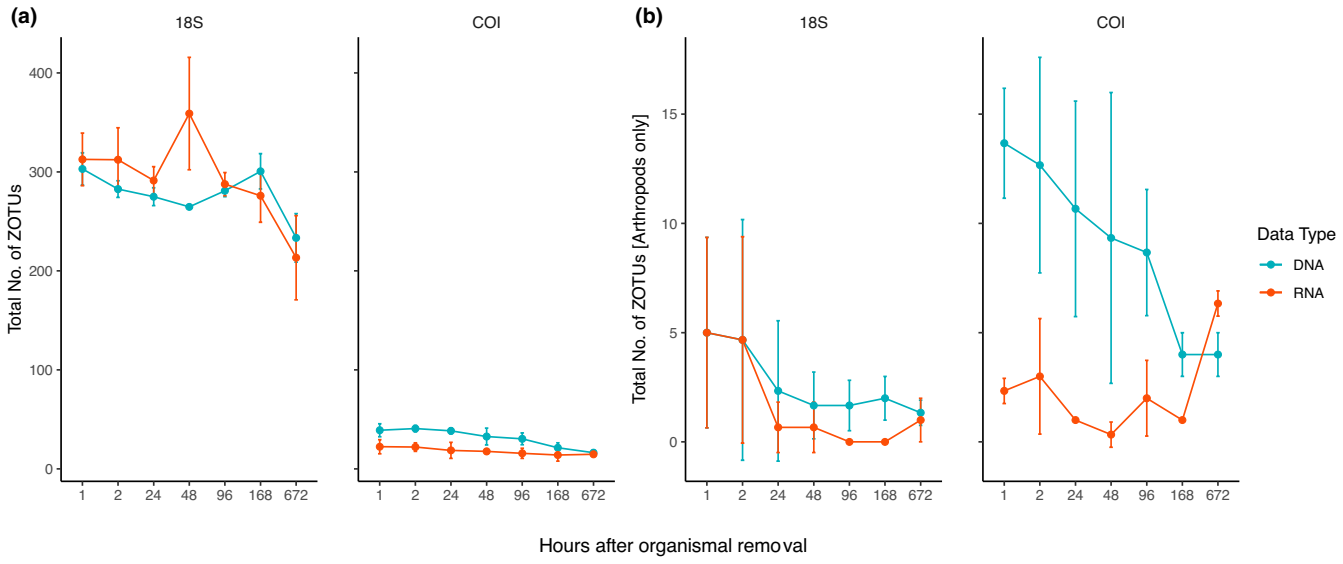

FIGURE 1 Total number (i.e., incidence) of (a) taxonomically assigned zero-radius operational taxonomic units (ZOTUs) and (b) *Arthropoda* ZOTUs (error bars: ±1 standard deviation) detected by environmental DNA (eDNA) and environmental RNA (eRNA) in three experimental microcosms using the V4 region of the nuclear 18S rRNA (18S) and *cytochrome c oxidase* subunit I (COI) barcode markers for each sampling time point. Note different y-axis scales.

3.4 | Community composition

At the family level, the COI barcode marker detected a total of 30 and 21 taxa using eDNA and eRNA, respectively (of which 15 and 8 were freshwater arthropods) (Figure 2; Table 1; Figure S1), while the 18S barcode marker detected 75 and 70 taxa using eDNA and eRNA, respectively (of which 3 were freshwater arthropods for both eDNA and eRNA; Figure 3; Table 1; Figure S2). Although the 18S marker detected fewer *Arthropoda* families overall for both eDNA and eRNA in comparison with the COI marker, eDNA and eRNA *Arthropoda* detections using 18S demonstrated higher frequency of overlap (i.e., detection of the same three families in the same microcosms) with one another over time than eDNA and eRNA detections using the COI marker (Table S1).

The PCoA conducted on presence/absence data of *Arthropoda* families showed that the primary axis explained 37.9% of total sample variance, while the secondary axis explained 21.2% (Figure 4). The ANOSIM test indicated differences between *Arthropoda* communities when grouped by molecular template (R statistic 0.1816; *p*-value 0.0001) and barcode marker (R statistic 0.5957; *p*-value 0.0001), but not microcosm (R statistic 0.0354; *p*-value 0.0734) or sampling time point (R statistic −0.0143; *p*-value 0.641). Within the degradation experiment, samples collected using both molecular templates (eDNA and eRNA) exhibited high overlap (with some differences) (Figure 4a), along with samples collected from all three microcosms (Figure 4b) and all time points (Figure 4c). There was little overlap between samples sequenced at the COI and 18S barcode markers, indicating that distinct community compositions were assigned by each marker (Figure 4d); thus, *Arthropoda* community composition was primarily influenced by marker choice (18S or COI), rather than by molecular template, sampling time point, or individual microcosm.

3.5 | Degradation of freshwater *Arthropoda* eDNA and eRNA

Using the 18S marker, an exponential decay model was fitted to 2 out of 3 *Arthropoda* families detected for both eDNA and eRNA (as families with a total of ≤30 reads detected were excluded from degradation estimates), with mean model-derived decay rate constants

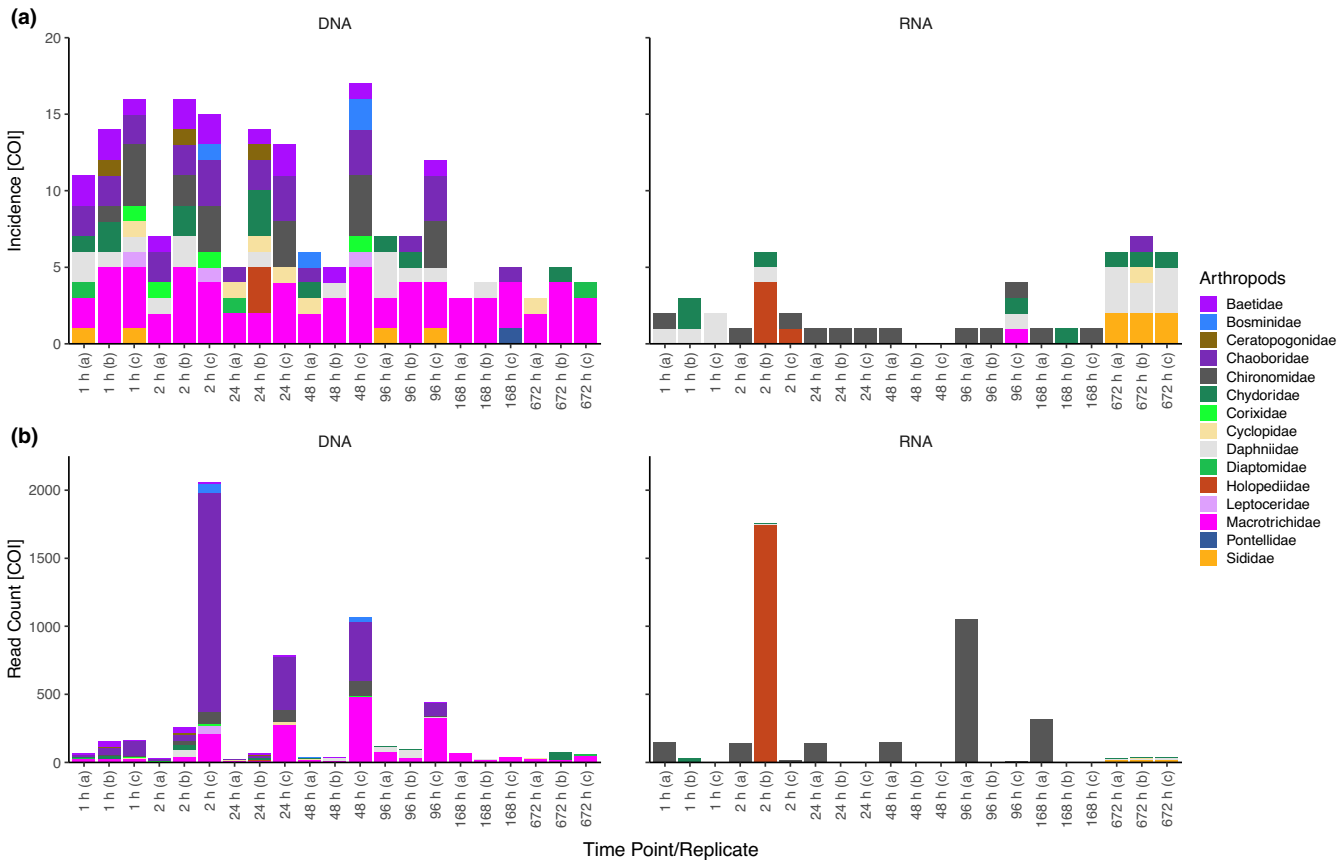

FIGURE 2 (a) Zero-radius operational taxonomic unit (ZOTU) incidence (i.e., total number of ZOTUs detected) and (b) read count (sum of all ZOTUs) of *Arthropoda* taxa detected by environmental DNA (eDNA) and environmental RNA (eRNA) at the family level using the *cytochrome c oxidase* subunit I (COI) barcode marker for each experimental time point and microcosm (designated on x-axis using lowercase a, b, or c).

TABLE 1 *Arthropoda* taxa detected across all experimental time points with the *cytochrome c oxidase* subunit I (COI) and V4 region of the nuclear 18S rRNA (18S) barcode markers using environmental DNA (eDNA) and environmental RNA (eRNA) metabarcoding following complete removal of *Arthropoda* organisms from microcosms. Taxa are sorted by Class, Order, and Family.

Class	COI		18S	
Order				
Family	eDNA	eRNA	eDNA	eRNA
Insecta				
Ephemeroptera				
Baetidae	+	−	+	+
Diptera				
Ceratopogonidae	+	−	−	−
Chaoboridae	+	+	−	−
Chironomidae	+	+	−	−
Hemiptera				
Corixidae	+	−	−	−
Trichoptera				
Leptoceridae	+	−	−	−
Branchiopoda				
Diplostraca				
Bosminidae	+	−	−	−
Chydoridae	+	+	+	+
Daphniidae	+	+	−	−
Holopediidae	+	+	−	−
Macrotrichidae	+	+	−	−
Sididae	+	+	−	−
Hexanauplia				
Cyclopoida				
Cyclopidae	+	+	+	+
Calanoida				
Diaptomidae	+	−	−	−
Pontellidae	+	−	−	−
TOTAL	15	8	3	3

ranging from −0.7359 h^{-1} to −0.0489 h^{-1} for eDNA, and −0.3336 h^{-1} to −0.0062 h^{-1} for eRNA. Using the COI marker, exponential decay curves were fitted to 6 out of 15 and 1 out of 8 *Arthropoda* families detected using eDNA and eRNA, respectively, with mean model-derived decay rate constants ranging from −0.0704 h^{-1} to −0.0022 h^{-1} for eDNA, and −0.0009 h^{-1} for eRNA (Figure 5; Table 2). The total number of reads detected per *Arthropoda* family for eRNA rapidly declined to zero within 24 to 96 h post-filtration for the 18S marker, and within up to 672 h for the COI marker. For eDNA using the 18S marker, while the total number of reads degraded to zero within 24 h in some instances, others exhibited non-zero detection for up to 672 h post-filtration. For eDNA using the COI marker, the total number of reads degraded to zero within 24 to up to 672 h post-filtration (Figures 2 and 3). The linear mixed-effects model revealed no significant differences in decay rates between sample groups (COI eDNA, 18S eDNA, 18S eRNA), with the exception of 18S eDNA and 18S eRNA ($p = 0.0229$), where *Arthropoda* eDNA decayed slightly more rapidly than eRNA using the 18S barcode marker (Tables S2–S4).

4 | DISCUSSION

As a growing number of studies advocate for the increased use of eRNA for various biomonitoring applications and ecosystem surveillance strategies (Cristescu, 2019; Yates et al., 2021), it is important to verify the utility and efficacy of eRNA-based surveys in providing users with real-time biodiversity estimates in comparison with eDNA. While such studies are important in advancing eNA methodologies, knowledge of eNA degradation rates across genomic origins in a metabarcoding context remains highly limited. Here, we present the first study, to the best of our knowledge, to comparatively assess the persistence and degradation of *Arthropoda* eDNA and eRNA using metabarcoding of two distinct barcode markers in a freshwater system.

4.1 | Degradation of eDNA and eRNA

As studies begin to comparatively estimate the degradation of eDNA and eRNA using single-species detection methods (Jo et al., 2022; Kagzi et al., 2022; Marshall et al., 2021; Qian et al., 2022; Wood et al., 2020), it is likewise important to verify whether eRNA decay patterns differ from those of eDNA in a metabarcoding context. In the present study, we found little to no difference between decay rate estimates among sample groups (18S eDNA, 18S eRNA, COI eDNA). While this contrasts with recent comparative studies assessing the decay rates of eDNA and eRNA (where eRNA was found to decay more rapidly than eDNA using single-species detection methods) (Jo et al., 2022; Kagzi et al., 2022; Marshall et al., 2021), our findings are similar to a previous study by Wood et al. (2020), who found no significant differences between the decay rates of eDNA and eRNA (quantified using ddPCR) collected from two marine organisms. The similarity in decay rates observed between eDNA and eRNA in our study—irrespective of marker choice—may be due to similarity in structure of both molecular markers, as they share target fragments of similar length (COI: 313 bp; 18S: ~400 bp) (Leray et al., 2013; Zhan et al., 2013). In addition, for the COI mRNA marker, its circular structure and location within the cell (i.e., packaged within the mitochondria) may reduce its susceptibility to degradation (Foran, 2006; Nielsen et al., 2007), while for the 18S rRNA marker, several factors including structural stability, location within ribosomes, and protection of rRNA via protein complexes may have shielded it from enzymatic degradation (Sidova et al., 2015).

It is important to note that the statistically insignificant result pertaining to the decay rates obtained in our study should be

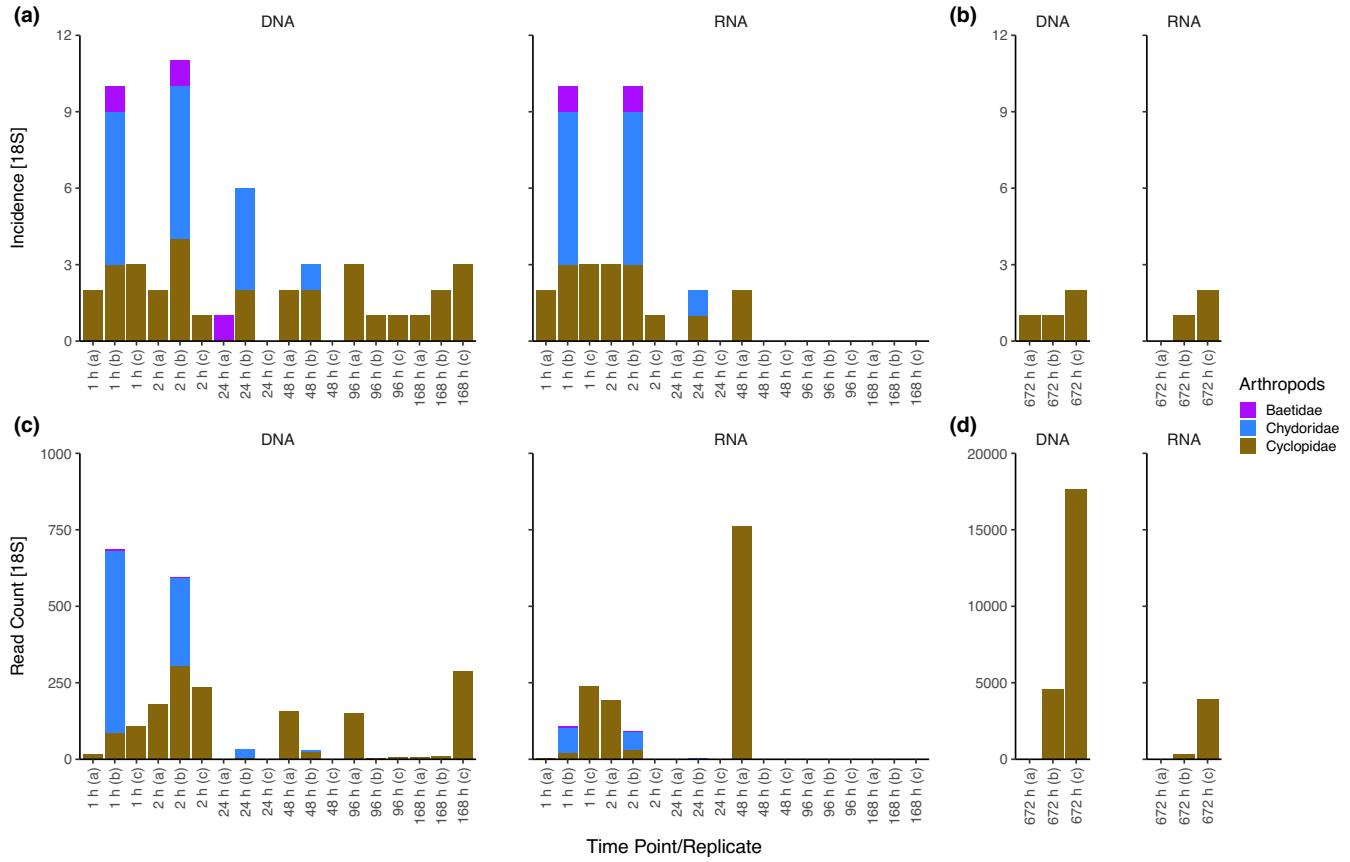

FIGURE 3 Zero-radius operational taxonomic unit (ZOTU) incidence (i.e., total number of ZOTUs; a, b) and read count (sum of all ZOTUs; c, d) of *Arthropoda* taxa detected by environmental DNA (eDNA) and environmental RNA (eRNA) at the family level using the V4 region of the nuclear 18S rRNA (18S) barcode marker for the first six sampling time points (a, c) and the last sampling time point (b, d). Microcosm replicates are designated on the x-axis using lowercase a, b, or c. Note different y-axis scales.

interpreted cautiously; for example, although not statistically significant, we found wide variability in the time taken to reach zero signal detectability among *Arthropoda* families both between and among sample groups. In particular, for eRNA we found that the total number of reads detected for *Arthropoda* families rapidly declined to zero within 96 h using the 18S marker, and within 672 h using the COI marker. This is similar to a previous study by Marshall et al. (2021), who found that the 18S nuclear rRNA gene had a faster decay rate than the COI mitochondrial mRNA gene; the authors suggested that this result may be due to the increased stability of the circular mitochondrial genome. For eDNA, however, we found wide variability in the time taken for total reads to degrade to zero for both markers; while some families exhibited rapid decay in <24 h, others were detected for up to 672 h post organismal removal. This is also in accordance with Marshall et al. (2021), who found that both nuclear and mitochondrial DNA genes had large overlap in decay constants. Previous studies have demonstrated that the concentration (and thus detectability) of eNAs in the environment is directly linked to the rate of eNA shedding (Sassoubre et al., 2016; Wood et al., 2020). Variability in production rates (i.e., shedding) may occur as a result of various factors, such as differences in species/organismal type, size, abundance, life stage, metabolic rate, and feeding activity (Goldberg et al., 2011; Sassoubre et al., 2016; Takahara et al., 2012; Thomsen et al., 2012; Wood et al., 2020). Accordingly, these differences in production rates (and thus initial eNA concentrations) may have contributed to the differences in persistence times (and thus decay rates) among *Arthropoda* families as observed in our study.

It is also important to consider that a stronger relationship between decay rates of eDNA and eRNA based on genomic origin will likely be observed using single-species detection methods that offer increased detection sensitivity (e.g., qPCR and/or ddPCR) (Jo et al., 2022; Kagzi et al., 2022; Marshall et al., 2021). Since several *Arthropoda* families were omitted from our decay curve analyses due to low copy numbers (thus diminishing statistical power due to lower sample size and replication), future studies employing species-specific primers targeting different genes may contrast from the findings herein. Indeed, metabarcoding has been shown to possess several limitations in comparison with qPCR/ddPCR, including losses in detection sensitivity due to PCR amplification bias (i.e., primer biases and taxa- or sample-specific variation in amplification efficiency), incomplete reference databases, and generation of sequencing artifacts (Brown et al., 2016; Feist & Lance, 2021; Valentini et al., 2016; von Ammon et al., 2019; Zinger et al., 2019). While some studies have reported a positive correlation between organismal

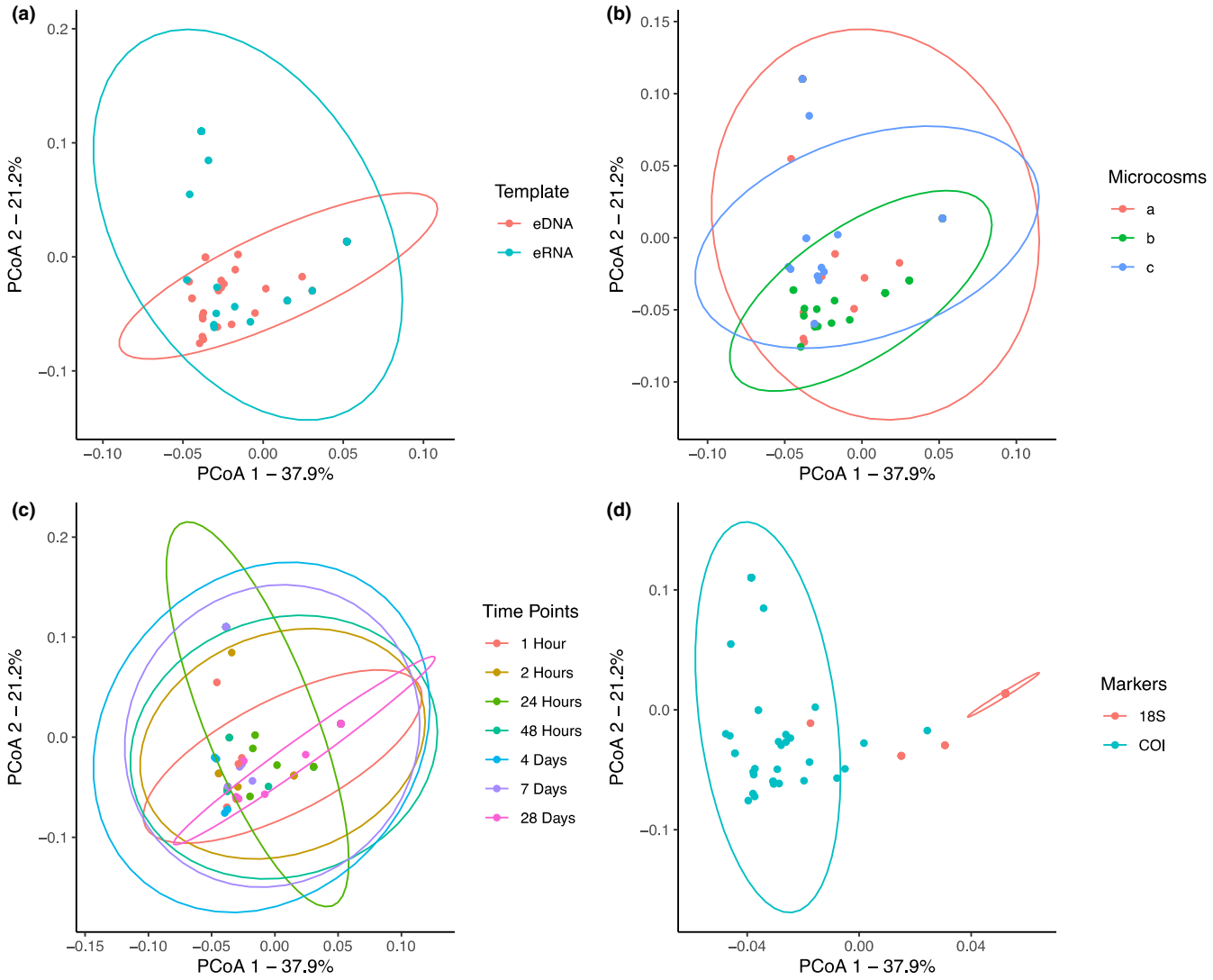

FIGURE 4 Principal coordinates analysis (PCoA) of Jaccard dissimilarity distances on presence/absence data of *Arthropoda* families detected throughout the experiment. Differences in community composition based on (a) molecular template, (b) microcosm, (c) sampling time point, and (d) marker are shown in a two-dimensional space. The primary and secondary axes show the percent variation explained by each dimension; ellipses are drawn with a 95% confidence level.

biomass estimates and metabarcoding reads (i.e., Yang et al., 2017), others have found a weak quantitative relationship between abundance/biomass and sequences produced (i.e., Lamb et al., 2019; Sun et al., 2015). The latter may partly explain the results obtained in our study, as we found little to no differences between decay rate estimates generated using different molecular templates and barcode markers. However, this result may also be explained by the relatively low sample volume filtered for eDNA/eRNA in the present study (i.e., 400 mL per filter; selected to optimize the trade-off between filtration time and volume filtered). Since a greater sample volume (i.e., 2 L rather than 400 mL) (Xing et al., 2022) may result in higher eDNA/eRNA copy numbers obtained (and thus greater sample size and replication for decay curve analyses), future studies employing a larger sample volume may contrast from the findings herein. Thus, to improve upon the use of eNA metabarcoding for quantitative applications, future studies investigating eDNA and eRNA decay should aim to compare single-species detection methods with metabarcoding using larger sample volumes to assess how decay rates differ based on genomic origin for a diverse assortment of taxa.

4.2 | Community composition

The PCoA analysis conducted on the presence/absence of *Arthropoda* taxa indicated that distinct community compositions were recovered using the 18S and COI barcode markers. Furthermore, although the 18S marker detected a higher number of total ZOTUs than the COI marker, the COI marker detected more *Arthropoda* families overall than the 18S marker. Fewer *Arthropoda* detections using the 18S marker could indicate a higher frequency of false negative detections; however, the higher number of *Arthropoda* detected using the COI marker may also indicate an increased susceptibility

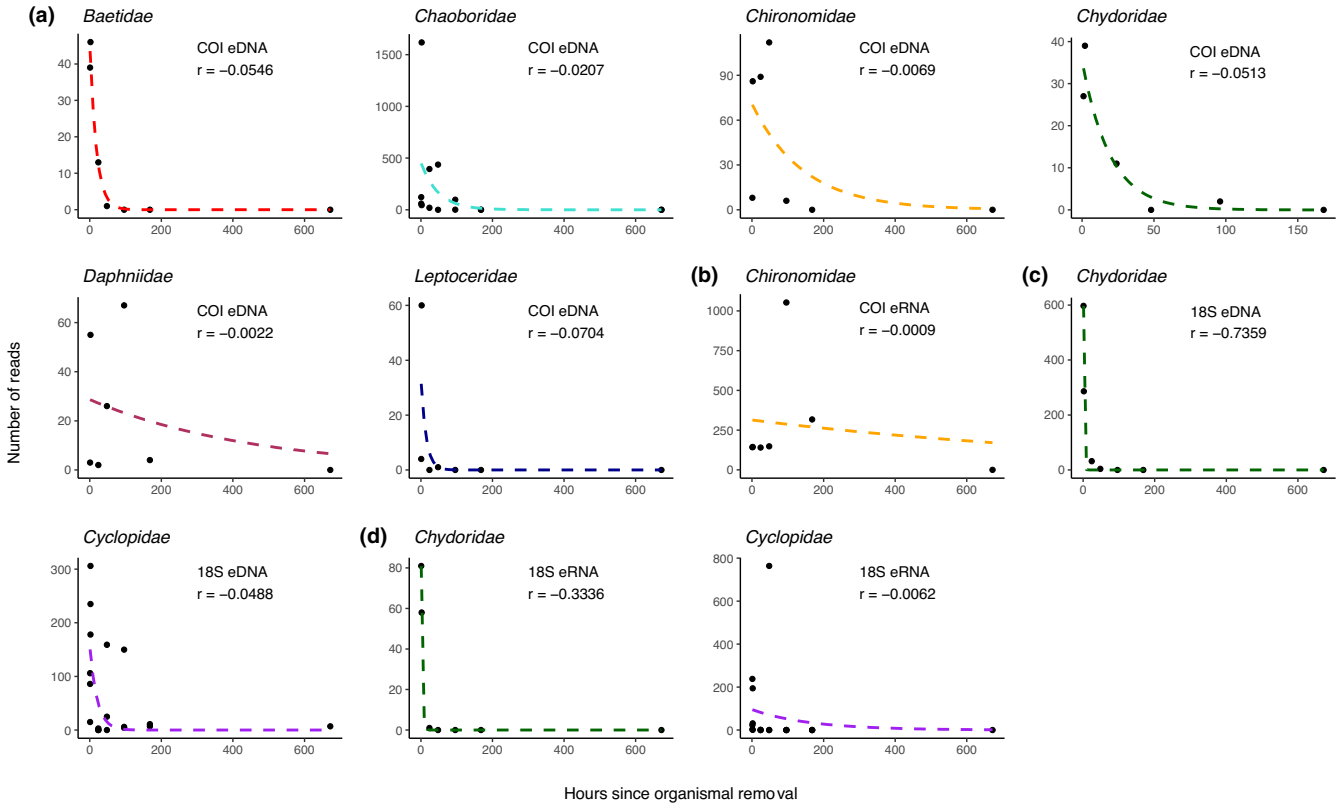

FIGURE 5 Exponential decay curves depicting the number of reads detected based on molecular template (environmental DNA [eDNA] or environmental RNA [eRNA]) and barcode marker (*cytochrome c oxidase* subunit I [COI] or V4 region of the nuclear 18S rRNA [18S]). Decay curves are grouped by (a) COI eDNA, (b) COI eRNA, (c) 18S eDNA, and (d) 18S eRNA (left to right) for each *Arthropoda* family for which a curve could be fitted. Decay curves were fitted to the data using the equation $N(t) = N_0 e^{rt}$, where r describes the mean model-derived decay rate constant. Note different y-axis scales.

of this marker to false-positive detections (e.g., the detection of *Chthamalidae*, a family of marine barnacles not detected using 18S). These differences can likely be attributed to differences in species distinguished by each marker, as well as the incompleteness of open-access reference databases (Zaiko et al., 2018). For example, a previous study by Cowart et al. (2015) revealed that the 18S marker uncovered higher species diversity in comparison with the COI marker. This finding was attributed to several factors, including the composition of the target community (high prevalence of taxa with a high affinity to 18S primers), length of target fragment, as well as a better-curated reference database for the 18S marker. In contrast, another study assessing the diversity of meiofauna using both COI and 18S found that eDNA surveys based on the 18S marker greatly underestimated species diversity relative to the COI marker (Tang et al., 2012). Thus, it is evident that due to differences in the intrinsic capacities of each marker (e.g., taxonomic-specific biases, resolution, reference databases, etc.), future studies should employ multi-marker assessments to unravel the broadest taxonomic diversity possible and limit taxonomic biases (Cowart et al., 2015; Lejzerowicz et al., 2015; Zaiko et al., 2018).

Overall, although *Arthropoda* community composition recovered using eDNA and eRNA overlapped with one another over time and across microcosms, eRNA detected fewer *Arthropoda* taxa than eDNA using the COI marker (but not the 18S marker). This suggests that although eRNA is capable of recovering similar diversity estimates as eDNA, the results may vary dependent on marker selection. While molecular methods for biodiversity surveillance have been shown to provide comparable estimates of species richness in comparison with traditional methods (Keck et al., 2022), both eRNA and eDNA may be prone to false negatives due to various factors such as unknown genetic variation in target taxa, insufficient target concentrations that fall below the detection threshold, environmental conditions, as well as the presence of inhibitors (Goldberg et al., 2013; 2016; Kagzi et al., 2022; Seymour et al., 2018; von Ammon et al., 2019). In contrast, false positives may occur as a result of lab/field contamination, inadequate primers that detect non-target taxa, as well as genetic mutations/variations in closely related species (Goldberg et al., 2013; 2016). Although we did not explicitly test for the rate of false positives/negatives generated by each molecular template and marker, future studies should nonetheless strive to assess and minimize these potential sources of data inaccuracies to improve survey results.

4.3 | Considerations for future sampling

Acknowledging the limitations of eNA-based survey methodologies is a critical step toward improving the efficiency, reproducibility, and

TABLE 2 Mean model-derived decay rate constants (and R^2 values) for *Arthropoda* environmental DNA (eDNA) and environmental RNA (eRNA) detected using the *cytochrome c oxidase* subunit I (COI) or V4 region of the nuclear 18S rRNA (18S) barcode marker.

Marker	Template	Family	Model-derived N_0	Mean model-derived decay rate constant h^{-1} (r)	Mean model-derived R^2 value	Estimated time for eDNA/eRNA to degrade below detection limits (hours)
COI	eDNA	*Baetidae*	45.9718	−0.0546	0.9795	112 (~5 days)
		Chaoboridae	456.8015	−0.0207	0.1835	407 (~17 days)
		Chironomidae	70.8003	−0.0069	0.3241	948 (~40 days)
		Chydoridae	35.4123	−0.0513	0.9204	114 (~5 days)
		Daphniidae	28.6713	−0.0022	0.1058	2590 (~108 days)
		Leptoceridae	33.6934	−0.0704	0.4246	101 (~4 days)
COI	eRNA	*Chironomidae*	313.6000	−0.0009	0.0339	8934 (~372 days)
18S	eDNA	*Chydoridae*	1246.1849	−0.7359	0.9967	13 (<1 day)
		Cyclopidae	157.9275	−0.0488	0.2828	151 (~6 days)
18S	eRNA	*Chydoridae*	113.0652	−0.3336	0.9999	21 (<1 day)
		Cyclopidae	95.1912	−0.0062	0.0294	1113 (~46 days)

Note: Decay rate constants and N_0 values were estimated using the exponential decay model $N(t) = N_0\, e^{-rt}$. The limit of detection was set at 0.1 copies of eDNA and eRNA in this study. Only *Arthropoda* families for which a decay curve could be fitted are shown.

reliability of survey results. In our study, spikes in *Arthropoda* eDNA and eRNA copy numbers were detected at the 28-day time point using both markers (with the 18S marker detecting larger spikes in eDNA/eRNA than the COI marker). This may be due to the passing of small eggs, resting cysts, or nauplii through the 50 μm Nytex mesh while siphoning water from the mesocosms, and subsequently growing over the span of the experiment. Lance et al. (2017) observed similar spikes in the late (Days 21–28) stages of their degradation experiment; they attributed these anomalies to a fraction of eDNA adhering to the walls of their polypropylene containers, and thus being temporarily protected from bacterial exonucleases. Subsequent desorption from the container walls would then have accounted for the spikes observed in later samples. This explanation may be extrapolated to our study, as we used polypropylene carboys to store water during our degradation experiment. Accordingly, future eNA-based studies may benefit from employing smaller mesh sizes to filter out target taxa (i.e., *Arthropoda*) for degradation-based experiments, as well as using meso/microcosms made of different materials (thus potentially minimizing eNA adherence to container walls). It is also possible that eNAs adhering to the walls of the carboys may have become more concentrated with the removal of water with each sample, thus resulting in the spikes observed. For example, an eDNA/eRNA decay study by Wood et al. (2020) detected eNAs in biofilms 21 days post-organismal removal. The authors of this study suggested that given enough time, it may be possible for eNAs to bind to organic and inorganic molecules and potentially stabilize within the matrix of the biofilm. Disturbance of the biofilm during sampling (more likely as water levels decreased with the progression of the experiment) may thus have further contributed to the spike in eNAs observed. Indeed, future studies using eNAs should also consider varying sampling strategies (i.e., larger meso/microcosms containing greater sample volumes) to minimize potential biofilm disturbance and reduce the probability of unexpected "spikes" in eNA copy numbers observed. Additionally, we detected an unusually high number of reads in the pooled blanks following bioinformatic processing—particularly in 18S eRNA samples—despite failing to amplify during PCR. This finding attests to the enhanced sensitivity of eNA metabarcoding; thus, besides the aforementioned suggestions, future studies should strive to minimize potential contamination via stringent "clean laboratory" and sampling protocols (Goldberg et al., 2016) to improve the accuracy and reliability of eNA metabarcoding results.

5 | CONCLUSIONS

In this study, we comparatively assessed the persistence and degradation of *Arthropoda* eDNA and eRNA using metabarcoding of two distinct barcode markers. Although we found little to no differences between *Arthropoda* decay rate estimates based on molecular template (eDNA or eRNA) or barcode marker (COI or 18S), these results are likely due to limitations in copy number estimates obtained for diverse taxa using the metabarcoding method. Moreover, we found that although eRNA is capable of recovering similar community diversity as eDNA (i.e., using 18S), this result may be marker-dependent and thus requires further investigation. Finally, we found that differences in *Arthropoda* community composition were largely due to differences in marker selection, rather than molecular template, sampling time point, or individual microcosm. Our findings highlight the need for further comparative studies using both metabarcoding and single-species detection methods to assess the persistence and degradation dynamics of eNAs using various markers

targeting a diverse range of taxa, and that marker selection—and not molecular template—drives estimates of species diversity and community composition. Collectively, these findings outline the importance of selecting an appropriate marker for eNA-based surveys while outlining the various limitations and potential future directions of eNA-based research for characterizing biological communities.

AUTHOR CONTRIBUTIONS

Katie L. Millette and Joanne E. Littlefair conducted the bioinformatics workflow. Kaushar Kagzi completed the data analysis and visualization. Kaushar Kagzi, Katie L. Millette, and Melania E. Cristescu wrote the manuscript. All authors reviewed drafts of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

Raw fastq files have been made available on the data repository Dryad (https://doi.org/10.5061/dryad.8931zcrw2).

ORCID

Kaushar Kagzi https://orcid.org/0000-0001-6745-1082
Katie L. Millette https://orcid.org/0000-0001-9638-1538
Joanne E. Littlefair https://orcid.org/0000-0002-4481-0211
Xavier Pochon https://orcid.org/0000-0001-9510-0407
Susanna A. Wood https://orcid.org/0000-0003-1976-8266
Gregor F. Fussmann https://orcid.org/0000-0001-9576-0122
Melania E. Cristescu https://orcid.org/0000-0002-0854-4040

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