Received: 23 February 2022 | Revised: 16 April 2022 | Accepted: 23 May 2022

DOI: 10.1111/1755-0998.13655

MOLECULAR ECOLOGY RESOURCES WILEY

RESOURCE ARTICLE

Environmental RNA degrades more rapidly than environmental DNA across a broad range of pH conditions

Kaushar Kagzi[1,2] | Robert M. Hechler[1,2] | Gregor F. Fussmann[1,2] | Melania E. Cristescu[1,2]

[1]Department of Biology, McGill University, Montreal, Quebec, Canada

[2]Groupe de Recherche Interuniversitaire en Limnologie (GRIL), Montreal, Quebec, Canada

Correspondence
Kaushar Kagzi, Department of Biology, McGill University, 1205 Docteur Penfield, Montreal, QC H3A 1B1, Canada.
Email: kaushar.kagzi@mail.mcgill.ca

Funding information
Canada First Research Excellence Fund, Grant/Award Number: CFREF-2015-0004; Natural Sciences and Engineering Research Council of Canada, Grant/Award Number: NSERC Discovery Grant 04331-2017

Handling Editor: Carla Martins Lopes

Abstract

Although the use and development of molecular biomonitoring tools based on environmental nucleic acids (eDNA and eRNA; collectively known as eNAs) have gained broad interest for the quantification of biodiversity in natural ecosystems, studies investigating the impact of site-specific physicochemical parameters on eNA-based detection methods (particularly eRNA) remain scarce. Here, we used a controlled laboratory microcosm experiment to comparatively assess the environmental degradation of eDNA and eRNA across an acid–base gradient following complete removal of the progenitor organism (*Daphnia pulex*). Using water samples collected over a 30-day period, eDNA and eRNA copy numbers were quantified using a droplet digital PCR (ddPCR) assay targeting the mitochondrial *cytochrome c oxidase* subunit I (COI) gene of *D. pulex*. We found that eRNA decayed more rapidly than eDNA at all pH conditions tested, with detectability—predicted by an exponential decay model—for up to 57 h (eRNA; neutral pH) and 143 days (eDNA; acidic pH) post organismal removal. Decay rates for eDNA were significantly higher in neutral and alkaline conditions than in acidic conditions, while decay rates for eRNA did not differ significantly among pH levels. Collectively, our findings provide the basis for a predictive framework assessing the persistence and degradation dynamics of eRNA and eDNA across a range of ecologically relevant pH conditions, establish the potential for eRNA to be used in spatially and temporally sensitive biomonitoring studies (as it is detectable across a range of pH levels), and may be used to inform future sampling strategies in aquatic habitats.

KEYWORDS
biomonitoring, droplet digital PCR, eDNA, eRNA, nucleic acid degradation

1 | INTRODUCTION

Reliable methods for the detection and quantification of species and community assemblages are essential for accurate biodiversity assessments, as they aid in deepening our understanding of the natural world and help inform current ecosystem management practices. Recently, environmental DNA (eDNA) has emerged as a powerful tool for biodiversity surveys, as it has been shown to improve the detectability of target organisms, is noninvasive, and does not require taxonomic expertise (Jerde et al., 2011; Piggott et al., 2021; Pilliod et al., 2013; Sengupta et al., 2019; Thomsen & Willerslev, 2015). Although eDNA-based surveys offer several advantages in ecosystem monitoring, uncertainties that may lead to inaccuracies in real-time biodiversity estimates remain prevalent (Cristescu & Hebert, 2018). In particular, several studies have previously outlined the ability of eDNA to persist in the environment

for weeks after organismal removal (Barnes et al., 2014; Dejean et al., 2011; Goldberg et al., 2013; Thomsen, Kielgast, Iversen, Wiuf, et al., 2012) as well as undergo transport downstream and/or in the water column (Deiner & Altermatt, 2014; Shogren et al., 2017), both of which may result in the detection of a species that is not present or living in a given sampled environment (i.e., false positive). To address these issues, environmental RNA (eRNA) has been proposed as a potential alternative biomolecule for species detection (Cristescu, 2019), as it is thought to break down rapidly following cell death (Eigner et al., 1961). If this assumption is valid, eRNA may be able to provide a finer spatiotemporal inference in surveying biodiversity, as it could have a more rapid turnover in comparison to eDNA. However, wide variability in eDNA/eRNA degradation estimates currently exist in published literature; for example, although eDNA may persist in the water column for several weeks in some instances, other studies have demonstrated that even small (<100 bp) eDNA fragments may degrade beyond detectable limits within hours to days after organismal removal (Thomsen, Kielgast, Iversen, Møller, et al., 2012). Similarly, several lines of evidence suggest that eRNA may persist in the environment for longer than expected under special circumstances (Cristescu, 2019); for example, Fordyce et al. (2013) suggested that due to factors such as reduced RNase activity, RNA may persist long-term (hours to years) in dehydrated post-mortem samples. However, such studies are often performed using organismal RNA (oRNA) collected directly from the target organism; in comparison, current knowledge on the persistence and degradation of RNA following release from the progenitor organism remains highly limited. To better understand the fate of environmental nucleic acids (eNAs; eDNA and eRNA) following organismal release, it is essential to consider the dynamics of the study organism, as well as local environmental parameters that affect the production, transport and degradation (and thus the persistence and detectability) of eDNA and eRNA molecules within the sampled environment (Barnes et al., 2014; Díaz-Ferguson & Moyer, 2014; Harrison et al., 2019). In this study, we exclusively refer to eNAs as molecules released into the environment in the absence of the progenitor organism (i.e., extra-organismal); these molecules may be cellular, vesicular or free-floating in form. We do not consider studies conducted using bulk samples containing whole organisms (organismal NAs or oNAs).

Knowledge on the abiotic and biotic factors affecting eDNA degradation has advanced rapidly over the past few years, with several studies investigating the impacts of pH (Seymour et al., 2018; Strickler et al., 2015; Tsuji et al., 2017), water temperature (Jo et al., 2019; Strickler et al., 2015), salinity (Dell'Anno & Corinaldesi, 2004), ultraviolet radiation (Strickler et al., 2015), microbial activity (Zulkefli et al., 2019), biomass (Jo et al., 2019), and DNA characteristics (Jo et al., 2017; Turner et al., 2014) on degradation rates. In particular, the pH of the sampled environment seems to have a significant effect on eDNA capture and degradation. Previous studies have suggested that site pH may influence the extent to which eDNA molecules are captured on glass fibre filters as it could impact their hydrophobicity and capacity for aggregation, especially for eDNA that is captured in free-form (Liang & Keeley, 2013; Lorenz & Wackernagel, 1994). While some studies demonstrate a negative relationship between eDNA yield and water pH ranging from pH 5 to 9 (i.e., eDNA yield is highest at low pH) (Lance et al., 2017; Tsuji et al., 2017), others indicate that degradation rates are lowest in alkaline rather than acidic conditions (Goldberg et al., 2018; Seymour et al., 2018; Strickler et al., 2015). This discrepancy may be due to differences in the predominant state of captured eDNA (i.e., contained within cells/organelles, vesicles, or free-floating) (Tsuji et al., 2017), as well as variation in sample-specific physicochemical parameters or microbial activity (Barnes et al., 2014; Liang & Keeley, 2013; Zulkefli et al., 2019). Although, to the best of our knowledge, no studies have yet investigated the influence of pH or any other abiotic or biotic environmental parameters on the degradation of eRNA specifically, some research indicates that RNA is more susceptible to hydrolysis under alkaline conditions, including those present naturally within the cell (Brown & Todd, 1952).

To date, two studies have comparatively explored the degradation rates of both eDNA and eRNA. Wood et al. (2020) investigated the production and degradation of eDNA and eRNA of the marine invertebrates *Sabella spallanzanii* and *Styela clava*, and found that although eDNA persisted in the environment for longer than eRNA following organismal removal (up to 94 and 13 h, respectively), there were no significant differences in decay rate constants between eDNA and eRNA. In contrast, Marshall et al. (2021) explored variation in degradation rates based on genomic origin of two *Dreissena* mussels and found significant differences in decay rates between eDNA and eRNA over three investigated gene regions. It is important to note, however, that neither of these studies assessed how various physicochemical parameters may impact degradation rates under different environmental conditions. Understanding these variables for both eDNA and eRNA will allow researchers to make a more informed choice regarding which survey method to employ depending on site-specific conditions, as well as provide greater accuracy in interpreting survey results; this is especially important in contexts such as early detection of non-indigenous species, or surveillance of rare or endangered populations.

In this study, we investigated whether eDNA and eRNA decay rates differ over time depending on the acidity of the sampled environment in a controlled laboratory microcosm experiment. We used *Daphnia pulex* as our target organism, as it is a keystone species in freshwater ecosystems and often serves as a model organism for various applications, including monitoring of water quality and ecological/evolutionary research (Stollewerk, 2010). Populations of *D. pulex* were cultured for two months in experimental microcosms prior to organismal removal and acidification to one of three distinct pH conditions (pH 4, 7 or 10). Water samples were collected over a period of 30 days post organismal removal, followed by the detection of the presence of *D. pulex* eDNA and eRNA using a droplet digital PCR assay targeting a 162 bp region of the mitochondrial *cytochrome c oxidase* subunit I (COI) gene. We hypothesized that (1) eDNA persists in the environment longer than eRNA following organismal removal, and (2) eDNA and eRNA decay rate constants will

differ depending on the detection molecule (eDNA or eRNA) and pH of the sampled environment.

2 | MATERIALS AND METHODS

2.1 | Experimental design

The degradation experiment involved 12 microcosms of 10 L each: nine experimental (pH 4, 7 or 10 with 3 replicates per treatment) and three positive controls (Figure 1). To obtain *D. pulex* eDNA and eRNA, 12 initial stock containers (polypropylene cylinders; 13 1/8″ D ×8″ H) were set up two months prior to the start of the experiment. Following the addition of artificial lake water (*D. pulex* growth medium, 10 L per container; Appendix A1, Tables S1–S3) (Celis-Salgado et al., 2008), each container was randomly inoculated with 30 *D. pulex* individuals (seven clones, all collected from Illinois, USA). These initial *D. pulex* populations were monitored and fed with 1.5 ml concentrated algae (*Ankistrodesmus* spp., *Scenedesmus* spp., and *Pseudokirchneriella* spp.) (Nichols & Bold, 1965; Stein, 1973) diluted in 200 ml of artificial lake water three times a week (Appendices A2–A3, Table S4) to ensure adequate population growth and eDNA/eRNA production.

At the beginning of the experiment, nine of the 12 initial stock containers were set up as experimental microcosms, while the remaining three containers were designated as positive controls and maintained as described above for the duration of the experiment. To set up experimental microcosms, stock container contents were carefully stirred and filtered through a 60 µm mesh into two large intermediate reservoirs; this step was performed to homogenize eDNA/eRNA starting concentrations across all experimental conditions. Once pooled, eDNA/eRNA-rich reservoir contents were stirred and refiltered through another 60 µm mesh to ensure complete removal of all *D. pulex* individuals, and subsequently evenly redistributed into nine new experimental microcosms. These microcosms were then immediately exposed to one of three pH levels (4, 7 or 10), with three replicates per treatment. The aforementioned pH conditions were chosen to represent the range of pH values found naturally in temperate freshwater ecosystems (Allan et al., 2021; Strickler et al., 2015), and were achieved using sterile 1.0 M HCl or 1.0 M NaOH (dropwise, whilst stirring) as required. All microcosms were kept in temperature-controlled (24°C) dark chambers for the duration of the experiment, with the pH levels of each microcosm monitored daily around sampling intervals to ensure stability. To eliminate all contaminating eDNA/eRNA, all experimental materials (10 L experimental containers/microcosms, intermediate reservoirs, 500 ml funnels) were bleached overnight in a 10% bleach solution, followed by a DI water rinse (7×) prior to the start of the experiment.

2.2 | Sample collection and filtration of eDNA and eRNA

Water samples containing *D. pulex* eDNA and eRNA were collected and filtered over a period of 30 days, beginning immediately after initial filtration of *D. pulex* through the 60 µm mesh (T0), followed by 1 h, 3 h, 5 h, 12 h, 24 h, 48 h, 4 days (96 h), 7 days (168 h), 14 days (336 h), 21 days (504 h), and 30 days (720 h) after initial filtration (corresponding to T0 through T11, respectively, for a total of 12 sampling time points). At each sampling time point, 400 ml of water was stirred and sampled using a 1″ PVC sampling tube from each experimental and positive control microcosm, of which 200 ml was filtered onto a 0.7 µm Millipore GF/F Glass Fibre filter, while the remaining 200 ml was filtered onto a 0.2 µm Millipore Express PLUS Polyethersulfone filter to capture both cellular and extracellular eDNA and eRNA (Matsui et al., 2001; Turner et al., 2014). Each filter

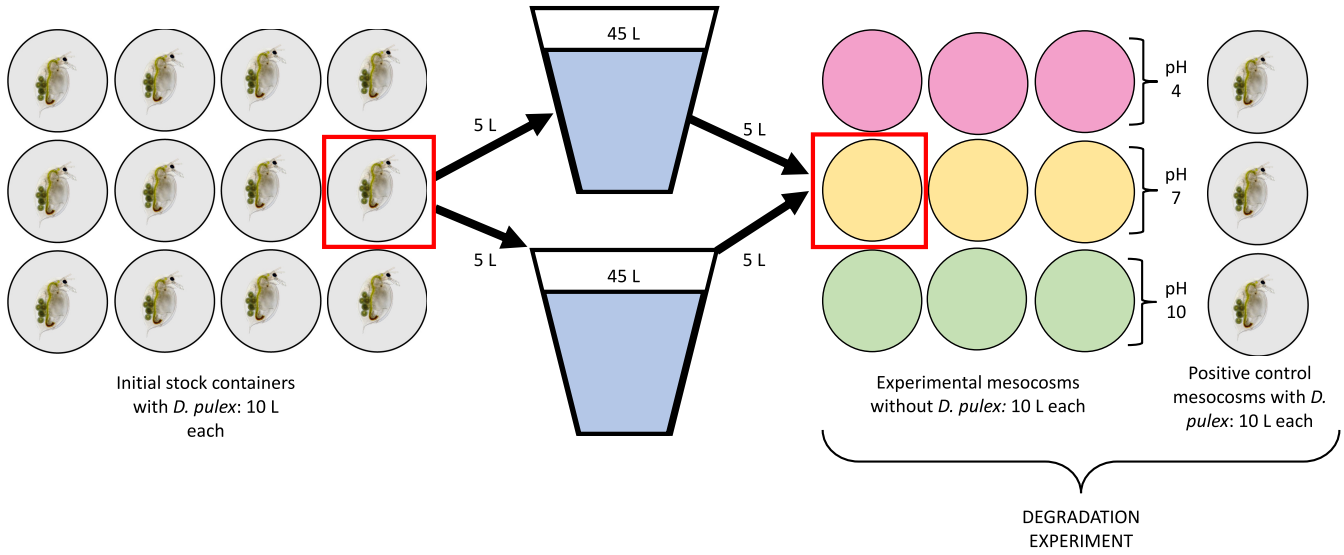

FIGURE 1 Schematic representation of experimental design, showing initial stock containers (12), intermediate reservoirs (2 buckets, 45 L each) and final experimental (9) and positive control (3) microcosms (10 L each) with or without live *Daphnia pulex* individuals

was cut in half using sterile scissors and forceps, with one half placed in an autoclaved 1.5 ml microcentrifuge tube containing either 370 µl ATL buffer (Qiagen) or 370 µl RLT buffer with β-mercaptoethanol (premixed in a 1 ml:10 µl ratio as per Qiagen RNEasy kit instructions) for eDNA or eRNA extractions respectively. For T0, 800 ml of water was collected from each of the intermediate reservoirs (with 400 ml from each reservoir) and filtered as described previously. Each time point included the filtration of 400 ml of autoclaved MilliQ water as a filtration blank. All sampling equipment was rinsed thoroughly between samples/time points in a series of three rinses with a 10% bleach solution, followed by two tap water rinses and a final DI water rinse to ensure the elimination of cross-contamination between samples. In addition, all experimental materials (1″ diameter PVC sampling tubes, graduated cylinders, filtration funnels, scissors and forceps) were bleached overnight in a 10% bleach solution, followed by a DI water rinse (7×) prior to use. Immediately following filtration, all eDNA and eRNA samples were placed in −80°C until extraction.

2.3 | Sampling of pH and other physicochemical parameters

Physicochemical parameters (pH, temperature, dissolved oxygen [concentration and saturation], atmospheric pressure and water conductivity) were measured and recorded for all microcosms at each sampling time point using a YSI Professional Plus Multiparameter instrument (YSI Inc.). The YSI was calibrated for pH, dissolved oxygen and conductivity prior to recording measurements at each time point.

2.4 | Molecular analysis

2.4.1 | Laboratory quality control

All eDNA/eRNA extractions, as well as DNA digestion and reverse transcription (for eRNA samples) were conducted in a dedicated clean eDNA/eRNA laboratory space, with separate areas designated for each sample type (eDNA or eRNA) and processing step (extraction, DNA digestion, or reverse transcription). All bench spaces and laboratory equipment were cleaned thoroughly with a 20% bleach solution, followed by wiping with RNase WiPER (iNtRON Biotechnology) to eliminate all RNase and DNA contamination from work surfaces. Aerosol barrier filter tips (FroggaBio) were used for all molecular steps to ensure no cross-contamination between samples. Researchers were required to wear the appropriate Personal Protective Equipment (PPE; laboratory coats, gloves, face masks, hairnets and shoe covers) in a separate decontamination room prior to entering the eDNA/eRNA laboratory; if previously working with high-quality DNA or in a post-PCR laboratory, researchers were further required to shower and change clothing before entry.

2.4.2 | Extraction of eDNA

Environmental DNA was extracted from filters using the DNeasy Blood and Tissue Kit (Qiagen, Montréal, QC) with the following modifications: samples were incubated at 56°C with 20 µl of proteinase K for 24 h prior to extraction; once incubation was complete, samples were removed from the incubator and vortexed for ~10 s prior to centrifugation for 3 min at 20,000*g*. Following centrifugation, the buffer surrounding the half filters (0.2 µm or 0.7 µm) from the same time point and sample source were pooled (for a total of ~800 µl) into a sterile 2 ml microcentrifuge tube, allowing for a more comprehensive sample consisting of both cellular and extracellular DNA. 400 µl of AL buffer was then added to each sample tube and incubated for 10 min at 56°C, followed by the addition of 400 µl of 100% ethanol. The resulting solution was pipette-mixed thoroughly and dispensed into a DNeasy spin column (600 µl at a time); spin columns were centrifuged for 1 min at 6000*g* three times due to the large volume of the filtrate. Contaminants and inhibitors were removed from spin column membranes using wash buffers AW1 and AW2 following the manufacturer's instructions. Purified DNA was eluted in 60 µl of AE buffer, and immediately stored in −20°C prior to total DNA quantification and droplet digital PCR analysis. Extraction blanks (370 µl ATL buffer) were processed alongside eDNA samples for each round of extractions.

2.4.3 | Extraction of eRNA, DNA digestion and reverse transcription

Environmental RNA was extracted from filters using the RNeasy Mini Kit (Qiagen) with the following modifications: eRNA samples were thawed on ice, and gently vortexed for ~5 s prior to centrifugation for 3 min at 20,000*g*. The buffer surrounding the 0.2 or 0.7 µm half filters from the same time point and sample source were pooled into a sterile 2 ml microcentrifuge tube containing 400 µl of 70% ethanol. The resulting solution was pipette mixed and transferred to an RNeasy spin column (700 µl at a time; repeated twice) and processed according to the manufacturer's instructions. Total RNA was eluted in 40 µl of DNase/RNase-free UltraPure distilled water (Invitrogen); 18 µl of RNA extract was plated immediately onto a 96-well plate for DNA digestion, while the remaining 22 µl was placed in −80°C. Extraction blanks consisting of 370 µl of RLT buffer were processed alongside eRNA samples for each round of extractions. Following extraction, two rounds of DNA digestion were completed using the Invitrogen DNA-free kit for DNase treatment and removal (Thermo Fisher Scientific) to ensure complete removal of contaminating DNA; for the first round, 1.8 µl of DNase I buffer (0.1 volume) and 1 µl of rDNase were added to each well, gently pipette mixed, and incubated at 37°C for 20 min. For the second round of DNA digestion, 2.7 µl (0.15 volume) of DNase I buffer and 1 µl of rDNase was added to each sample and gently pipette mixed, followed by incubation at 37°C for 20 min. Once incubation was complete, 2 µl of DNase inactivation reagent was added to each well and incubated for 2 min at room temperature, followed by centrifugation at 2000*g* for 5 min

to pellet the DNase inactivation reagent. After centrifuging, 10 µl of the supernatant was carefully transferred to a new 96-well plate for immediate cDNA synthesis, while the remaining 10 µl was transferred to a separate 96-well plate and placed in −80°C for later RNA quantification and PCR to confirm complete DNA removal. Digestion blanks consisting of 10 µl of DNase/RNase-free UltraPure distilled water (Invitrogen) were included on each plate. To verify complete removal of contaminating DNA, RNA extracts post-digestion were PCR amplified using previously described universal COI primers known to amplify *D. pulex* DNA: forward mlCOIintF 5′-GGWACW GGWTGAACWGTWTAYCCYCC-3′ (Leray et al., 2013) and reverse HCO2198 5′TAAACTTCAGGGTGACCAAAAAATCA-3′ (Folmer et al., 1994). Cycling conditions were as follows: 16 cycles of 95°C for 10 s, 62°C for 30 s (−1°C/cycle), and 72°C for 60 s, followed by 25 cycles of 95°C for 10 s, 46°C for 30 s, and 72°C for 60 s. This PCR amplification yielded no amplified products (verified on a 1% agarose gel), indicating complete removal of DNA from RNA extracts. Post DNA-digested RNA was reverse transcribed to cDNA using the high capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's protocol. Reverse transcription blanks (reagents only) were included on each plate. All cDNA (henceforth referred to as eRNA) was immediately stored at −20°C prior to total RNA quantification and droplet digital PCR analysis.

2.4.4 | Quantification of eDNA and eRNA samples

Total eDNA and eRNA quantifications were conducted prior to ddPCR using a Qubit 4 fluorometer (Thermo Fisher Scientific). For total sample eDNA, the Qubit 1X dsDNA HS Assay Kit was used with a 5 µl eDNA sample volume, and quantified according to manufacturer's instructions. For total sample eRNA, the Qubit RNA HS Assay Kit (Thermo Fisher Scientific) was used with a 2 µl eRNA sample volume, using a modified protocol to allow for a lower quantification limit as described in Li et al. (2015). All samples were measured three times to obtain the average reading for downstream analyses (Figure S1).

2.4.5 | Droplet digital polymerase chain reaction

All droplet digital PCR (ddPCR) reactions were performed in triplicate using a QIAcuity digital PCR instrument on an 8.5 K 96-well QIAcuity Nanoplate (Qiagen). Copy numbers (per µl) of the COI marker were determined for each sample using the novel *D. pulex* specific primers and probe as described in the present study (Table 1, Appendix A4). TaqMan probes were dual-labelled with a 5′ 6-carboxyfluorescein (6-FAM) fluorescent tag and a 3′ Iowa Black FQ quencher. Each reaction contained 1.2 µl of 800 nM of each primer and 400 nM of probe, 3 µl of 1X QIAcuity Probe PCR Master Mix, 1 µl of 0.025–0.25 U/µl restriction enzyme (for eDNA samples only), 2 µl of eDNA or cDNA, and RNase-free water for a total reaction volume of 12 µl. All sample volumes/concentrations used were according to manufacturer's recommendations. Following sample preparation, nanoplates were loaded into the QIAcuity digital PCR instrument for amplification using the following thermal cycling conditions: PCR initial heat activation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 s and combined annealing/extension at 60°C for 30 s. Plates were then analysed using the QIAcuity Software Suite for absolute quantification. For each ddPCR plate run, at least one negative (no template) control and one positive control (genomic DNA extracted from *D. pulex*) were included.

2.5 | Statistical analysis

All statistical analyses were conducted in the R version 4.0.1 statistical computing environment (R Core Team, 2020). The degradation rate of eDNA and eRNA was estimated using the exponential decay model $N(t) = N_0\, e^{-\lambda t}$, where $N(t)$ is the concentration of eDNA or eRNA at time t, N_0 is the initial concentration of eDNA or eRNA (taken immediately after removal of *D. pulex* from microcosms), and λ is the decay rate constant. This model has previously been used in several similar studies (Dejean et al., 2011; Strickler et al., 2015; Wood et al., 2020; Zulkefli et al., 2019). Decay rate models with optimized starting values were fitted to raw data using the packages easynls and minpack.lm implemented in R (Arnhold, 2017; Elzhov et al., 2016; Kaps & Lamberson, 2009; R Core Team, 2020). Estimated decay rate constants were used to calculate the time taken for *D. pulex* eDNA and eRNA to degrade to undetectable levels (limit of detection = 0.1 copies/µl in this study, as described in Strickler et al. (2015) and Wood et al. (2020)). Differences in decay rate constants between eDNA and eRNA at each pH condition were estimated using a linear mixed-effects model generated using the R package lme4 (Bates et al., 2015). This model included

TABLE 1 Droplet digital PCR assay developed for *Daphnia pulex* using the COI barcode marker

Primer/probe	Sequence (5′ → 3′)	Template strand	Length	Start	End	T_m (°C)	GC (%)	Hairpin	Dimer ΔG (kcal/mole) For	Rev	Probe
Forward	CAGCATTTGTTTTGGTTCTTTGG	Plus	23	580	602	61.8	39.1	0	−2.90	−6.19	−4.69
Reverse	GAAGCCTAACACTCCGATAGC	Minus	21	741	721	62.0	52.4	0		−4.48	−3.54
Probe	CGTAAATTATCCCAAGCGTGCCGA	Minus	24	712	689	66.9	50.0	−0.31			−5.22
Product length	162			580	741						

$\log_e$-transformed copies/μl of eDNA and eRNA as the response variable, sampling time (h), sample group (eDNA or eRNA at pH 4, 7 or 10) and the two-way interaction between sampling time and sample group as fixed effects, and samples nested within replicate microcosms as a random effect (Appendix A5). A post hoc Tukey's test using the R package emmeans (Lenth et al., 2022) was performed to evaluate differences in decay constants between sample groups.

3 | RESULTS

3.1 | Physicochemical parameters

The pH, temperature and pressure readings taken from each microcosm per time point remained relatively stable throughout the experiment (Figures S2 and S3). However, for dissolved oxygen ([mg/L] and %), an unexplained drop occurred between T5 and T9 (24 h and 14 days post organismal removal), while for conductivity, measurements taken from pH 10 microcosms increased over time (Figure S3). This observed increase in conductivity is probably a result of higher volumes of NaOH required to maintain constant alkaline pH conditions with the progression of the experiment.

3.2 | Droplet digital PCR results and pH-dependent eDNA/eRNA decay rates

Successful amplification of positive control microcosms occurred across all experimental time points (mean copies/μl ± standard deviation for eDNA = 44,461.7 ± 44,296.9, and 54,356.2 ± 58,445.7 for eRNA). The high variability in observed results is probably due to natural cycles of population growth in the microcosms, resulting in changes in *D. pulex* population sizes over time. For negative controls, the mean copies/μl detected were 4.2 ± 6.7 for eDNA, and 0.4 ± 0.6 for eRNA (Table S5). The mean initial copies/μl detected at T0 (prior to acid/base addition) were 603.3 ± 177.8 and 25.5 ± 12.9 for eDNA and eRNA respectively. Immediately following acid/base addition (T1), the mean copies/μl detected by eDNA were 406.0 ± 114.9 (pH 4), 449.1 ± 59.2 (pH 7), and 356.9 ± 145.4 (pH 10), while for eRNA, the mean copies/μl detected at T1 were 13.4 ± 7.1 (pH 4), 11.3 ± 6.9 (pH 7), and 25.1 ± 19.3 (pH 10) (Figure 2, Table S5). The average decay rate constants (derived from the exponential decay model) for eDNA and eRNA, respectively were 0.0025/h and 0.5723/h at pH 4, 0.0348/h and 0.0932/h at pH 7, and 0.0203/h and 0.1342/h at pH 10 (Figure 3, Table 2). For eDNA, the average decay rate constant at pH 4 was significantly lower than that of pH 7 and pH 10 ($p < .001$ and $p = .001$ respectively), while for eRNA, no significant difference in decay rates was observed among pH levels (Tables 2 and S6–S8). The estimated time for eDNA to degrade beyond detection limits (set at 0.1 copies/μl in this study) ranged from 136 to 151 days at pH 4 (mean = 143 days), 9 to 14 days at pH 7 (mean = 10 days), and 15 to 19 days at pH 10 (mean = 17 days). In contrast, the estimated time for eRNA to degrade beyond detection limits ranged from 4 to 13 h at pH 4 (mean = 10 h), 35 to 70 h at pH 7 (mean = 57 h), and 37 to 50 h at pH 10 (mean = 42 h) (Table 2, Figures 2 and 3). When comparing the average decay rates observed for eDNA and eRNA at each pH, eRNA decay rates were significantly higher than eDNA decay rates at all pH conditions ($p < .001$; Tables 2 and S6–S8).

4 | DISCUSSION

Although eDNA has revolutionized biomonitoring capabilities in aquatic ecosystems, current advancements in eRNA-based methodologies are also rapidly gaining interest due to the comparatively rapid turnover rate of eRNA, as well as its proposed ability to characterize metabolically active communities (Cristescu, 2019; Yates et al., 2021). Despite growing evidence citing the importance of understanding the influence of site-specific environmental parameters prior to designing and conducting eNA-based surveys (Barnes et al., 2014), knowledge on how environmental variables comparatively influence eRNA capture and detection is severely limited. Here, we present the first study (to the best of our knowledge) to comparatively assess the persistence and degradation of both eRNA and eDNA across a range of ecologically relevant pH conditions, using *Daphnia pulex* as a target organism.

4.1 | pH-dependent degradation of eDNA and eRNA

Although several studies have investigated the influence of acidity and other environmental conditions on the degradation of eDNA (Seymour et al., 2018; Strickler et al., 2015; Tsuji et al., 2017), no studies have yet been conducted assessing the impact of various pH conditions on the degradation of eRNA. In the present study, we found that eDNA persisted in experimental microcosms for a longer time span and had a lower decay rate than eRNA at all observed pH conditions. Traditionally, the molecular instability of RNA has been attributed to several factors including its single stranded structure (Eigner et al., 1961), sensitivity to oxidation from reactive oxygen species (Fabre et al., 2014), and susceptibility to spontaneous cleavage of its phosphodiester bond (Fabre et al., 2014; Oivanen et al., 1998). This cleavage occurs via nucleophilic attack on the phosphorus atom from the neighbouring 2′OH, which is absent in DNA (Fabre et al., 2014). In addition, the double-stranded configuration of DNA is thought to reduce the susceptibility of its sensitive oxygen atom to hydrolytic attack (Eigner et al., 1961), which may partly explain the difference in the degradation rates observed. Notably, although eRNA degradation occurred more rapidly than eDNA at all pH conditions, estimated eRNA decay rates were not 50-fold higher than those of eDNA as suggested by previous literature (Eigner et al., 1961). This finding is probably because eRNA is present in the environment in a complex assortment of states (e.g., protected within cells, organelles, other binding molecules, etc) rather than solely in a free form directly exposed to enzymatic

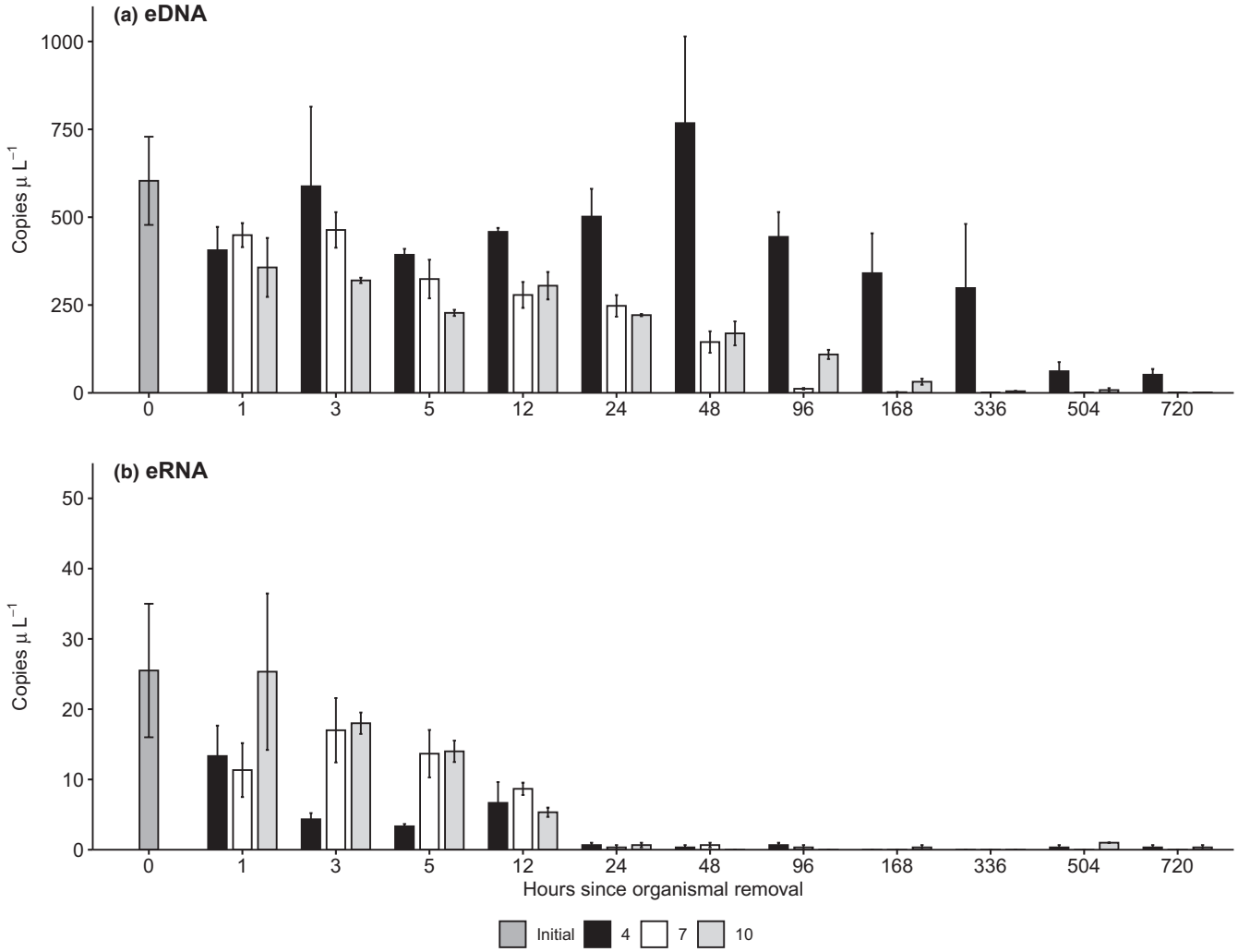

FIGURE 2 COI copy number · µl of (a) eDNA and (b) eRNA detected using droplet digital PCR at each pH level (4, 7 and 10) through time. The "initial" value corresponds to the initial sample reading taken prior to acid/base addition. Note different *y*-axis scales

degradation (Cristescu, 2019). Further, it is also important to note that starting concentrations of eRNA were lower than those of eDNA across all experimental conditions. This result is similar to the findings of a previous study by Wood et al. (2020), who suggested that the difference in starting concentrations may be due to loss of eRNA during extraction and laboratory processing, as it is particularly exposed and labile during these stages. However, confirmation of this assumption warrants further investigation. In summary, eRNA seems to exhibit more rapid decay in comparison to eDNA (but not too rapid as to incur nondetection of the target organism); this propensity for rapid decay demonstrates the capacity of eRNA to respond rapidly to ecosystem changes, thus making it highly amenable for assessing biodiversity changes and ecosystem health.

Further, decay rates for eDNA were highest in neutral/alkaline conditions, and lowest in acidic conditions. This finding is similar to that of a previous study by Strickler et al. (2015), who found that neutral conditions were not associated with eDNA persistence, and instead had shorter time to <5% eDNA remaining than the other pH levels tested. The authors of this study postulated that neutral conditions may have provided a more favourable environment for microbial growth and diversity, consequently resulting in higher exonuclease activity and increased eDNA degradation. This explanation may also be extrapolated to our study, as any microbial communities present were probably adapted to the neutral pH of the artificial lake water used. If this is the case, it may partially explain the lower decay rate of eDNA at low pH as microbial activity may not have been a significant factor accelerating eDNA degradation under these conditions. It is also important to note that in natural systems, bacterial communities adapted to local acidic or alkaline pH conditions may promote the degradation of eNAs in an equivalent manner, and thus differ from the decay rates observed in this study (Strickler et al., 2015). However, since we did not assess bacterial community composition and its impact on eDNA and eRNA decay rates, further research is required to test this assumption. In addition, a higher observed eDNA yield in acidic conditions has also been previously described in a study by Tsuji et al. (2017), who noted that their findings may be due to a change in the state of eDNA

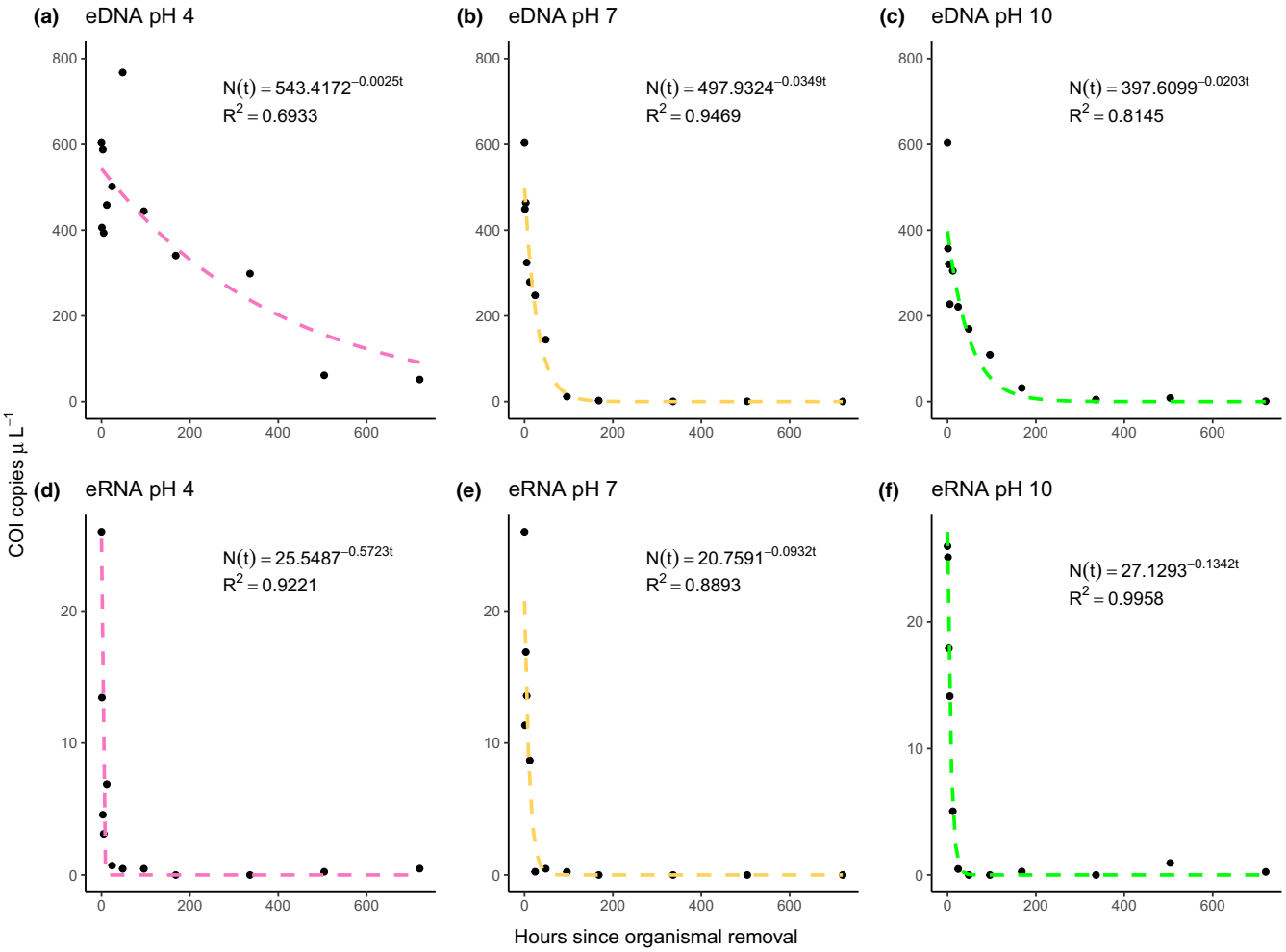

FIGURE 3 Exponential decay curves depicting mean COI copies/µl detected over time for (a) eDNA at pH 4, (b) eDNA at pH 7, (c) eDNA at pH 10, (d) eRNA at pH 4, (e) eRNA at pH 7, and (f) eRNA at pH 10. Decay curves were fitted to data using the equation $N(t) = N_0\, e^{-\lambda t}$, and R^2 values indicate the goodness of fit of the model to the data. Note different *y*-axis scales

TABLE 2 Mean decay rate constants for *Daphnia pulex* eDNA/eRNA based on the detection of the *cytochrome c oxidase* subunit I (COI) gene using droplet digital PCR at three pH conditions: 4, 7 and 10

	Treatment	N_0 (COI copies · µl)	Mean model-derived decay rate constant h^{-1} (λ)	Estimated time for eDNA/eRNA to degrade below detection limits (h)
eDNA	pH 4	543	0.0025	3440 (~143 days)
	pH 7	498	0.0348	245 (~10 days)
	pH 10	398	0.0203	408 (~17 days)
eRNA	pH 4	26	0.5723	10 (~0.4 days)
	pH 7	21	0.0932	57 (~2 days)
	pH 10	27	0.1342	42 (~2 days)

Notes: Decay rate constants and N_0 values were estimated by fitting an exponential decay curve using the equation $N(t) = N_0\, e^{-\lambda t}$ to all raw data. The limit of detection was defined as 0.1 copies/µl in this study.

molecules in low pH environments. They suggested that in acidic conditions, H^+ ions act to increase the molecular size of negatively charged DNA molecules and promote adsorption to suspended particles, thereby increasing the trapping efficiency of filters (Liang & Keeley, 2013; Lorenz & Wackernagel, 1994). The adherence of eDNA to filters may have also been promoted due to an increase in hydrophobicity as a result of deionization in low pH conditions (Bratby, 2015). In contrast, decreased hydrophobicity of DNA molecules in alkaline conditions may result in a lower binding rate of H^+ ions, which could have possibly contributed to the higher observed decay rate (and lower yield over time) of eDNA in high pH conditions.

In contrast to eDNA, the estimated decay rate constants for eRNA did not differ significantly across pH conditions. This consistent decay pattern may be due to the state of eRNA present in each sample. Metabolically active organisms release eRNA encapsulated in protective structures such as cells, extracellular vesicles, or retrovirus-like protein capsids (Cristescu, 2019). These protective structures may have partially shielded eRNA from pH-mediated decay; however, once released from these structures, pH-dependent decay processes acting on free-form or molecular complexes of eRNA may have facilitated the rapid decay patterns as observed in our study. For example, in alkaline conditions, the instability of the free-form RNA molecule is attributed to the presence of its 2′ hydroxyl group which acts as an intramolecular nucleophile; this alkaline hydrolysis often occurs at pH > 6 (Bernhardt & Tate, 2012; Oivanen et al., 1998). In addition, the calculated half-life of ribose at 25°C is highest at pH 3.5 and lowest at pH 10 (i.e., ribose becomes less stable as the pH of the solution increases), while the calculated half-life of the phosphodiester bond and cytosine (the least stable nucleobase) respectively at 25°C has been shown to be highest in the pH 5.4–8 range, and confers far less stability at pH 3.5 and 10 (Bernhardt & Tate, 2012; Kua & Bada, 2011). We found that eRNA exhibits rapid decay regardless of local pH, suggesting that most eRNA may initially be captured in a protected state prone to pH-independent degradation; however, once in free-form (and thus exposed to pH-dependent degradation) eRNA molecules are extremely short-lived. Our findings provide further evidence that extra-organismal RNA is able to persist in the environment long enough to be safely captured and extracted irrespective of sample pH; accordingly, due to its rapid decay rate and shorter persistence time, eRNA may serve as a superior proxy to eDNA in instances where high spatial and temporal acuity is essential (e.g., assessing rapid ecosystem and community-level changes, marine/freshwater biosecurity, discriminating between living and dead organisms, etc) (Pochon et al., 2017; Veilleux et al., 2021). In contrast, the differential persistence and degradation rates of eDNA depending on local pH conditions may introduce higher variability and reduced accuracy in sampling results; if this is the case, eRNA may serve as a more reliable biomonitoring tool in comparison to eDNA in aquatic environments. It is important to note, however, that experiments conducted in a laboratory setting may oversimplify the fate of eNAs in natural environments, as other site-specific variables (e.g., temperature, UV radiation, microbial activity, etc.) may interact with pH to influence the persistence and degradation rates observed (Marshall et al., 2021; Strickler et al., 2015). Moreover, although we did not specifically test for the effect of various states (e.g., protected by extracellular vesicles, protein capsids or other binding molecules, free-form, etc.) and types (mRNA, long non-coding RNA or lncRNA, rRNA, etc.) of captured eRNA on observed decay rates, these factors may nonetheless act to influence estimated decay rate patterns in future studies (Cristescu, 2019; Marshall et al., 2021; Yates et al., 2021). Accordingly, future eRNA-based research should strive to incorporate samples consisting of various states and types of eRNA collected in a diverse range of environmental conditions, thereby promoting our understanding of the complex ecology of eRNA in natural systems.

5 | CONCLUSION

To the best of our knowledge, this study is the first to comparatively assess the persistence and degradation of both eRNA and eDNA across a range of ecologically relevant pH conditions in a freshwater system. Our results indicate that eRNA persists for a shorter time span and decays more rapidly than eDNA at all pH conditions, which can probably be attributed to differences in molecular composition and stability. This finding further highlights the potential of eRNA to detect organismal presence with a finer spatiotemporal resolution in comparison to eDNA. Additionally, we show that eDNA decay rates were highest in neutral/alkaline conditions and lowest in acidic conditions, while eRNA decay rates did not differ significantly across pH levels. Collectively, these findings outline the importance of evaluating site-specific environmental conditions and eDNA/eRNA decay rates prior to the development and implementation of eNA-based surveys, provide further support for the increased use of eRNA-based methodologies, and may be used to inform present and future biomonitoring strategies.

AUTHOR CONTRIBUTIONS

Kaushar Kagzi conceptualized, designed and performed the experiment, protocol optimization, laboratory work (eDNA and eRNA extractions, DNA digestion, reverse transcription to cDNA and DNA/RNA quantification, PCR), data analysis and visualization, and wrote the manuscript. Robert M. Hechler aided in sample collection and performed optimization of eRNA extraction/DNA digestion protocols. Gregor Fussmann and Melania E. Cristescu provided feedback on experimental design. All authors reviewed drafts of the manuscript.

ACKNOWLEDGEMENTS

We are grateful to the research assistants that helped prepare for and execute this project (Elif Irmak Bektas, Aviva Zhao, Jihane Benbahtane and Charles Bazerghi). We thank Geneviève Bourret for her work in optimizing and processing eDNA/eRNA samples using droplet digital PCR. We would like to thank Djibril Soumahoro for his assistance in data analysis and visualization, and Julien Beaulieu for providing valuable guidance and advice on statistical methods. This study was funded by an NSERC Discovery grant (04331-2017), as well as CFREF Food from Thought Agricultural Systems for a Healthy Planet (CFREF-2015-0004) awarded to MEC and GFF.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at 10.5061/dryad.4qrfj6qc0.

DATA AVAILABILITY STATEMENT

The raw droplet digital PCR data generated for this study have been made available on the public data repository Dryad (doi: 10.5061/dryad.4qrfj6qc0).

ORCID

Kaushar Kagzi https://orcid.org/0000-0001-6745-1082
Robert M. Hechler https://orcid.org/0000-0002-9961-7179
Gregor F. Fussmann https://orcid.org/0000-0001-9576-0122
Melania E. Cristescu https://orcid.org/0000-0002-0854-4040

REFERENCES

Allan, J., Castillo, M., & Capps, K. (2021). *Stream ecology: Structure and function of running waters*. Springer International Publishing.

Arnhold, E. (2017). *Easynls: Easy nonlinear model* (5.0) [computer software]. https://CRAN.R-project.org/package=easynls

Barnes, M. A., Turner, C. R., Jerde, C. L., Renshaw, M. A., Chadderton, W. L., & Lodge, D. M. (2014). Environmental conditions influence eDNA persistence in aquatic systems. *Environmental Science & Technology*, *48*(3), 1819–1827. https://doi.org/10.1021/es404734p

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*(1), 1–48. https://doi.org/10.18637/jss.v067.i01

Bernhardt, H. S., & Tate, W. P. (2012). Primordial soup or vinaigrette: Did the RNA world evolve at acidic pH? *Biology Direct*, *7*(1), 4. https://doi.org/10.1186/1745-6150-7-4

Bratby, J. (2015). Coagulation and flocculation in water and wastewater treatment—Second edition. *Water Intelligence Online*, *5*(0), 9781780402321. https://doi.org/10.2166/9781780402321

Brown, D. M., & Todd, A. R. (1952). 13. Nucleotides. Part X. some observations on the structure and chemical behaviour of the nucleic acids. *Journal of the Chemical Society (Resumed)*, *52*, 52–58. https://doi.org/10.1039/jr9520000052

Celis-Salgado, M. P., Cairns, A., Kim, N., & Yan, N. D. (2008). The FLAMES medium: A new, soft-water culture and bioassay medium for Cladocera. *SIL Proceedings, 1922–2010*, *30*(2), 265–271. https://doi.org/10.1080/03680770.2008.11902123

Cristescu, M., & Hebert, P. (2018). Uses and misuses of environmental DNA in biodiversity science and conservation. *Annual Review of Ecology, Evolution, and Systematics*, *49*(1), 209–230. https://doi.org/10.1146/annurev-ecolsys-110617-062306

Cristescu, M. E. (2019). Can environmental RNA revolutionize biodiversity science? *Trends in Ecology & Evolution*, *34*(8), 694–697. https://doi.org/10.1016/j.tree.2019.05.003

Deiner, K., & Altermatt, F. (2014). Transport distance of invertebrate environmental DNA in a natural river. *PLoS One*, *9*(2), e88786. https://doi.org/10.1371/journal.pone.0088786

Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., Taberlet, P., & Miaud, C. (2011). Persistence of environmental DNA in freshwater ecosystems. *PLoS One*, *6*(8), e23398. https://doi.org/10.1371/journal.pone.0023398

Dell'Anno, A., & Corinaldesi, C. (2004). Degradation and turnover of extracellular DNA in marine sediments: Ecological and methodological considerations. *Applied and Environmental Microbiology*, *70*(7), 4384–4386. https://doi.org/10.1128/AEM.70.7.4384-4386.2004

Díaz-Ferguson, E. E., & Moyer, G. R. (2014). History, applications, methodological issues and perspectives for the use environmental DNA (eDNA) in marine and freshwater environments. *Revista de Biología Tropical*, *62*(4), 1273–1284. https://doi.org/10.15517/rbt.v62i4.13231

Eigner, J., Boedtker, H., & Michaels, G. (1961). The thermal degradation of nucleic acids. *Biochimica et Biophysica Acta*, *51*(1), 165–168. https://doi.org/10.1016/0006-3002(61)91028-9

Elzhov, T. V., Mullen, K. M., Spiess, A-N., Bolker, B. (2016). *Minpack.lm: R Interface to the Levenberg-Marquardt Nonlinear Least-Squares Algorithm Found in MINPACK, Plus Support for Bounds (1.2–1)* [Computer software]. https://CRAN.R-project.org/package=minpack.lm

Fabre, A.-L., Colotte, M., Luis, A., Tuffet, S., & Bonnet, J. (2014). An efficient method for long-term room temperature storage of RNA. *European Journal of Human Genetics*, *22*(3), 379–385. https://doi.org/10.1038/ejhg.2013.145

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, *3*(5), 294–299.

Fordyce, S. L., Kampmann, M.-L., van Doorn, N. L., & Gilbert, M. T. P. (2013). Long-term RNA persistence in postmortem contexts. *Investigative Genetics*, *4*(1), 7. https://doi.org/10.1186/2041-2223-4-7

Goldberg, C. S., Sepulveda, A., Ray, A., Baumgardt, J., & Waits, L. P. (2013). Environmental DNA as a new method for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*). *Freshwater Science*, *32*(3), 792–800. https://doi.org/10.1899/13-046.1

Goldberg, C. S., Strickler, K. M., & Fremier, A. K. (2018). Degradation and dispersion limit environmental DNA detection of rare amphibians in wetlands: Increasing efficacy of sampling designs. *Science of the Total Environment*, *633*, 695–703. https://doi.org/10.1016/j.scitotenv.2018.02.295

Harrison, J. B., Sunday, J. M., & Rogers, S. M. (2019). Predicting the fate of eDNA in the environment and implications for studying biodiversity. *Proceedings of the Royal Society B: Biological Sciences*, *286*(1915), 20191409. https://doi.org/10.1098/rspb.2019.1409

Jerde, C. L., Mahon, A. R., Chadderton, W. L., & Lodge, D. M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA: eDNA surveillance of rare aquatic species. *Conservation Letters*, *4*(2), 150–157. https://doi.org/10.1111/j.1755-263X.2010.00158.x

Jo, T., Murakami, H., Masuda, R., Sakata, M. K., Yamamoto, S., & Minamoto, T. (2017). Rapid degradation of longer DNA fragments enables the improved estimation of distribution and biomass using environmental DNA. *Molecular Ecology Resources*, *17*(6), e25–e33. https://doi.org/10.1111/1755-0998.12685

Jo, T., Murakami, H., Yamamoto, S., Masuda, R., & Minamoto, T. (2019). Effect of water temperature and fish biomass on environmental DNA shedding, degradation, and size distribution. *Ecology and Evolution*, *9*(3), 1135–1146. https://doi.org/10.1002/ece3.4802

Kaps, M., & Lamberson, W. R. (2009). *Biostatistics for animal science: An introductory text* (2nd ed.). CABI Publishing. 504p.

Kua, J., & Bada, J. L. (2011). Primordial Ocean chemistry and its compatibility with the RNA world. *Origins of Life and Evolution of Biospheres*, *41*(6), 553–558. https://doi.org/10.1007/s11084-011-9250-5

Lance, R., Klymus, K., Richter, C., Guan, X., Farrington, H., Carr, M., Thompson, N., Chapman, D., & Baerwaldt, K. (2017). Experimental observations on the decay of environmental DNA from bighead and silver carps. *Management of Biological Invasions*, *8*(3), 343–359. https://doi.org/10.3391/mbi.2017.8.3.08

Lenth, R. V., Buerkner, P., Herve, M., Love, J., Miguez, F., Riebl, H., & Singmann, H. (2022). *Emmeans: Estimated marginal means, aka*

least-squares means (1.7.3) [computer software]. https://CRAN.R-project.org/package=emmeans

Leray, M., Yang, J. Y., Meyer, C. P., Mills, S. C., Agudelo, N., Ranwez, V., Boehm, J. T., & Machida, R. J. (2013). A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: Application for characterizing coral reef fish gut contents. *Frontiers in Zoology*, *10*(1), 34. https://doi.org/10.1186/1742-9994-10-34

Li, X., Ben-Dov, I. Z., Mauro, M., & Williams, Z. (2015). Lowering the quantification limit of the qubit™ RNA HS assay using RNA spike-in. *BMC Molecular Biology*, *16*(1), 9. https://doi.org/10.1186/s12867-015-0039-3

Liang, Z., & Keeley, A. (2013). Filtration recovery of extracellular DNA from environmental water samples. *Environmental Science & Technology*, *47*(16), 9324–9331. https://doi.org/10.1021/es401342b

Lorenz, M. G., & Wackernagel, W. (1994). Bacterial gene transfer by natural genetic transformation in the environment. *Microbiological Reviews*, *58*(3), 563–602. https://doi.org/10.1128/mr.58.3.563-602.1994

Marshall, N. T., Vanderploeg, H. A., & Chaganti, S. R. (2021). Environmental (e)RNA advances the reliability of eDNA by predicting its age. *Scientific Reports*, *11*(1), 2769. https://doi.org/10.1038/s41598-021-82205-4

Matsui, K., Honjo, M., & Kawabata, Z. (2001). Estimation of the fate of dissolved DNA in thermally stratified lake water from the stability of exogenous plasmid DNA. *Aquatic Microbial Ecology*, *26*, 95–102. https://doi.org/10.3354/ame026095

Nichols, H. W., & Bold, H. C. (1965). *Trichosarcina polymorpha* gen. Et Sp. Nov. *Journal of Phycology*, *1*(1), 34–38. https://doi.org/10.1111/j.1529-8817.1965.tb04552.x

Oivanen, M., Kuusela, S., & Lönnberg, H. (1998). Kinetics and mechanisms for the cleavage and isomerization of the phosphodiester bonds of RNA by Brønsted acids and bases. *Chemical Reviews*, *98*(3), 961–990. https://doi.org/10.1021/cr960425x

Piggott, M. P., Banks, S. C., Broadhurst, B. T., Fulton, C. J., & Lintermans, M. (2021). Comparison of traditional and environmental DNA survey methods for detecting rare and abundant freshwater fish. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *31*(1), 173–184. https://doi.org/10.1002/aqc.3474

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., & Waits, L. P. (2013). Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. *Canadian Journal of Fisheries and Aquatic Sciences*, *70*(8), 1123–1130. https://doi.org/10.1139/cjfas-2013-0047

Pochon, X., Zaiko, A., Fletcher, L. M., Laroche, O., & Wood, S. A. (2017). Wanted dead or alive? Using metabarcoding of environmental DNA and RNA to distinguish living assemblages for biosecurity applications. *PLOS ONE*, *12*(11), e0187636. https://doi.org/10.1371/journal.pone.0187636

R Core Team. (2020). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. https://www.R-project.org/

Sengupta, M. E., Hellström, M., Kariuki, H. C., Olsen, A., Thomsen, P. F., Mejer, H., Willerslev, E., Mwanje, M. T., Madsen, H., Kristensen, T. K., Stensgaard, A.-S., & Vennervald, B. J. (2019). Environmental DNA for improved detection and environmental surveillance of schistosomiasis. *Proceedings of the National Academy of Sciences*, *116*(18), 8931–8940. https://doi.org/10.1073/pnas.1815046116

Seymour, M., Durance, I., Cosby, B. J., Ransom-Jones, E., Deiner, K., Ormerod, S. J., Colbourne, J. K., Wilgar, G., Carvalho, G. R., de Bruyn, M., Edwards, F., Emmett, B. A., Bik, H. M., & Creer, S. (2018). Acidity promotes degradation of multi-species environmental DNA in lotic mesocosms. *Communications Biology*, *1*(1), 4. https://doi.org/10.1038/s42003-017-0005-3

Shogren, A. J., Tank, J. L., Andruszkiewicz, E., Olds, B., Mahon, A. R., Jerde, C. L., & Bolster, D. (2017). Controls on eDNA movement in streams: Transport, retention, and resuspension. *Scientific Reports*, *7*(1), 5065. https://doi.org/10.1038/s41598-017-05223-1

Stein, J. (1973). *Handbook of phycological methods. Culture methods and growth measurements*. Cambridge University Press.

Stollewerk, A. (2010). The water flea daphnia—A "new" model system for ecology and evolution? *Journal of Biology*, *9*(2), 21. https://doi.org/10.1186/jbiol212

Strickler, K. M., Fremier, A. K., & Goldberg, C. S. (2015). Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. *Biological Conservation*, *183*, 85–92. https://doi.org/10.1016/j.biocon.2014.11.038

Thomsen, P. F., Kielgast, J., Iversen, L. L., Møller, P. R., Rasmussen, M., & Willerslev, E. (2012). Detection of a diverse marine fish fauna using environmental DNA from seawater samples. *PLoS One*, *7*(8), e41732. https://doi.org/10.1371/journal.pone.0041732

Thomsen, P. F., Kielgast, J., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., Orlando, L., & Willerslev, E. (2012). Monitoring endangered freshwater biodiversity using environmental DNA: Species monitoring by environmental DNA. *Molecular Ecology*, *21*(11), 2565–2573. https://doi.org/10.1111/j.1365-294X.2011.05418.x

Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA—An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation*, *183*, 4–18. https://doi.org/10.1016/j.biocon.2014.11.019

Tsuji, S., Yamanaka, H., & Minamoto, T. (2017). Effects of water pH and proteinase K treatment on the yield of environmental DNA from water samples. *Limnology*, *18*(1), 1–7. https://doi.org/10.1007/s10201-016-0483-x

Turner, C. R., Barnes, M. A., Xu, C. C. Y., Jones, S. E., Jerde, C. L., & Lodge, D. M. (2014). Particle size distribution and optimal capture of aqueous macrobial eDNA. *Methods in Ecology and Evolution*, *5*(7), 676–684. https://doi.org/10.1111/2041-210X.12206

Veilleux, H. D., Misutka, M. D., & Glover, C. N. (2021). Environmental DNA and environmental RNA: Current and prospective applications for biological monitoring. *Science of the Total Environment*, *782*, 146891. https://doi.org/10.1016/j.scitotenv.2021.146891

Wood, S. A., Biessy, L., Latchford, J. L., Zaiko, A., von Ammon, U., Audrezet, F., Cristescu, M. E., & Pochon, X. (2020). Release and degradation of environmental DNA and RNA in a marine system. *Science of the Total Environment*, *704*, 135314. https://doi.org/10.1016/j.scitotenv.2019.135314

Yates, M. C., Derry, A. M., & Cristescu, M. E. (2021). Environmental RNA: A revolution in ecological resolution? *Trends in Ecology & Evolution*, *36*(7), 601–609. https://doi.org/10.1016/j.tree.2021.03.001

Zulkefli, N. S., Kim, K.-H., & Hwang, S.-J. (2019). Effects of microbial activity and environmental parameters on the degradation of extracellular environmental DNA from a eutrophic lake. *International Journal of Environmental Research and Public Health*, *16*(18), 3339. https://doi.org/10.3390/ijerph16183339

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Kagzi, K., Hechler, R. M., Fussmann, G. F., & Cristescu, M. E. (2022). Environmental RNA degrades more rapidly than environmental DNA across a broad range of pH conditions. *Molecular Ecology Resources*, *22*, 2640–2650. https://doi.org/10.1111/1755-0998.13655