Received: 30 November 2023 | Revised: 19 June 2024 | Accepted: 25 June 2024

DOI: 10.1002/edn3.589

Environmental DNA
Open Access
Dedicated to the study and use of environmental DNA for basic and applied sciences

WILEY

METHOD

Optimization and application of bacterial environmental DNA and RNA isolation for qualitative and quantitative studies

Daniel J. Browne[1] | Catherine M. Miller[2] | Emily P. O'Hara[3] | Robert Courtney[3] | Jamie Seymour[3] | Denise L. Doolan[1,4,5] | Ryan Orr[6,7]

[1]Centre for Molecular Therapeutics, Australian Institute of Tropical Health & Medicine, James Cook University, Cairns, Queensland, Australia

[2]College of Medicine and Dentistry, James Cook University, Cairns, Queensland, Australia

[3]Tropical Australian Stinger Research Unit, Australian Institute of Tropical Health & Medicine, James Cook University, Cairns, Queensland, Australia

[4]Centre for Tropical Bioinformatics and Molecular Biology, Australian Institute of Tropical Health & Medicine, James Cook University, Cairns, Queensland, Australia

[5]Institute for Molecular Bioscience, The University of Queensland, St Lucia, Queensland, Australia

[6]College of Science and Engineering, James Cook University, Cairns, Queensland, Australia

[7]Queensland Department of Agriculture and Fisheries, Mareeba, Queensland, Australia

Correspondence
Ryan Orr, College of Science and Engineering, James Cook University, Cairns, QLD, Australia.
Email: ryan.orr@daf.qld.gov.au

Daniel J. Browne, Centre for Molecular Therapeutics, Australian Institute of Tropical Health & Medicine, James Cook University, Cairns, QLD 4870, Australia.
Email: daniel.browne@jcu.edu.au

Funding information
NHMRC Principal Research Fellowship, Grant/Award Number: 1137285

Abstract

Molecular detection of environmental DNA (eDNA) and RNA (eRNA) allows highly sensitive qualitative (i.e., presence or absence) and quantitative (i.e., abundance) monitoring of aquatic bacteria. However, bacterial molecular diagnostics are limited by low positive predictive values. Protocols for bacterial eDNA and eRNA molecular monitoring have primarily focused on optimizing specimen collection, and the optimal method to purify bacterial nucleic material from postcollection aquatic specimens to maximize the analytical sensitivity of molecular diagnostics remains poorly defined. Accordingly, strategies to isolate bacterial eDNA and eRNA from fresh and saltwater were investigated. We evaluated two filtration and four nucleic acid purification systems as representative of current generation bacterial eDNA and eRNA isolation strategies for capacity to isolate bacterial eDNA and eRNA from prelysed (i.e., free-nucleic acids) and viable (i.e., colony forming units, CFU) bacterial cells. We also compared the sensitivities of reverse transcription quantitative PCR (RT-qPCR) and metagenomic shotgun microbiome sequencing. The optimal protocol used 0.7 μm borosilicate glass filters (Whatman plc) followed by extraction with the RNeasy PowerWater kit (Qiagen). The protocol had a very high analytical sensitivity (10^{-3}–10^{0} ng and 10^{2}–10^{1} CFU detected in 500 mL) across multiple species of bacteria, when tested with either RTqPCR or metagenomic sequencing. Importantly, this study highlighted several limitations which are restrictive to both qualitative and quantitative bacterial eDNA and eRNA studies. First, a 12-h time course between sampling and extraction revealed significant species-specific changes in cell number and free-nucleic acid concentrations can occur postspecimen collection. Second, we found Gram-positive bacteria yielded less nucleic material compared to Gram-negative bacteria suggesting bacterial eDNA and eRNA studies could be biased by microorganism genome stability and extraction efficiency. This study highlights the need to define the species-specific diagnostic sensitivity of a protocol when monitoring aquatic bacterial eDNA and eRNA with molecular diagnostics.

Daniel J. Browne and Ryan Orr contributed equally to this work.

KEYWORDS
analytical sensitivity, bacterial, diagnostic sensitivity, environmental DNA, environmental RNA, microbiome, RT-qPCR

1 | INTRODUCTION

Molecular monitoring techniques to detect microbial environmental DNA (eDNA) or RNA (eRNA) are now used across various applications, including aquaculture, environmental conservation, and human health (Ciesielski et al., 2021; Farrell et al., 2021). Techniques have been developed to identify eDNA or eRNA from a range of microorganisms, including bacteria (Sato et al., 2019), fungi (Gonzalez et al., 2021), algae (Knudsen et al., 2022), protists (Galvani et al., 2019), helminths (Sengupta et al., 2019), and viruses (Medema et al., 2020; Nieuwenhuijse et al., 2020). Molecular monitoring methods, such as reverse transcription-qPCR (RT-qPCR), high-throughput metabarcoding, or next-generation sequencing, are highly sensitive and specific (Kralik & Ricchi, 2017) and can provide a qualitative (i.e., presence or absence) (Ruppert et al., 2019) or a quantitative (i.e., abundance) (Pont et al., 2023; Tillotson et al., 2018; Tsuji et al., 2022) estimation of a specific organism. However, despite the high theoretical sensitivity of molecular diagnostics, monitoring of microbial eDNA and eRNA is not common practice. While cost is an important factor (Browne, Kelly, et al., 2022), the major limitation of current molecular diagnostic protocols is their generally low positive predictive values (i.e., high false-positive and false-negative rates) (Browne et al., 2022; Farrell et al., 2021; Grumaz et al., 2020; van de Groep et al., 2019). The ability of a molecular diagnostic to correctly identify a true positive, known as diagnostic sensitivity (Saah & Hoover, 1997), is generally dependent upon optimal specimen collection and nucleic acid isolation, which require very high analytical sensitivity (i.e., the ability to detect very small quantities of analyte) (Browne et al., 2022; Saah & Hoover, 1997). Thus, enhancing the analytical sensitivity of a molecular diagnostic, either during specimen collection or nucleic acid isolation, could significantly enhance the effectiveness of molecular microbial monitoring.

A very high level of analytical sensitivity can be achieved when collecting eDNA or eRNA specimens from aquatic environments, as eDNA and eRNA can persist within ecosystems for long periods of time (Barnes et al., 2014; Wood et al., 2020), and large volumes of water can be filtered to detect very low abundance targets (Handley, 2015; Yates et al., 2021). The analytical sensitivity of eDNA and eRNA molecular diagnostics has been further enhanced by a variety of microorganism-specific specimen collection strategies. These strategies have been designed to address the challenges associated with collecting microorganisms, such as small cell sizes, species-specific crypticity, and diverse metabolic states (Bowers et al., 2021; Dickie et al., 2018; Spens et al., 2017; Xing et al., 2022). In contrast, optimization of bacterial eDNA and eRNA purification from aquatic specimens is limited (Bowers et al., 2021). Efficient isolation of bacterial eDNA and eRNA may be challenged by small cells that evade filtration, robust cell wall, or biofilm structures that resist lysis. Furthermore, all nucleic acid isolation techniques are highly susceptible to technical variations such as contamination (Ruppert et al., 2019), degradation (Bustin et al., 2009), and co-isolation of PCR inhibitors (Williams et al., 2017), which can significantly influence the analytical sensitivity of an assay. Indeed, studies across multiple disciplines have demonstrated that the expected recovery of purified nucleic acids differs between isolation protocols (Bowers et al., 2021; Browne et al., 2020; Djurhuus et al., 2017; Gosiewski et al., 2014; Muha et al., 2019; Williams et al., 2017).

To maximize the analytical sensitivity of molecular diagnostics, both DNA and RNA can be simultaneously isolated from a single sample. Simultaneously isolating DNA and RNA from a specimen is a routine strategy that enhances the speed and cost efficiency of molecular analyses (Moen et al., 2016; Okazaki et al., 2023; Xu et al., 2008) and allows assessment of matched genomic and transcriptomic datasets (Biedka et al., 2024; Grima et al., 2022; Valledor et al., 2014; Zhang et al., 2020). Following isolation, specific nucleases are used to purify DNA and RNA for accurate quantification. However, excluding DNase or RNase enzymes and targeting detection of both DNA and RNA is a strategy that has been demonstrated to significantly increase the sensitivity of molecular diagnostics (Deleu et al., 2022; He et al., 2017; Kajiura et al., 2015; Kotorashvili et al., 2012), and previous studies have successfully employed this technique during environmental sampling studies (DeAngelis et al., 2010; Gillies et al., 2015). These studies suggest that simultaneously targeting eDNA and eRNA through co-isolation could improve the analytical performance of environmental bacterial molecular diagnostics.

Postcollection degradation of bacterial eDNA and eRNA is also an important consideration. The quantity and quality of isolated eDNA and eRNA can be profoundly influenced by specimen storage (Bivins et al., 2021) and transportation (Goldberg et al., 2016; Holman et al., 2022). Nucleic acid degradation has been observed when conducting aquatic biosecurity surveillance (Bowers et al., 2021; Darling et al., 2020; Kumar et al., 2020), and degradation reduction strategies such as in situ filtration (Govindarajan et al., 2022), specimen cold storage (Bowers et al., 2021), or chemical treatment (Yamanaka et al., 2017) have been proposed as potential solutions. However, in situ filtration and an effective site-to-laboratory cold chain may be impractical or costly, and transportation of aquatic specimens from remote sites for filtration may take many hours in suboptimal conditions. Therefore, defining factors that influence postcollection bacterial species-specific degradation rates is crucial for assessing the sensitivity of bacterial eDNA and eRNA studies.

Accurate assessment of eDNA and eRNA bacterial molecular diagnostics requires the determination of species-specific diagnostic sensitivities (Saah & Hoover, 1997), as the concentration of

detectable nucleic acids obtained from pathogenic microorganisms can vary significantly between species (Gosiewski et al., 2014; Klaschik et al., 2002; Kralik & Ricchi, 2017). Such variations are influenced by organism-intrinsic factors like genomic copy number or lysis resistance (de Bruin & Birnboim, 2016; Luk et al., 2018), as well as extrinsic factors such as the conditions of the molecular diagnostic. Additionally, while RT-qPCR remains the gold standard (Browne et al., 2020), high-throughput technologies are increasingly being deployed as tools for unbiased microorganism detection and monitoring (Batovska et al., 2021; Zaiko et al., 2015). The comparative analytical sensitivities of high-throughput technologies and traditional RT-qPCR for both quantitative and qualitative bacterial molecular diagnostics remain unclear (De Brauwer et al., 2023; Zaiko et al., 2018). While many studies have described the relative abundance of aquatic microbial communities (Lee et al., 2020; Liu et al., 2022; Xie et al., 2018), no publication has directly compared the cross-species, cross-platform relative analytical sensitivity of bacteria purified from aquatic eDNA or eRNA specimens.

Herein, we optimized a protocol to isolate bacterial eDNA and eRNA to improve aquatic microorganism diagnostics. Specifically, we assessed the capacity of several current generation eDNA and eRNA isolation techniques to simultaneously isolate DNA and RNA from captured viable cells (i.e., colony forming units (CFU)) and free nucleic acids (NA) of two Gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis*) and three Gram-negative (*Escherichia coli*, *Serratia marcescens*, and *Klebsiella pneumoniae*) species of bacteria from salt and freshwater. Ultimately, this study sought to optimize the postcollection purification phase of eDNA and eRNA molecular diagnostics for bacterial nucleic material from aquatic samples to inform future qualitative and quantitative microbial eDNA and eRNA studies.

2 | METHODS

2.1 | Specimens

2.1.1 | Water samples

In all experiments, specimens of 20 L volume were collected from the surface of 20,000 L freshwater or 60,000 L saltwater research aquariums located at James Cook University (Cairns, Australia) in a 20 L plastic water carry can (SCA Water, Smithfield, Australia). In all experiments, aside from the eDNA and eRNA degradation experiments, aquarium water specimens were aliquoted as 500 mL volume into 500 mL bleach-treated and autoclaved Schott bottles and extracted within an hour postcollection. Type I water (MilliQ) negative controls were processed in parallel. The aquariums were monitored weekly for water pH, hardness, salinity, and the concentration of ammonia, nitrite, nitrate, phosphate, and calcium using commercial test kits (Sera, Heinsberg, Germany) following the manufacturer's instructions. Conditions were relatively stable over the testing period (Figure S1).

2.1.2 | Bacterial culture

To generate characterized bacterial isolates for use as water sample recovery controls (i.e., spikes), single colony glycerol stocks were isolated from five common bacterial clinical isolates (Pathology Queensland) as previously described (Browne et al., 2022; Espy et al., 2006). Briefly, single colonies from two Gram-positive bacteria, *S. aureus* and *E. faecalis*, and three Gram-negative bacteria: *E. coli*, *S. marcescens*, and *K. pneumoniae*, were isolated with streak plate technique and cultured in 15 mL of antibiotic-free Lennox formulation lysogeny broth (LB), made in house with 10 g of tryptone (Sigma-Aldrich), 5 g of yeast extract (Sigma-Aldrich), and 5 g of NaCl (Sigma-Aldrich) in a 50-mL conical tube (Corning) overnight (~16 h) at 37°C. Genomic DNA (gDNA) was isolated from viable cells with a MagMAX™ Total Nucleic Acid Isolation Kit (Thermo Fisher), following the manufacturer's instructions. The identity of bacterial single-colony isolates was confirmed as previously published (Browne et al., 2022). Briefly, PCR amplicons of isolate gDNA intervening variable regions of the 16S/rRNA gene underwent Sanger sequencing (Australian Genomics Research Facility, University of Queensland), allowing species-specific Basic Local Alignment Search Tool (National Center for Biotechnology Information) identification. Viable cells were frozen as glycerol stocks in 20% sterilized Ultra-Pure™ Glycerol (Thermo Fisher).

2.1.3 | NA and CFU sample recovery controls

Total nucleic acid (NA) spikes were generated from DNA and RNA extracted from viable cells grown from glycerol stocks with a MagMAX™ Total Nucleic Acid Isolation Kit (Thermo Fisher), following the manufacturer's instructions. The concentration of total NA was determined using a NanoPhotometer® N60 (Implen, München, Germany) (Sarathkumara et al., 2022) and stored at −20°C before use. Colony forming unit (CFU) spikes were quantified from viable cells grown from glycerol stocks with the aerobic plate count method, as previously described (Feldsine et al., 2002). Briefly, the CFU/mL of stationary-phase LB broths was determined using CFU standard curves generated from an aliquot of LB broth diluted Log_{10} and grown on agar. Stationary-phase viable cells were stored no longer than 24 h at 4°C before use.

2.2 | Nucleic acid isolation

2.2.1 | Filtration

CFU or NA were spiked into 500 mL of fresh or saltwater, mixed briefly by inversion, and, unless otherwise stated, immediately filtered with either a 0.45 μm mixed cellulose ester (HA) (Sigma-Aldrich) or 0.70 μm borosilicate glass (GF/F) filter (Whatman plc) using a Büchner funnel, rubber seal, and vacuum flask vacuum filtration system as previously described (Jensen, 2006).

2.2.2 | Homogenization

Filters were rolled into PowerWater™ Bead Tubes (QIAGEN) as per the manufacturer's instructions with bleach-treated forceps. Each tube received 1 mL of extraction kit-specific lysis buffer. The filters were homogenized within the PowerWater Bead Tubes, which were shaken at a frequency of 30 beats per second for 5 min with a TissueLyser II (QIAGEN). Approximately 600 µL of lysis buffer was recovered from both GF/F and HA filters, as expected from the manufacturer's instructions. As advised by the extraction kit manufacturer, GF/F filter lysis buffer was squeezed from the filter homogenate with a 10 mL uncapped disposable syringe (Livingstone International). The recovered lysis buffer was dispensed into PCR-clean, purity-grade 1.5-mL tubes (Eppendorf) for immediate extraction.

2.2.3 | eDNA and eRNA filtration evaluation

To identify the optimal filter to isolate NA and CFU from an aquatic source, HA or GF/F systems were used to isolate either eDNA and eRNA from *E. coli* and *S. aureus* in freshwater (Figure 1a). Triplicate replicate samples and spikes were prepared as described above and extracted with a DNeasy Power H_2O kit (QIAGEN) and evaluated with RT-qPCR.

2.2.4 | eDNA and eRNA isolation kit evaluation

For evaluation of eDNA and eRNA yield, two silica column-based kits: DNeasy PowerWater kit (QIAGEN) and RNeasy PowerWater kit (QIAGEN), and two magnetic bead-based kits: MagMAX™ Total Nucleic Acid Isolation Kit (Thermo Fisher) and MagMAX™ *mirvanna* RNA Isolation Kit (Thermo Fisher) were evaluated to isolate either eDNA and eRNA from *E. coli* and *S. aureus* CFU and NA in salt and freshwater (Figure 1b). All extractions followed the manufacturer's instructions, with the exception that no DNase or RNase enzymes were used. Triplicate replicate samples and spikes were prepared as described above and filtered with GF/F filters. All samples were eluted in 50 µL of kit-specific elution buffer and evaluated with RT-qPCR.

2.2.5 | eDNA and eRNA degradation testing

To evaluate eDNA and eRNA degradation, CFU and NA were spiked into 10 L of salt or freshwater, from which a single 500 mL sample was collected every 2 h and processed over 12 h. To ensure a consistent and homogeneous mix, the 10-L containers of salt and freshwater were inverted several times hourly, and again moments before each specimen was collected. Once collected, bacterial eDNA and eRNA were isolated from the specimen using GF/F filtration and a RNeasy PowerWater kit (Figure 1c). To evaluate degradation in a sterilized sample, 1% Didecyldimethylammonium chloride (DDAC) antimicrobial chemical (Sipcam Pacific Australia Pty Ltd) was added to 10 L samples of spiked fresh and saltwater, which were processed in parallel and evaluated with RT-qPCR.

2.2.6 | Analytical sensitivity testing

To determine analytical sensitivity, titrations of CFU (10^6 to 10^1 CFU) or NA (10^2 ng to 10^{-4} ng NA) were spiked into triplicate replicate 500 mL samples of salt and freshwater and immediately extracted using GF/F filtration and a RNeasy PowerWater kit (Figure 1d). The analytical sensitivity of *S. aureus*, *E. faecalis*, *E. coli*, and *S. marcescens* was determined with RT-qPCR, and pooled technical replicates of freshwater samples were analyzed with microbiome shotgun sequencing. Microbiome shotgun sequencing evaluated all detected taxonomic families and the analytical sensitivity of *S. aureus*, *E. faecalis*, *E. coli*, *S. marcescens*, and *K. pneumoniae*.

2.3 | Molecular analysis

2.3.1 | RT-qPCR

Reverse transcription

RNA was converted to cDNA with the SuperScript™ IV First-Strand Synthesis System (Thermo Fisher) with samples treated with five enzymatic units (U) of Superscript™ IV (Invitrogen) reverse-transcriptase (RT) per 1 µL of eluent, as previously described (Browne, Kelly, et al., 2022). These samples represented a combined eDNA and eRNA (DNA + RNA) signal. An RT negative control sample (i.e., 0 U/µL eluent) was processed in parallel, which represents equivalent eDNA-only measurements (DNA). Before PCR, all samples were diluted 1:4 in Ultra-Pure™ H_2O (Invitrogen).

Quantitative PCR

DNA + RNA and DNA samples were evaluated with SYBR® chemistry quantitative PCR (qPCR), which was conducted with 500 nM desalt-grade (Sigma-Aldrich) primers (Table 1) as previously described (Browne et al., 2020) using SsoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green Supermix (Bio-Rad). Five microliters of total volume reactions were run in technical triplicate in accordance with MIQE guidelines (Bustin et al., 2009). The signal from the samples was quantified as cycle threshold (C_t) values determined with the threshold set in exponential phase amplification at ΔRn0.3. All reactions were followed by a melt curve analysis, ensuring primer specificity. Data were acquired using a QuantStudio5 Real-Time PCR system running QuantStudio Design and Analysis Software (v1.4.3, Applied Biosystems). Primer

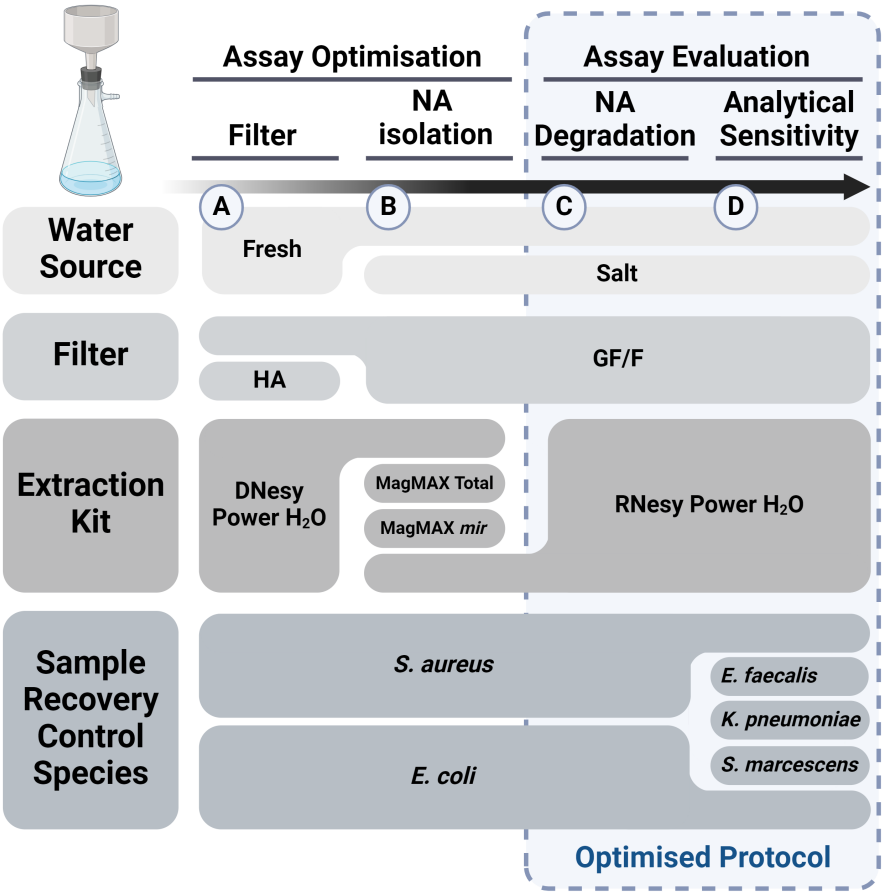

FIGURE 1 Experimental workflow. Overview of the study, optimizing a protocol to isolate bacterial eDNA and eRNA from freshwater (Fresh) and saltwater (Salt) using a Büchner funnel, rubber seal, and vacuum flask vacuum filtration system (Top Left). When performing filter optimization (A) 0.7-μm borosilicate glass fiber (GF/F) filters were found to be superior to 0.45-μm mixed cellulose ester (HA) filters. When performing nucleic acid (NA) isolation optimization (B) the RNeasy PowerWater kit was found to be superior to the DNeasy PowerWater kit, the MagMAX™ Total Nucleic Acid Isolation Kit (MagMAX Total), and the MagMAX™ *mirvanna* RNA Isolation Kit (MagMAX *mir*). The optimized protocol (blue shaded area) was used when evaluating eDNA and eRNA degradation rates (C) and the limit of detection (analytical sensitivity) of the assay (D). Shown are the species *Staphylococcus aureus* (*S. aureus*) and *Enterococcus faecalis* (*E. faecalis*), *Escherichia coli* (*E. coli*), *Serratia marcescens* (*S. marcescens*), and *Klebsiella pneumoniae* (*K. pneumoniae*) used as sample recovery controls. Image created with BioRender™.

reaction efficiency was calculated by amplification of $\log_2$ titrations of NA from 1 ng/μL.

2.3.2 | Microbiome shotgun sequencing

Triplicate replicate extractions from freshwater analytical sensitivity CFU titrations were pooled and commercially shotgun sequenced (TransnetYX™, Cordova, TN, USA). Briefly, library preparation was performed using the Watchmaker DNA library preparation with fragmentation protocol. Sequencing was performed using the Illumina NovaSeq instrument and protocol, and raw data (FASTQ files) were analyzed using the One Codex database, where read alignment and similarity analysis resulting in taxonomic family- and species-specific total reads were determined as previously described (Minot et al., 2015). The relative abundance of each microbial species was estimated based on the depth and coverage of sequencing across reference genomes within the One Codex database. Normalized species abundance was calculated as species-specific reads relative to the total number of reads.

2.4 | Gram stain

Bacteria were concentrated from 500 mL to 1 mL of water by centrifugation at 4000 relative centrifugal force (RCF) for 15 min in an Avanti J-26 XPI centrifuge (Beckman Coulter). Bacteria were further concentrated from 1 mL to 100 μL by centrifugation at 4000 RCF for 15 min in a benchtop centrifuge (Sigma). Specimens were dried onto a glass slide and Gram-stained with a Gram Stain Kit (Thermo Fisher), which includes an initial methanol fixation, a primary stain of crystal violet, and a safranin counterstain. Slides were imaged on an Axioscope 5 (Zeiss) with an objective adapter and a D850 DSLR

camera (Nikon). The examiner recorded co-localized Gram-positive and Gram-negative bacteria, aiming to minimize bias due to over or under decolorization.

2.5 | Data analysis

When testing protocol optimization and analytical sensitivities, RT-qPCR data (cycle threshold values) were assessed using a repeated-measures two-way ANOVA. Where the main effects were significant, a Bonferroni-corrected multiple comparisons test was used to compare the means of all test conditions. When testing eDNA or eRNA degradation over time, the strength of the association between time and cycle threshold was tested with Pearson's correlation. Analysis was conducted using GraphPad Prism version 10 for Windows (GraphPad Software). p Values were reported, with $\alpha = 0.05$ in all cases.

3 | RESULTS

3.1 | Borosilicate glass filtration (GF/F) significantly enhanced the detection of bacterial eDNA and eRNA

To develop a sensitive protocol to detect aquatic bacteria, we first tested the capability of mixed cellulose ester (HA) and borosilicate glass filtration (GF/F) filtration systems to isolate *S. aureus* and *E. coli* eDNA and eRNA from freshwater (Figure 1a). GF/F filtration significantly lowered the C_t (i.e., increased the signal of isolated bacterial nucleic material; Figure 2) when isolating DNA and DNA + RNA (Table 2). When interrogated with multiple comparison testing, the yield of nucleic material from CFU and NA in all tested conditions was significantly increased by GF/F filtration, except for when isolating *S. aureus* DNA + RNA from CFU, where no significant difference was observed ($p = 0.1700$, Table 2). These data demonstrate that GF/F filtration significantly enhanced, or was equally effective, when isolating bacterial eDNA and eRNA from all tested conditions.

3.2 | A silica column-based isolation strategy significantly increased bacterial eDNA and eRNA isolation

We assessed the performance of four extraction kits to isolate eDNA and eRNA from *E. coli* and *S. aureus* when collected with the optimal filter (GF/F), as identified earlier, from salt and freshwater specimens (Figure 1b). The signal of nucleic acid from the specimen was significantly influenced by the extraction kit when isolating *E. coli* DNA (fresh water $p < 0.0001$, salt water $p < 0.0097$; Table 3), *S. aureus* DNA from fresh water ($p < 0.0001$; Table 3), and *E. coli* DNA + RNA from salt water ($p = 0.0009$; Table 3). When investigated with multiple comparison testing there was

TABLE 1 Primer list.

Target species	GOI	GenBank accession number	Forward sequence (5′-3′)	Reverse sequence (5′-3′)	Amp size (bp)	E′ (%)	Ref
EC	*16S*	J01859	GTTAATACCTTTGCTCATTGA	ACCAGGGTATCTAATCCTGTT	350	103.4	Gao et al. (2011)
SA	*16S*	OR167048.1	ACGGTCTTGCTGTCACTTATA	TACACATATGTTCTTCCCTAATAA	257	98.1	Matsuda et al. (2007)
SM	*16S*	M59160	GGGAGCTTGCTCACTGGGTG	GCGAGTAACGTCAGTTGATGAGCGTATTA	417	93.5	Wilson et al. (1999)
EF	*16S*	AB012212.1	CCCGAGTGCTTGCACTCAATTGG	AGGGGACGTTCAGTTACTAACGT	419	98.9	Kubota et al. (2010)

Note: Characteristics of primers. Primer efficiency (E′) was calculated in accordance with MIQE guidelines (Bustin et al., 2009).
Abbreviations: AMP, amplicon; EC, *Escherichia coli*; EF, *Enterococcus faecalis*; GOI, gene of interest; Ref, reference; SA, *Staphylococcus aureus*; SM, *Serratia marcescens*.

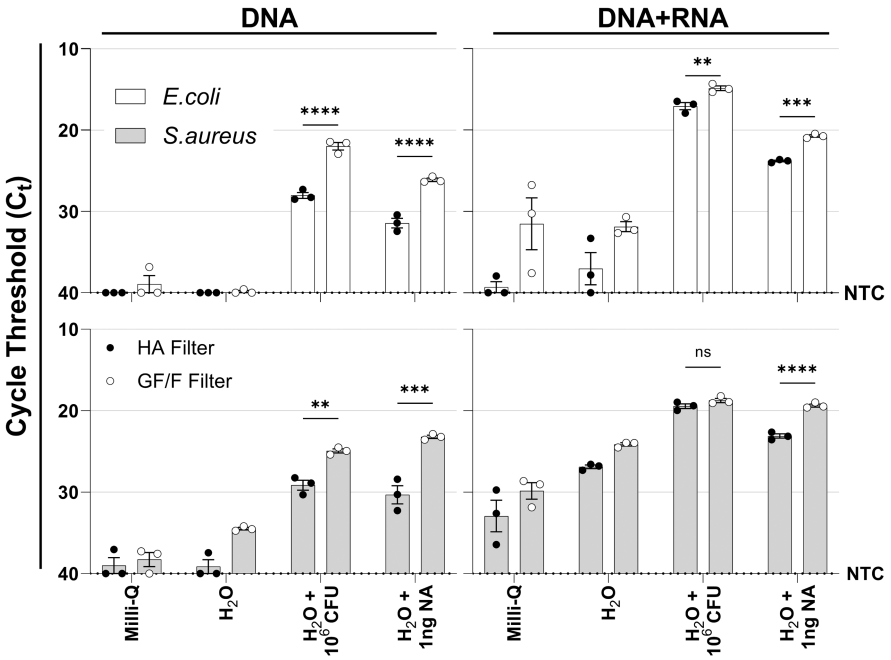

FIGURE 2 Filter optimization for pathogenic microorganism detection with eDNA and eRNA molecular diagnostics: *E. coli* (white bars) and *S. aureus* (gray bars) 10^6 colony forming units (CFU) or 1 ng of nucleic acid (NA) were spiked into 500 mL of freshwater (H_2O), filtered with either mixed cellulose ester (HA) or borosilicate glass filtration (GF/F) filters, extracted with the DNeasy PowerWater kit (QIAGEN), reverse-transcribed with Superscript IV (Invitrogen), and tested with *16S* species-specific qPCR alongside a sterile filtered water (MILIQ) negative control. Samples were either (i) reverse-transcriptase negative representative of genomic DNA (DNA) or (ii) reverse-transcriptase positive representative of genomic DNA and *16S* RNA expression (DNA + RNA). qPCR signal cycle threshold (C_t Value; Log_2 scale) shown. *Undefined* results were given a value of 40. The no-template control (NTC) of the PCR was *undefined* for both *E. coli* and *S. aureus* (dotted line). Data were compared with a two-way ANOVA with post-hoc Bonferroni's multiple-comparisons test (NS $p > 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$). Triplicate replicated extractions with single reverse transcription reactions per extraction were performed. The mean ± SEM of three technical replicates is shown.

no significant difference between the column-based DNeasy and RNeasy PowerWater kits (Table 3). However, column-based kits increased the signal of nucleic acid when extracting from *E. coli* DNA and DNA + RNA (Figure 3) and when extracting *S. aureus* DNA (Figure S2) relative to the MagMAX kits. These data demonstrate that column-based extraction kits can significantly increase the signal of bacterial eDNA and eRNA from CFU and NA, however, not in all conditions. Nevertheless, the RNeasy PowerWater kit either significantly enhanced or was equally effective when isolating bacterial eDNA and eRNA from all tested conditions. Taken together, the above data provided an optimized protocol for the isolation of bacterial eDNA and eRNA from viable cells and free-NAs.

3.3 | The signal of eDNA and eRNA recoverable from viable cells in postcollection specimens exhibited species-specific and specimen-specific variation over time

There is often a delay between the time of sampling and laboratory processing. Therefore, we tested if the signal of eDNA and eRNA isolatable from *E. coli* and *S. aureus* varied over 12 h postsample collection and if this differed between specimens (fresh vs saltwater) and species (Figure 1c). The signal of viable cells did not change in saltwater (*S. aureus* CFU $p = 0.4062$, *E. coli* CFU $p = 0.0973$; Figure 4a) but decreased significantly in freshwater (*S. aureus* CFU $p = 0.0007$, *E. coli* CFU $p = 0.0052$; Figure 4c), while the signal of *E. coli* CFU decreased so dramatically that it was undetectable ($C_t > 40$) at 10 h post spike. DNA + RNA from CFU spikes were significantly correlated with time in both salt (*S. aureus* $p = 0.0047$, *E. coli* $p = 0.0076$; Figure 4a) and freshwater (*S. aureus* $p = 0.0001$, *E. coli* $p = 0.0014$; Figure 4c). When considering the difference between DNA and DNA + RNA (ΔC_t), each sample was less than two standard deviations from the condition mean (Table 4), demonstrating the signal of eRNA isolated from CFU generally followed eDNA concentration trends. These data demonstrate that the signal from viable cells in postcollection specimens varied between both the bacterial species and the specimen.

3.4 | Species and specimen-specific variation occurs over time in the signal of eDNA and eRNA recoverable from free nucleic acids

When considering the signal of NA spikes over 12 h in postcollection specimens, we found *E. coli* DNA samples were significantly correlated to time in both water types (salt $p = 0.0026$; Figure 4b, and fresh $p < 0.0001$; Figure 4d), while NA in *S. aureus* DNA samples

TABLE 2 Analysis of Variance (ANOVA) and multiple comparisons testing of bacterial eDNA and eRNA filtration optimization.

	ANOVA result			Multiple comparisons testing	
Detecting	**Organism**	**Water type**	**(*p*) Filter**	**Spike**	**(*p*) HA vs GF/F**
DNA	*E. coli*	Fresh	<0.0001	Colony Forming Units	<0.0001
				Nucleic Acid	<0.0001
	S. aureus	Fresh	<0.0001	Colony Forming Units	0.0038
				Nucleic Acid	0.0001
DNA + RNA	*E. coli*	Fresh	<0.0001	Colony Forming Units	0.0010
				Nucleic Acid	0.0001
	S. aureus	Fresh	0.0003	Colony Forming Units	0.1700
				Nucleic Acid	<0.0001

Abbreviation: *p*, *p*-value.

TABLE 3 Analysis of Variance (ANOVA) and multiple comparisons testing of bacterial eDNA and eRNA optimization of nucleic acid isolation kit.

	ANOVA results			Multiple comparisons testing			
Detecting	**Organism**	**Water type**	**(*p*) Kit**	**Spike**	**(*p*) DNeasy vs RNeasy**	**(*p*) DNeasy vs MagMAX Total**	**(*p*) DNeasy vs MagMAX *mirvanna***
DNA	*E. coli*	Fresh	<0.0001	Colony Forming Units	>0.9999	<0.0001	<0.0001
				Nucleic Acid	>0.9999	<0.0001	<0.0001
		Salt	0.0097	Colony Forming Units	>0.9999	0.1510	0.2778
				Nucleic Acid	>0.9999	0.1748	0.3579
	S. aureus	Fresh	<0.0001	Colony Forming Units	>0.9999	<0.0001	0.0002
				Nucleic Acid	>0.9999	0.0001	0.0007
		Salt	0.1309	Colony Forming Units	NA	NA	NA
				Nucleic Acid	NA	NA	NA
DNA + RNA	*E. coli*	Fresh	0.0534	Colony Forming Units	NA	NA	NA
				Nucleic Acid	NA	NA	NA
		Salt	0.0009	Colony Forming Units	>0.9999	0.0019	0.0055
				Nucleic Acid	0.7697	>0.9999	>0.9999
	S. aureus	Fresh	0.2552	Colony Forming Units	NA	NA	NA
				Nucleic Acid	NA	NA	NA
		Salt	0.1597	Colony Forming Units	NA	NA	NA
				Nucleic Acid	NA	NA	NA

Abbreviations: NA, non applicable; *p*, *p*-value.

was not affected by time in either (salt $p = 0.0717$; Figure 4b, and fresh $p = 0.8393$; Figure 4d). NA spiked DNA + RNA samples were significantly correlated with time in freshwater (*S. aureus* $p = 0.0278$, *E. coli* $p = 0.0343$; Figure 4a); however, only *E. coli* DNA + RNA was significantly correlated with time in saltwater (*S. aureus* $p = 0.3077$, *E. coli* $p = 0.0419$; Figure 4d). NA ΔC_t remained within two standard deviations of the mean for all tested conditions (Table 4), indicating a parallel trend in DNA + RNA signal with DNA signal similar to CFU. These findings highlight that substantial specimen- and species-specific variations of detectable eDNA and eRNA from viable cells and free-nucleic material can occur postcollection. We hypothesized that the species-specific differential rates of CFU and NA degradation were driven by microbiological activity.

3.5 | Sample sterilization can reduce eDNA degradation but may degrade the signal from DNA + RNA samples

To test if species-specific differences in degradation rates were caused by microbial activity and if degradation rates could be reduced through a reduction in microbial activity, we examined the degradation rate of CFU and NA in a freshwater sample containing 1% of the commercial sterilant Didecyldimethylammonium chloride (DDAC). The C_t between DNA and DNA + RNA isolated from freshwater containing DDAC was almost identical for both *S. aureus* (mean ΔC_t CFU = 0.124 and NA = 1.429; Table 4) and *E. coli* (mean ΔC_t CFU = 0.306 and NA = 1.147; Table 4), demonstrating DDAC rapidly

degraded the signal from DNA + RNA samples. In contrast, DDAC prevented eDNA degradation, as the C_t values of DNA samples did not significantly change with time in CFU spiked samples (*S. aureus* $p = 0.4124$, *E. coli* $p = 0.3279$, Figure 4e), nor in *E. coli* NA spiked samples ($p = 0.2122$; Figure 4f). However, the C_t values of DNA samples from *S. aureus* NA spiked samples decreased significantly over time ($p = 0.0244$; Figure 4f). These data indicate that DDAC, as a sterilizing reagent, is not suitable for preserving the signal from DNA + RNA samples; however, it can slow DNA sample degradation, although some degradation may still occur. Taken together, these data demonstrate that eRNA and eDNA signals in field samples can vary significantly over time and be affected by chemical and microbial degradation, and care must be taken to develop preservation strategies.

3.6 | The optimized protocol can detect as few as 10 bacterial cells above background in both DNA and DNA + RNA samples

We next determined the analytical sensitivity (i.e., the limit of detection) of several representative bacteria when extracted with our optimized protocol (Figure 1d). When isolating *E. coli* CFU from salt or fresh water, 10 cells (10^1 CFU/500 mL) were detected above the background in DNA (saltwater $p = 0.0469$, freshwater $p < 0.0001$; Table 5) and DNA + RNA samples (salt and freshwater $p < 0.0001$; Table 5). As demonstrated by a flattening titration curve, we found *S. marcescens* and *E. faecalis* had a relatively high background in freshwater (10^3 CFU; Figure 5), while *S. aureus* background (10^4 CFU) was present in fresh and saltwater (Figure 5). Nevertheless, 10 cells were detected above background in DNA + RNA samples when isolating *S. marcescens* ($p < 0.0001$; Table 5), and 100 cells above background were detected in DNA + RNA samples when isolating *E. faecalis* ($p = 0.0007$; Table 5). These data demonstrate that this protocol has an analytical sensitivity that can distinguish as few as 10 to 100 cells above the background for Gram-positive and Gram-negative species, respectively. We found the C_t values of all DNA and DNA + RNA samples with CFU titrations above 10^2 CFU/500 mL were significantly lower in Gram-negative species when compared to Gram-positive species (*S. marcescens* vs. *E. faecalis*; and *S. aureus* vs. *E. coli* 10^{7-2}, $p < 0.0001$ Figure 5). This suggested that the protocol was more efficient at isolating both eDNA and eRNA from Gram-negative species of bacteria.

3.7 | The extraction efficiency from viable bacteria cells is gram-stain-specific

To assess whether the difference between Gram-stain recovery was an artifact of our optimized protocol, we titrated *E. coli* and *S. aureus* purified nucleic acids (10^2 ng NA to 10^{-4} ng NA) into fresh and saltwater. When quantifying *E. coli* with RT-qPCR, we reliably detected as little as 10 picograms (10^{-2} ng NA) of DNA above background ($p < 0.0001$; Table 5) and 1 picogram of DNA + RNA above background ($p = 0.0059$; Table 5) in freshwater. A relatively high background of *S. aureus* was detected again. There was no significant difference in C_t values between DNA or DNA + RNA samples for most NA titrations in both salt and freshwater (Figure 5). These data demonstrate that our optimized protocol is equally efficient at purifying and quantifying NA from Gram-positive and gram-negative bacteria, and therefore, there is a bias toward the isolation of nucleic material from Gram-negative CFU. Taken together these data demonstrate that this protocol has a very high analytical sensitivity and is capable of isolating as little as 10 CFU or a picogram of nucleic material.

3.8 | The relative abundance of bacteria *16S* is gram-stain-specific

To investigate further if the extraction efficiency bias toward Gram-negative bacteria extended to other species, we performed an unbiased screen of the microbiome from the freshwater samples spiked with titrated CFU. We considered normalized species abundance above background as a positive detection and found the species-specific analytical sensitivity of shotgun microbiome sequencing to be relatively high (10^4 CFU/mL *E. faecalis*, 10^3 CFU/mL *E. coli* and *S. aureus*, 10^1 CFU/mL both *S. marcescens* and *K. pneumoniae*; Figure 6). These data demonstrate that microbiome shotgun sequencing allows detection of 10 CFU above background, which is equivalent to the analytical sensitivity of RT-qPCR. The abundance of the spiked Gram-negative bacteria was higher than the spiked Gram-positive bacteria (e.g., normalized species abundance from 10^7 CFU spike: 1.3×10^6 vs 4.4×10^3 reads, respectively, Figure 6). Furthermore, of the 21 taxonomic families that were present in greater than 1% of the total reads, none were Gram-positive (Figure 6). These data demonstrate that the bias toward the detection of Gram-negative bacteria is not specific to RT-qPCR. To investigate if the relative abundance of Gram-negative bacteria in the background sample reflected Gram-stain conditions, we visualized Gram-stained samples collected from the freshwater source. We found abundant Gram-positive bacteria (Figure S2A), and when Gram-negative and Gram-positive bacteria were co-localized, an even ratio was observed (Figure S2B). Taken together, these data demonstrate that there is a bias toward the isolation of bacterial eDNA and eRNA from viable Gram-negative cells. Our observations revealed a high repeatability in the normalized read number of the top three nonspiked background species: *Aeromonas hydrophila* (*A. hydrophila*), *Herbaspirillum seropedicae* (*H. seropedicae*), and *Chromobacterium violaceum* (*C. violaceum*; Figure 6). These findings suggest that although there is an extraction bias toward Gram-negative bacteria when determining relative abundance, shotgun sequencing consistently produces reliable data. The high repeatability in the normalized read number of the top three nonspiked background species supports the validity of studies that have reported changes to relative microbial populations.

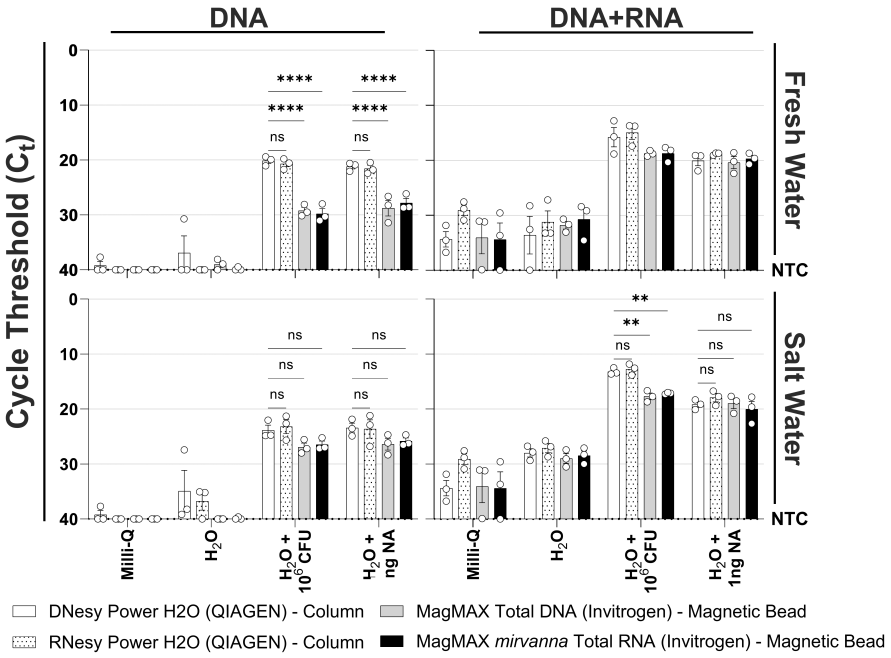

FIGURE 3 Optimization of the nucleic acid isolation kit for eDNA and eRNA microorganism detection: 10^6 *E. coli* colony forming units (CFU) or 1 ng of nucleic acids (NA) were spiked into 500 mL of freshwater or salt water, filtered with borosilicate glass filtration (GF/F) filters, and extracted with one of either the DNeasy PowerWater kit (white bars), the RNeasy PowerWater kit (white/spotted bars), the MagMAX™ Total Nucleic Acid Isolation Kit (gray bars), or the MagMAX™ *mirvanna* RNA Isolation Kit (black bars), then reverse-transcribed with Superscript IV (Invitrogen) and tested with *16S* species-specific qPCR, alongside a sterile filtered water (MILIQ), and background (H_2O) negative controls. Samples were either reverse-transcriptase negative representative of genomic DNA (DNA) or reverse-transcriptase positive representative of genomic DNA and *16S* RNA expression (DNA + RNA). qPCR signal cycle threshold (C_t Value; Log_2 scale) shown. *Undefined* results were given a value of 40. The no-template control (NTC) of the PCR was *undefined*. Data were compared with a two-way ANOVA with post-hoc Bonferroni's multiple-comparisons test; (NS $p > 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$). Triplicate replicated extractions with single reverse transcription reactions per extraction were performed. The mean ± SEM of three technical replicates are shown.

4 | DISCUSSION

In this study, we have identified a highly sensitive protocol for isolating bacterial eDNA and eRNA from aquatic specimens as a valuable tool for both qualitative and quantitative analysis of aquatic bacteria. The protocol detected very low numbers of viable cells and picograms of nucleic material. Such high analytical sensitivity is essential for high-accuracy qualitative species-specific pathogen diagnostics. Additionally, we have demonstrated several significant limitations of current common eDNA and eRNA technologies with important implications for microbiome-wide or quantitative studies. Specifically, we demonstrated that the time between sample collection and extraction can affect species-specific CFU and NA abundance. Furthermore, we found a bias toward the relative abundance of Gram-negative species of bacteria when isolating nucleic material from viable cells, in agreement with previous studies (Fernandez-Pato et al., 2024; Guo & Zhang, 2013). These data indicate that eDNA and eRNA aquatic bacterial abundance studies may be biased by the relative stability of CFUs and NAs within a specimen and the efficiency with which DNA and RNA can be isolated from viable cells. Furthermore, this highlights the need to define molecular diagnostic protocols for species-specific diagnostic sensitivity when monitoring aquatic bacterial eDNA and eRNA.

Most eDNA methodological studies have aimed to improve the sensitivity of their assays by improving field sampling methods (Burian et al., 2021). Herein, we have focused on optimization of postsampling laboratory processing, which, once optimized, reproducibly detected as little as 1 picogram of species-specific total nucleic acids (Figure 5). This likely represents a detection limit between 1 and 10 genomic copies (Land et al., 2015) from the total nucleic acids isolated from the *E. coli* and *S. aureus* sample recovery controls. During assay optimization, we found no amplification in our PCR no-template control (NTC). However, we routinely detected relatively low background (>30 C_t) amplification in the MilliQ negative control and H_2O background controls (Figures 2 and 3). This detection is likely due to the ubiquity of the model microorganisms (*S. aureus* and *E. coli*) targeted in these experiments and the potential for minor contamination of equipment and reagents. The diagnostic significance of the presence of microorganisms (DNA and RNA) in assay equipment and reagents is likely to vary between studies and target pathogens. We recommend reporting background amplification rather than using normalization methods such as subtracting these values from test results to ensure full transparency.

We evaluated the ability of several protocols to simultaneously isolate eDNA and eRNA from CFU and NA within aquatic specimens without the use of DNase or RNase nucleases. Multiple studies have

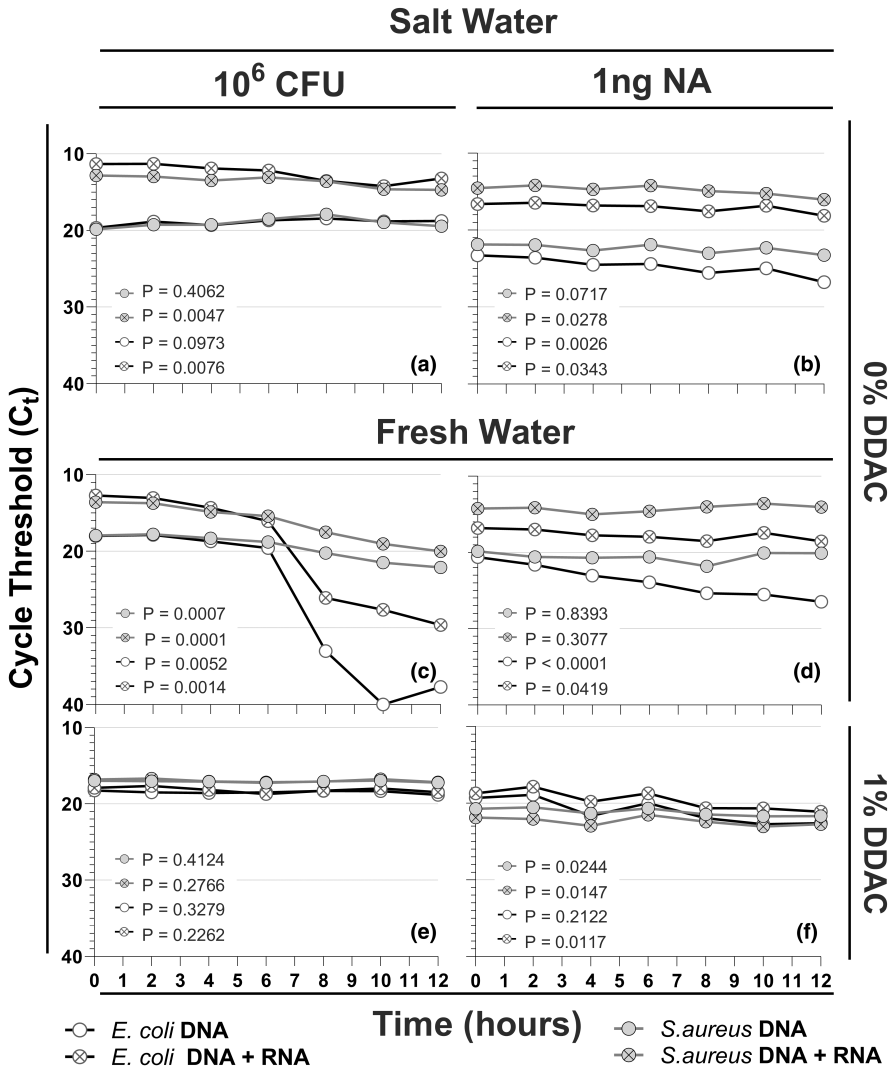

FIGURE 4 eDNA and eRNA sample degradation over time: 10^6 CFU/500 mL (a, c, and e) or 1 ng NA/500 mL (b, d, and f) of *E. coli* and *S. aureus* were added to 10 L of saltwater (a and b), or freshwater (c, d, e, and f), containing either 0% (a, b, c, and d) or 1% (e and f) sterilizing Didecyldimethylammonium chloride (DDAC) from which 500 mL was sampled at timepoints 0, 2, 4, 6, 8, 10, and 12 h "post spike." Samples were filtered with borosilicate glass filtration (GF/F) filters, extracted with either the RNeasy PowerWater kit (QIAGEN), reverse-transcribed with Superscript IV (Invitrogen), and tested with *16S* species-specific qPCR. Samples were either reverse-transcriptase negative representative of genomic DNA (DNA) or reverse-transcriptase positive representative of genomic DNA and *16S* RNA expression (DNA + RNA). qPCR signal cycle threshold (C_t Value; Log_2 scale, lower C_t is higher eDNA) is shown. *Undefined* results were given a value of 40. The no-template control of the PCR was *undefined*. Single extractions with single reverse transcription reactions per extraction were performed. The sample mean shown is calculated from technical triplicate qPCR.

TABLE 4 Relative signal over time.

Time (h)		0	2	4	6	8	10	12	Mean	±2 SD
CFU (ΔC_t)										
Salt	*E. coli*	8.339	7.536	7.422	6.494	4.890	4.618	5.543	6.406	2.863
	S. aureus	7.012	6.278	5.785	5.449	4.305	4.334	4.755	5.417	2.044
Fresh	*E. coli*	5.281	4.813	4.361	3.524	6.923	12.370	8.097	6.481	6.052
	S. aureus	4.347	4.049	3.411	3.364	2.732	2.433	2.083	3.203	1.666
Fresh + DDAC	*E. coli*	0.342	0.868	0.402	−0.219	0.026	0.368	0.352	0.306	0.676
	S. aureus	0.153	0.371	0.040	0.087	−0.035	0.183	0.070	0.124	0.261
NA (ΔC_t)										
Salt	*E. coli*	6.689	7.129	7.683	7.526	8.005	8.136	8.648	7.854	1.306
	S. aureus	7.307	7.712	7.964	7.671	8.076	7.046	7.216	7.614	0.779
Fresh	*E. coli*	3.817	4.634	5.287	5.969	6.856	8.098	7.915	6.460	3.255
	S. aureus	5.606	6.444	5.669	5.987	7.795	6.474	6.034	6.401	1.491
Fresh + DDAC	*E. coli*	2.570	3.148	1.313	1.531	0.464	0.288	0.141	1.147	2.328
	S. aureus	2.018	2.675	1.524	1.939	0.807	1.054	0.577	1.429	1.500

Note: Difference in RT-qPCR C_t values (ΔC_t) between DNA and DNA + RNA samples for colony forming units (CFU) or nucleic acids (NA) spiked into salt water (Salt), fresh water (Fresh), or fresh water and 1% Didecyldimethylammonium chloride (Fresh + DDAC).

Abbreviation: SD, standard deviation.

TABLE 5 The analytical sensitivity of RT-qPCR for the detection of bacteria's eDNA and eRNA.

	CFU								NA				
	DNA				DNA + RNA				DNA			DNA + RNA	
(0) vs	EC	SA	SM	EF	EC	SA	SM	EF	(0) vs	EC	SA	EC	SA
Salt													
10^7	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	10^2	<0.0001	<0.0001	<0.0001	<0.0001
10^6	<0.0001	<0.0001	<0.0001	0.0024	<0.0001	<0.0001	<0.0001	<0.0001	10^1	<0.0001	<0.0001	<0.0001	<0.0001
10^5	<0.0001	0.0108	<0.0001	>0.9999	<0.0001	0.0018	<0.0001	<0.0001	10^0	<0.0001	0.0013	<0.0001	<0.0001
10^4	<0.0001	0.8922	<0.0001	>0.9999	<0.0001	>0.9999	<0.0001	<0.0001	10^{-1}	0.0011	>0.9999	0.0005	0.1438
10^3	<0.0001	>0.9999	<0.0001	>0.9999	<0.0001	>0.9999	<0.0001	<0.0001	10^{-2}	0.6161	0.1133	0.0305	>0.9999
10^2	<0.0001	0.6269	<0.0001	>0.9999	<0.0001	0.8108	<0.0001	0.0007	10^{-3}	>0.9999	0.0400	>0.9999	>0.9999
10^1	0.0469	0.1962	0.0631	>0.9999	<0.0001	>0.9999	<0.0001	>0.9999	10^{-4}	>0.9999	>0.9999	>0.9999	>0.9999
Fresh													
10^7	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	10^2	<0.0001	0.0001	<0.0001	<0.0001
10^6	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	10^1	<0.0001	0.1117	<0.0001	<0.0001
10^5	<0.0001	0.0013	<0.0001	<0.0001	<0.0001	0.0002	<0.0001	0.0340	10^0	<0.0001	>0.9999	<0.0001	<0.0001
10^4	<0.0001	>0.9999	<0.0001	>0.9999	<0.0001	>0.9999	0.0985	>0.9999	10^{-1}	<0.0001	>0.9999	<0.0001	0.1933
10^3	<0.0001	>0.9999	0.2943	0.2713	<0.0001	>0.9999	>0.9999	>0.9999	10^{-2}	<0.0001	>0.9999	<0.0001	>0.9999
10^2	<0.0001	>0.9999	>0.9999	>0.9999	<0.0001	>0.9999	>0.9999	>0.9999	10^{-3}	0.2449	>0.9999	0.0059	>0.9999
10^1	<0.0001	>0.9999	>0.9999	0.9904	<0.0001	0.0834	0.7096	>0.9999	10^{-4}	>0.9999	>0.9999	>0.9999	>0.9999

Note: The limit of detection of saltwater (Salt) and freshwater (Fresh) samples spiked with colony forming units (CFU) or nucleic acids (NA), exhibiting a statistically significant increase in measured nucleic material (C_t values) as compared to the background (0). Shown are the *p* value of Bonferroni-corrected multiple comparisons tests between C_t values and the background.

Abbreviations: EC, *Escherichia coli*; EF, *Enterococcus faecalis*; SA, *Staphylococcus aureus*; SM, *Serratia marcescens*.

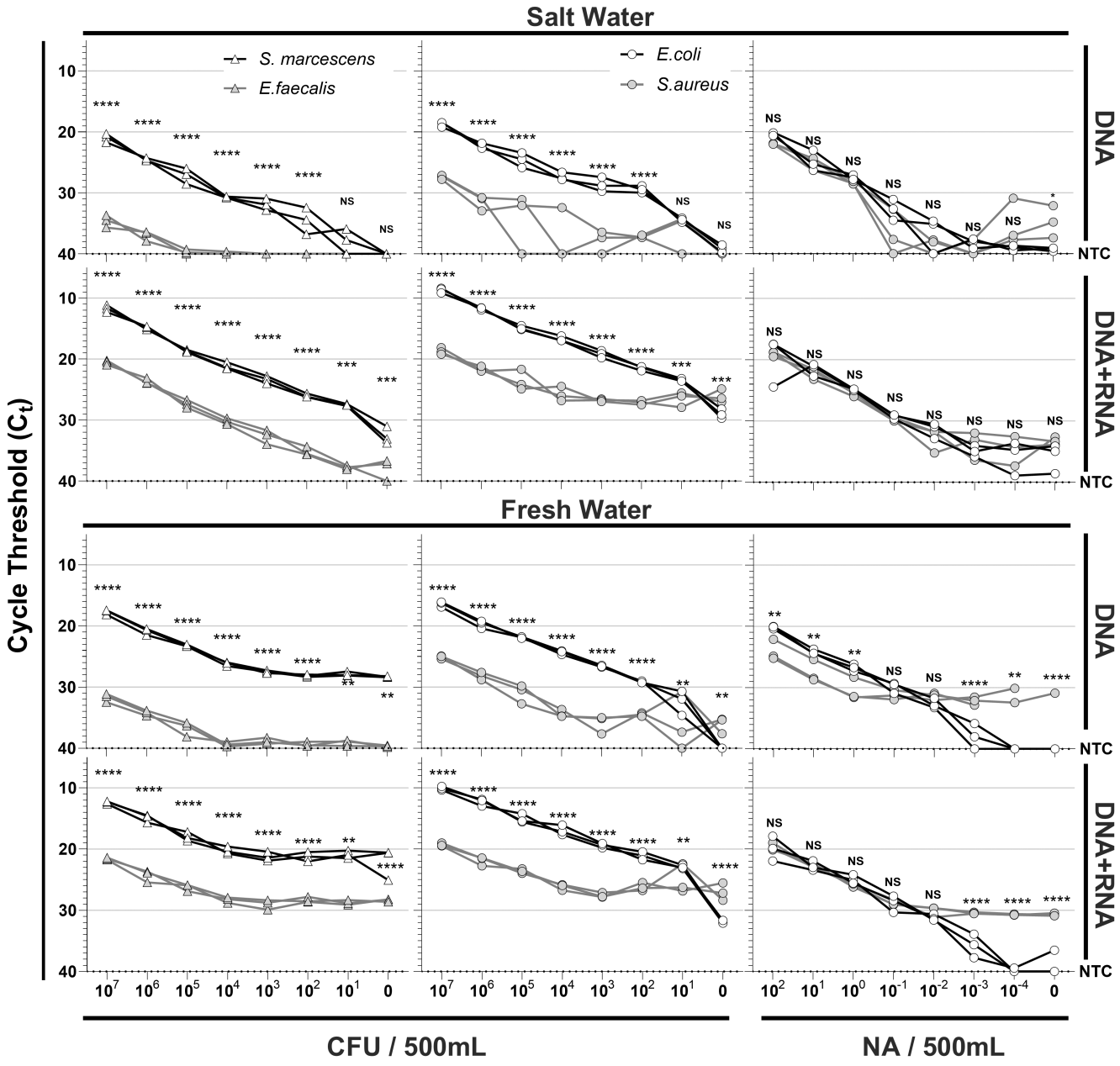

FIGURE 5 RT-qPCR analytical sensitivity. Titrated colony forming units (10^6 CFU to 10^1 CFU) or purified nucleic acids (10^2 ng NA to 10^{-4} ng NA) of the Gram-negative bacteria *E. coli* (white circles) and the Gram-positive bacteria *S. aureus* (gray circles) were spiked into salt or freshwater, filtered with borosilicate glass filtration (GF/F) filters, extracted with the RNeasy PowerWater kit, reverse-transcribed with Superscript IV (Invitrogen), and tested with *16S* (Bacteria) species-specific qPCR, alongside a background (0) control. The titrated CFU of *S. marcescens* (white triangles) and *E. faecalis* (gray triangles) are shown. Samples were either reverse-transcriptase negative representative of genomic DNA (DNA) or reverse-transcriptase positive representative of genomic DNA and *16S* RNA expression (DNA + RNA). qPCR signal cycle threshold (C_t Value; Log_2 scale, lower C_t is higher eDNA) is shown. *Undefined* results were given a value of 40. The no-template control (NTC) of the PCR was *undefined* for all species tested. Data were compared with a two-way ANOVA with post-hoc Bonferroni's multiple-comparisons test; shown are *E. coli* vs *S. aureus* (NS $p > 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$). Triplicate replicated extractions with single reverse transcription reactions per extraction were performed. The mean of the sample technical triplicate shown.

indicated that silica-based isolation methods can successfully extract both DNA and RNA without mutual inhibition (Mirna Lorena et al., 2023; Sanchez et al., 2015). Following co-isolation, genomic and transcriptomic studies often remove RNA or DNA with DNase or RNase endonucleases to ensure accurate quantification. In this study, we did not quantify eDNA or eRNA but rather sought to optimize the analytical sensitivity of the molecular diagnostic for bacterial detection by maximizing the RTqPCR signal. We assessed the RTqPCR signal from two reverse transcription reactions from each specimen; one reaction lacked reverse-transcriptase and contained only genomic eDNA (DNA), while the other included reverse-transcriptase and contained both eDNA and eRNA as

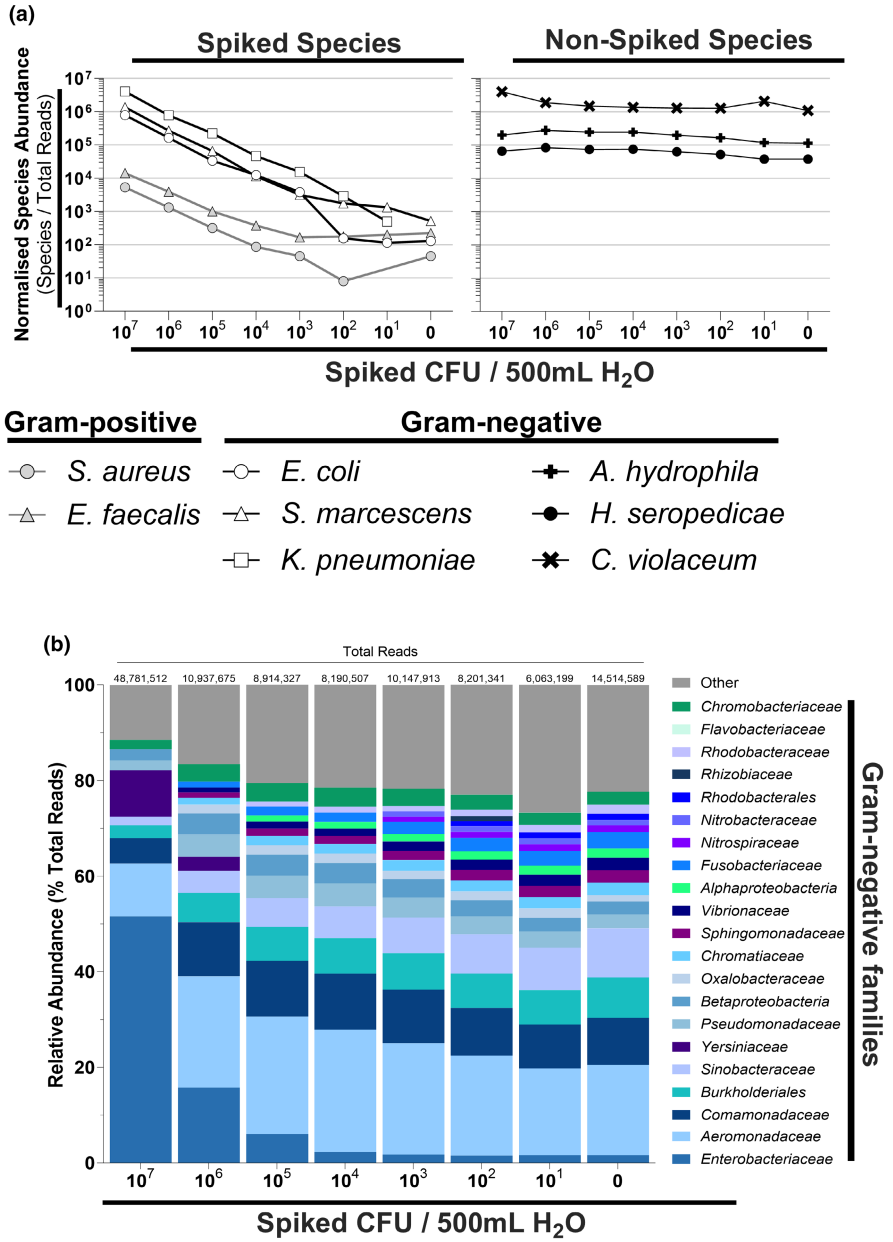

FIGURE 6 The analytical sensitivity of shotgun sequencing of the optimized protocol for pathogenic microorganism detection. (a) Titrated colony forming units (10^6 CFU to 10^1 CFU) of three Gram-negative bacteria, *E. coli* (white circles), *S. marcescens* (white triangles), *K. pneumoniae* (white squares), two Gram-positive bacteria, *S. aureus* (gray circles), and *E. faecalis* (gray triangles), were spiked into 500 mL of freshwater (H_2O), filtered with borosilicate glass filtration (GF/F) filters, extracted with the RNeasy PowerWater kit, and sequenced with unbiased *16S*, *18S*, and *ITS* shotgun sequencing, alongside a background H_2O (0) control. (b) Triplicate replicate extractions were pooled into a single sample for sequencing. Shown are microorganism taxonomic families with >1% relative abundance, and the species-specific abundance is normalized to total reads. The technical mean is shown.

complementary DNA (DNA + RNA). Following optimization, as expected, the analytical sensitivity of DNA + RNA samples was higher than the DNA-only samples by at least 10-fold across several species of bacteria in both fresh and saltwater specimens (Table 5). This demonstrates that a strategy of generating DNA + RNA samples can improve the analytical sensitivity of bacterial environmental monitoring. However, great care should be taken when inferring abundance with DNA + RNA samples, as bacterial gene expression can vary greatly (Sharkey et al., 2004) and studies quantifying bacterial eRNA expression may require the use of DNase enzymes for optimal RNA analysis.

High diagnostic sensitivity is perceived as a major advantage of species-specific molecular diagnostics (Furlan et al., 2016). However, few studies have determined the LOD of their target species following laboratory sample processing, despite calls to provide this information (Borchardt et al., 2021; Ciesielski et al., 2021; Klymus et al., 2020). Of the studies that have investigated the LOD of eDNA technologies with sample recovery controls (such as those detecting freshwater mussels (Mauvisseau et al., 2019), human coronavirus (SARS-Cov2) (Philo et al., 2021), or aquatic vertebrates (Brys et al., 2021; Mauvisseau et al., 2020)), most have reported LOD between 1 and 10 picograms or between 1 and 100 copies of the target. In addition to the collection of NA, microbiological eDNA studies have the capacity to collect viable cells within the specimen. Herein, we report an analytical sensitivity of between 10 and 100 CFU in 500 mL (Figure 5). Studies investigating micro-parasites (Merou et al., 2020), parasitic helminths (Sengupta et al., 2019), and algae (Knudsen et al., 2022) have reported LOD ranges between 1 and 100 viable cells. A bacterial eDNA and eRNA extraction protocol with equivalent analytical

sensitivity to best practice eDNA methodological studies is expected to be a valuable tool as a qualitative diagnostic, such as when screening for specific pathogens in aquaculture (Peters et al., 2018; Shea et al., 1937).

The research aquariums used in this study were designed to simulate large, complex, and diverse aquatic ecosystems while providing a stable and controlled setting. These aquariums are indicative of a range of low-turbidity conditions applicable to this technology, including in aquaculture, in industrial settings such as drinking water systems or cooling towers, and in recreational and wild habitats characterized by low turbidity. Nevertheless, the results of this study and the diagnostic sensitivity of each target pathogen must be independently validated in each environment. We found silica-column isolation techniques to be the most effective strategy to isolate nucleic acids from CFU and NA spiked into these specimens. Previous studies had indicated that bead-based isolation methods might enhance nucleic acid extraction from turbid or otherwise contaminated aquatic samples (Byrne et al., 2022; Williams et al., 2017). Consequently, it is plausible that bacterial CFU and NA extractions could be more efficient using alternative isolation kits designed for different environmental conditions, and further optimization will likely be required when isolating bacteria from turbid specimens.

All assays inherently have some degree of imperfect sensitivity, which should be considered when interpreting their results (Furlan et al., 2016). Quantitative studies are especially susceptible to variations in sensitivity. Indeed, the interpretation of eDNA abundance remains controversial, as some studies have found the correlation between eDNA and species abundance to be high (Di Muri et al., 2020; Olds et al., 2016), while others have demonstrated a very low or no significant correlation (Bradley et al., 2022; Danziger et al., 2022). Degradation of eDNA and eRNA contributes significantly to lost sensitivity and, therefore, a reduced correlation between eDNA and species abundance (Kumar et al., 2020). This report identified species-specific loss of bacterial eDNA and eRNA signals in a postcollection sample (Figure 4). It has been noted that conditions where aquatic eDNA and eRNA most rapidly degrade (e.g., high temperature (Tsuji et al., 2017), neutral pH (Seymour et al., 2018)) are those most favorable for microbial growth (Kumar et al., 2020). Consistent with the literature, our findings indicate that 1% DDAC significantly reduced eDNA degradation (Yamanaka et al., 2017). However, 1% DDAC rapidly degraded eRNA, making this preservation strategy unsuitable for eRNA studies. We speculate that when a specimen cannot be filtered in situ or cold-chain collection is impractical, the introduction of a sterilizing agent that does not degrade nucleic material will help maintain the correlation between bacterial eDNA and species abundance.

This report identified that when isolating eDNA and eRNA from viable cells, the concentration of nucleic material extracted is higher from Gram-negative than Gram-positive bacteria (Figure 5). It is possible that differential extraction efficiencies between bacteria, due to cell wall lysis resistance, are contributing significantly to variations between bacterial eDNA abundance studies. Variable isolation of nucleic material from viable cells is recognized as a key contributor to the low diagnostic sensitivities of bacterial molecular diagnostics for human diseases (Gosiewski et al., 2014; Liang et al., 2018; Petralia & Conoci, 2017). Strategies such as enzymatic (e.g., lysozyme, proteinase K) (Yuan et al., 2012) or mechanical (e.g., bead-beating, heat, pressure) (Raja et al., 2005) lysis have been developed, which have improved the sensitivity of several microbial diagnostic assays (Petralia & Conoci, 2017). It has been suggested that the implementation of such strategies during bacterial eDNA and eRNA isolation would likely improve the precision of microbiome-wide relative abundance screens (Albertsen et al., 2015; Pollock et al., 2018). We speculate that effective eDNA or eRNA molecular diagnostics will require the development of pathogen-specific detection protocols.

When considering high-throughput technologies, our data demonstrates that both RT-qPCR and shotgun sequencing have high analytical sensitivity. RT-qPCR is widely considered the gold standard molecular diagnostic (Browne et al., 2020), and when screening eDNA samples, RT-qPCR typically has a higher positive detection rate than the sequencing-based technique metabarcoding (Harper et al., 2018; Yu et al., 2022). However, others have shown equivalent sensitivity between RT-qPCR and high-throughput platforms (Devonshire et al., 2013). The rapid integration of high-throughput systems into biosecurity screening (DAFF, 2022; Lebas et al., 2022) highlights the need to define species-specific analytical sensitivity, particularly as our data suggests a Gram-type bias may be present due to variable extraction efficiency. Despite this, consistent measurements of nonspiked background species validate the reliability of population variations detected by high-throughput microbiome sequencing studies.

In conclusion, we report herein a highly sensitive protocol for the detection of microorganism eDNA and eRNA in aquatic samples. This protocol defines GF/F filters and silica-column-based nucleic acid isolation as an optimal postcollection protocol to purify low abundance microbial CFU and NA. We report that relatively rapid changes in eDNA and eRNA can occur in specimens containing both CFU and NA, and that sample sterilization with DDAC reduces eDNA degradation. When comparing the cross-species relative analytical sensitivity of pathogenic microorganisms in this protocol, we found the extraction efficiency of Gram-negative bacteria was significantly higher than that of Gram-positive bacteria, which was confirmed with unbiased sequencing and Gram staining. These data demonstrate several key limitations that must be considered to produce accurate data for eDNA and eRNA screening diagnostics with RT-qPCR and microbiome analysis with high-throughput sequencing. Especially, the importance of establishing a protocols species-specific diagnostic sensitivity when monitoring aquatic bacterial eDNA and eRNA with molecular diagnostics.

AUTHOR CONTRIBUTIONS

DJB, CMM, EPO'H, and RO performed experiments. DJB, DLD, RC, JS, and RO designed the study. DJB and RO wrote the manuscript, with editorial input from all authors.

ACKNOWLEDGMENTS

The authors thank Dr Andreas Kupz (JCU) and Pathology Queensland for the kind gift of clinical bacterial isolates, Ms. Jessica Watt (JCU), and Ms. Sharron Long (JCU) for assistance with Gram-staining. We also thank the efforts and support of the many staff and volunteers who maintain JCU's marine and freshwater research aquariums.

FUNDING INFORMATION

RO was supported by a Department of Agriculture, Water and Environment's Science and Innovation Awards for Young People in Agriculture, Fisheries and Forestry grant. DJB was supported by a James Cook University (JCU) Prestige Research Training Program Stipend (RTPS). DLD was supported by an NHMRC Principal Research Fellowship (#1137285). The research was supported by funding from the JCU Centre for Molecular Therapeutics Researcher Funding Scheme.

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The microbiome shotgun sequencing data generated herein, which supports the findings of this study, is openly available in the National Center for Biotechnology Information's Sequencing Read Archive, Accession PRJNA1046649. All other datasets for this study are presented in this publication.

ORCID

Daniel J. Browne https://orcid.org/0000-0003-1840-8904

REFERENCES

Albertsen, M., Karst, S. M., Ziegler, A. S., Kirkegaard, R. H., & Nielsen, P. H. (2015). Back to basics – The influence of DNA extraction and primer choice on phylogenetic analysis of activated sludge communities. *PLoS One*, *10*(7), e0132783.

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.

Batovska, J., Piper, A. M., Valenzuela, I., Cunningham, J. P., & Blacket, M. J. (2021). Developing a non-destructive metabarcoding protocol for detection of pest insects in bulk trap catches. *Scientific Reports*, *11*(1), 7946.

Biedka, S., Alkam, D., Washam, C. L., Yablonska, S., Storey, A., Byrum, S. D., & Minden, J. S. (2024). One-pot method for preparing DNA, RNA, and protein for multiomics analysis. *Communications Biology*, *7*(1), 324.

Bivins, A., Kaya, D., Bibby, K., Simpson, S. L., Bustin, S. A., Shanks, O. C., & Ahmed, W. (2021). Variability in RT-qPCR assay parameters indicates unreliable SARS-CoV-2 RNA quantification for wastewater surveillance. *Water Research*, *203*, 117516.

Borchardt, M. A., Boehm, A. B., Salit, M., Spencer, S. K., Wigginton, K. R., & Noble, R. T. (2021). The environmental microbiology minimum information (EMMI) guidelines: qPCR and dPCR quality and reporting for environmental microbiology. *Environmental Science & Technology*, *55*(15), 10210–10223.

Bowers, H. A., Pochon, X., von Ammon, U., Gemmell, N., Stanton, J-A. L., Jeunen, G-J., Sherman, C. D. H., & Zaiko, A. (2021). Towards the optimization of eDNA/eRNA sampling Technologies for Marine Biosecurity Surveillance. *Water*, *13*(8), 1113. https://doi.org/10.3390/w13081113

Bradley, D. L., Morey, K. C., Bourque, D. A., Fost, B., Loeza-Quintana, T., & Hanner, R. H. (2022). Environmental DNA detection and abundance estimates comparable to conventional methods for three freshwater larval species at a power plant discharge. *Environmental DNA*, *4*(3), 700–714.

Browne, D. J., Brady, J. L., Waardenberg, A. J., Loiseau, C., & Doolan, D. L. (2020). An analytically and diagnostically sensitive RNA extraction and RT-qPCR protocol for peripheral blood mononuclear cells. *Frontiers in Immunology*, *11*, 402.

Browne, D. J., Kelly, A. M., Brady, J. L., & Doolan, D. L. (2022). A high-throughput screening RT-qPCR assay for quantifying surrogate markers of immunity from PBMCs. *Frontiers in Immunology*, *13*, 962220.

Browne, D. J., et al. (2022). Multiplex microsphere PCR (mmPCR) allows simultaneous gram typing, detection of fungal DNA, and antibiotic resistance genes. *Laboratoriums Medizin*, *53*(5), 459–464.

Brys, R., Halfmaerten, D., Neyrinck, S., Mauvisseau, Q., Auwerx, J., Sweet, M., & Mergeay, J. (2021). Reliable eDNA detection and quantification of the European weather loach (Misgurnus fossilis). *Journal of Fish Biology*, *98*(2), 399–414.

Burian, A., Mauvisseau, Q., Bulling, M., Domisch, S., Qian, S., & Sweet, M. (2021). Improving the reliability of eDNA data interpretation. *Molecular Ecology Resources*, *21*(5), 1422–1433.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, *55*(4), 611–622.

Byrne, R. L., Cocker, D., Alyayyoussi, G., Mphasa, M., Charles, M., Mandula, T., Williams, C. T., Rigby, J., Hearn, J., Feasey, N., Adams, E. R., & Edwards, T. (2022). A novel, magnetic bead-based extraction method for the isolation of antimicrobial resistance genes with a case study in river water in Malawi. *Journal of Applied Microbiology*, *133*(5), 3191–3200.

Ciesielski, M., Blackwood, D., Clerkin, T., Gonzalez, R., Thompson, H., Larson, A., & Noble, R. (2021). Assessing sensitivity and reproducibility of RT-ddPCR and RT-qPCR for the quantification of SARS-CoV-2 in wastewater. *Journal of Virological Methods*, *297*, 114230.

DAFF. (2022). *National biosecurity strategy*. F.a.F. Department of Agriculture.

Danziger, A. M., Olson, Z. H., & Frederich, M. (2022). Limitations of eDNA analysis for *Carcinus maenas* abundance estimations. *BMC Ecology and Evolution*, *22*(1), 14.

Darling, J. A., Pochon, X., Abbott, C. L., Inglis, G. J., & Zaiko, A. (2020). The risks of using molecular biodiversity data for incidental detection of species of concern. *Diversity and Distributions*, *26*(9), 1116–1121.

De Brauwer, M., et al. (2023). Best practice guidelines for environmental DNA biomonitoring in Australia and New Zealand. *Environmental DNA*, *5*(3), 417–423.

de Bruin, O. M., & Birnboim, H. C. (2016). A method for assessing efficiency of bacterial cell disruption and DNA release. *BMC Microbiology*, *16*(1), 197.

DeAngelis, K. M., Silver, W. L., Thompson, A. W., & Firestone, M. K. (2010). Microbial communities acclimate to recurring changes in soil redox potential status. *Environmental Microbiology*, *12*(12), 3137–3149.

Deleu, J., Schoofs, K., Decock, A., Verniers, K., Roelandt, S., Denolf, A., Verreth, J., de Wilde, B., van Maerken, T., de Preter, K., & Vandesompele, J. (2022). Digital PCR-based evaluation of nucleic

acid extraction kit performance for the co-purification of cell-free DNA and RNA. *Human Genomics*, *16*(1), 73.

Devonshire, A. S., Sanders, R., Wilkes, T. M., Taylor, M. S., Foy, C. A., & Huggett, J. F. (2013). Application of next generation qPCR and sequencing platforms to mRNA biomarker analysis. *Methods*, *59*(1), 89–100.

Di Muri, C., et al. (2020). Read counts from environmental DNA (eDNA) metabarcoding reflect fish abundance and biomass in drained ponds. *Metabarcoding and Metagenomics*, *4*, 56959.

Dickie, I. A., Boyer, S., Buckley, H. L., Duncan, R. P., Gardner, P. P., Hogg, I. D., Holdaway, R. J., Lear, G., Makiola, A., Morales, S. E., Powell, J. R., & Weaver, L. (2018). Towards robust and repeatable sampling methods in eDNA-based studies. *Molecular Ecology Resources*, *18*(5), 940–952.

Djurhuus, A., Port, J., Closek, C. J., Yamahara, K. M., Romero-Maraccini, O., Walz, K. R., Goldsmith, D. B., Michisaki, R., Breitbart, M., Boehm, A. B., & Chavez, F. P. (2017). Evaluation of filtration and DNA extraction methods for environmental DNA biodiversity assessments across multiple trophic levels. *Frontiers in Marine Science*, *4*, 314.

Espy, M. J., Uhl, J. R., Sloan, L. M., Buckwalter, S. P., Jones, M. F., Vetter, E. A., Yao, J. D. C., Wengenack, N. L., Rosenblatt, J. E., Cockerill, F. R., III, & Smith, T. F. (2006). Real-time PCR in clinical microbiology: Applications for a routine laboratory testing. *Clinical Microbiology Reviews*, *19*(1), 165–256.

Farrell, J. A., Whitmore, L., & Duffy, D. J. (2021). The promise and pitfalls of environmental DNA and RNA approaches for the monitoring of human and animal pathogens from aquatic sources. *Bioscience*, *71*(6), 609–625.

Feldsine, P., Abeyta, C., & Andrews, W. H. (2002). AOAC INTERNATIONAL methods committee guidelines for validation of qualitative and quantitative food microbiological official methods of analysis. *Journal of AOAC International*, *85*(5), 1187–1200.

Fernandez-Pato, A., et al. (2024). Choice of DNA extraction method affects stool microbiome recovery and subsequent phenotypic association analyses. *Scientific Reports*, *14*(1), 3911.

Furlan, E. M., Gleeson, D., Hardy, C. M., & Duncan, R. P. (2016). A framework for estimating the sensitivity of eDNA surveys. *Molecular Ecology Resources*, *16*(3), 641–654.

Galvani, A. T., Christ, A. P. G., Padula, J. A., Barbosa, M. R. F., de Araújo, R. S., Sato, M. I. Z., & Razzolini, M. T. P. (2019). Real-time PCR detection of *Toxoplasma gondii* in surface water samples in Sao Paulo, Brazil. *Parasitology Research*, *118*(2), 631–640.

Gao, W., Zhang, W., & Meldrum, D. R. (2011). RT-qPCR based quantitative analysis of gene expression in single bacterial cells. *Journal of Microbiological Methods*, *85*(3), 221–227.

Gillies, L. E., Thrash, J. C., deRada, S., Rabalais, N. N., & Mason, O. U. (2015). Archaeal enrichment in the hypoxic zone in the northern Gulf of Mexico. *Environmental Microbiology*, *17*(10), 3847–3856.

Goldberg, C. S., Turner, C. R., Deiner, K., Klymus, K. E., Thomsen, P. F., Murphy, M. A., Spear, S. F., McKee, A., Oyler-McCance, S. J., Cornman, R. S., Laramie, M. B., Mahon, A. R., Lance, R. F., Pilliod, D. S., Strickler, K. M., Waits, L. P., Fremier, A. K., Takahara, T., Herder, J. E., & Taberlet, P. (2016). Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution*, *7*(11), 1299–1307.

Gonzalez, D. L., Baláž, V., Vojar, J., & Chajma, P. (2021). Dual detection of the chytrid fungi Batrachochytrium spp. with an enhanced environmental DNA approach. *Journal of Fungi*, *7*(4), 258. https://doi.org/10.3390/jof7040258

Gosiewski, T., Szała, L., Pietrzyk, A., Brzychczy-Włoch, M., Heczko, P. B., & Bulanda, M. (2014). Comparison of methods for isolation of bacterial and fungal DNA from human blood. *Current Microbiology*, *68*(2), 149–155. https://doi.org/10.1007/s00284-013-0451-1

Govindarajan, A. F., McCartin, L., Adams, A., Allan, E., Belani, A., Francolini, R., Fujii, J., Gomez-Ibañez, D., Kukulya, A., Marin, F., Tradd, K., Yoerger, D. R., McDermott, J. M., & Herrera, S. (2022). Improved biodiversity detection using a large-volume environmental DNA sampler with in situ filtration and implications for marine eDNA sampling strategies. *Deep Sea Research Part I: Oceanographic Research Papers*, *189*, 103871.

Grima, N., Henden, L., Watson, O., Blair, I. P., & Williams, K. L. (2022). Simultaneous isolation of high-quality RNA and DNA from post-mortem human central nervous system tissues for omics studies. *Journal of Neuropathology and Experimental Neurology*, *81*(2), 135–145.

Grumaz, C., Hoffmann, A., Vainshtein, Y., Kopp, M., Grumaz, S., Stevens, P., Decker, S. O., Weigand, M. A., Hofer, S., Brenner, T., & Sohn, K. (2020). Rapid next-generation sequencing-based diagnostics of bacteremia in septic patients. *The Journal of Molecular Diagnostics*, *22*(3), 405–418.

Guo, F., & Zhang, T. (2013). Biases during DNA extraction of activated sludge samples revealed by high throughput sequencing. *Applied Microbiology and Biotechnology*, *97*(10), 4607–4616.

Handley, L. L. (2015). How will the "molecular revolution" contribute to biological recording? *Biological Journal of the Linnean Society*, *115*(3), 750–766.

Harper, L. R., Lawson Handley, L., Hahn, C., Boonham, N., Rees, H. C., Gough, K. C., Lewis, E., Adams, I. P., Brotherton, P., Phillips, S., & Hänfling, B. (2018). Needle in a haystack? A comparison of eDNA metabarcoding and targeted qPCR for detection of the great crested newt (*Triturus cristatus*). *Ecology and Evolution*, *8*(12), 6330–6341.

He, H., Li, R., Chen, Y., Pan, P., Tong, W., Dong, X., Chen, Y., & Yu, D. (2017). Integrated DNA and RNA extraction using magnetic beads from viral pathogens causing acute respiratory infections. *Scientific Reports*, *7*(1), 45199.

Holman, L. E., Chng, Y., & Rius, M. (2022). How does eDNA decay affect metabarcoding experiments? *Environmental DNA*, *4*(1), 108–116.

Jensen, W. B. (2006). The origins of the Hirsch and Buchner vacuum filtration funnels. *Journal of Chemical Education*, *83*(9), 1283.

Kajiura, L. N., Stewart, S. D., Dresios, J., & Uyehara, C. F. T. (2015). Simultaneous extraction of viral and bacterial nucleic acids for molecular diagnostic applications. *Journal of Biomolecular Techniques*, *26*(4), 118–124.

Klaschik, S., Lehmann, L. E., Raadts, A., Book, M., Hoeft, A., & Stuber, F. (2002). Real-time PCR for detection and differentiation of gram-positive and gram-negative bacteria. *Journal of Clinical Microbiology*, *40*(11), 4304–4307.

Klymus, K. E., Merkes, C. M., Allison, M. J., Goldberg, C. S., Helbing, C. C., Hunter, M. E., Jackson, C. A., Lance, R. F., Mangan, A. M., Monroe, E. M., Piaggio, A. J., Stokdyk, J. P., Wilson, C. C., & Richter, C. A. (2020). Reporting the limits of detection and quantification for environmental DNA assays. *Environmental DNA*, *2*(3), 271–282.

Knudsen, S. W., Hesselsøe, M., Thaulow, J., Agersnap, S., Hansen, B. K., Jacobsen, M. W., Bekkevold, D., Jensen, S. K. S., Møller, P. R., & Andersen, J. H. (2022). Monitoring of environmental DNA from nonindigenous species of algae, dinoflagellates and animals in the North East Atlantic. *Science of the Total Environment*, *821*, 153093.

Kotorashvili, A., Ramnauth, A., Liu, C., Lin, J., Ye, K., Kim, R., Hazan, R., Rohan, T., Fineberg, S., & Loudig, O. (2012). Effective DNA/RNA Co-extraction for analysis of MicroRNAs, mRNAs, and genomic DNA from formalin-fixed paraffin-embedded specimens. *PLoS One*, *7*(4), e34683.

Kralik, P., & Ricchi, M. (2017). A basic guide to real time PCR in microbial diagnostics: Definitions, parameters, and everything. *Frontiers in Microbiology*, *8*, 108.

Kubota, H., Tsuji, H., Matsuda, K., Kurakawa, T., Asahara, T., & Nomoto, K. (2010). Detection of human intestinal catalase-negative, gram-positive cocci by rRNA-targeted reverse transcription-PCR. *Applied and Environmental Microbiology*, *76*(16), 5440–5451.

Kumar, G., Eble, J. E., & Gaither, M. R. (2020). A practical guide to sample preservation and pre-PCR processing of aquatic environmental DNA. *Molecular Ecology Resources*, *20*(1), 29–39.

Land, M., Hauser, L., Jun, S. R., Nookaew, I., Leuze, M. R., Ahn, T. H., Karpinets, T., Lund, O., Kora, G., Wassenaar, T., Poudel, S., & Ussery, D. W. (2015). Insights from 20 years of bacterial genome sequencing. *Functional & Integrative Genomics*, *15*(2), 141–161.

Lebas, B., Adams, I., al Rwahnih, M., Baeyen, S., Bilodeau, G. J., Blouin, A. G., Boonham, N., Candresse, T., Chandelier, A., de Jonghe, K., Fox, A., Gaafar, Y. Z. A., Gentit, P., Haegeman, A., Ho, W., Hurtado-Gonzales, O., Jonkers, W., Kreuze, J., Kutjnak, D., … Massart, S. (2022). Facilitating the adoption of high-throughput sequencing technologies as a plant pest diagnostic test in laboratories: A step-by-step description. *EPPO Bulletin*, *52*(2), 394–418.

Lee, A. H., Lee, J., Noh, J., Lee, C., Hong, S., Kwon, B. O., Kim, J. J., & Khim, J. S. (2020). Characteristics of long-term changes in microbial communities from contaminated sediments along the west coast of South Korea: Ecological assessment with eDNA and physicochemical analyses. *Marine Pollution Bulletin*, *160*, 111592.

Liang, F., Browne, D. J., Gray, M. J., Gartlan, K. H., Smith, D. D., Barnard, R. T., Hill, G. R., Corrie, S. R., & Markey, K. A. (2018). Development of a multiplexed microsphere PCR for culture-free detection and gram-typing of bacteria in human blood samples. *ACS Infectious Diseases*, *4*(5), 837–844.

Liu, Q., Zhang, H., Chang, F., Xie, P., Zhang, Y., Wu, H., Zhang, X., Peng, W., & Liu, F. (2022). eDNA revealed in situ microbial community changes in response to *Trapa japonica* in Lake Qionghai and Lake Erhai, southwestern China. *Chemosphere*, *288*(Pt 3), 132605.

Luk, A. W., Beckmann, S., & Manefield, M. (2018). Dependency of DNA extraction efficiency on cell concentration confounds molecular quantification of microorganisms in groundwater. *FEMS Microbiology Ecology*, *94*(10). https://doi.org/10.1093/femsec/fiy146

Matsuda, K., Tsuji, H., Asahara, T., Kado, Y., & Nomoto, K. (2007). Sensitive quantitative detection of commensal bacteria by rRNA-targeted reverse transcription-PCR. *Applied and Environmental Microbiology*, *73*(1), 32–39.

Mauvisseau, Q., Burian, A., Gibson, C., Brys, R., Ramsey, A., & Sweet, M. (2019). Influence of accuracy, repeatability and detection probability in the reliability of species-specific eDNA based approaches. *Scientific Reports*, *9*(1), 580.

Mauvisseau, Q., Kalogianni, E., Zimmerman, B., Bulling, M., Brys, R., & Sweet, M. (2020). eDNA-based monitoring: Advancement in management and conservation of critically endangered killifish species. *Environmental DNA*, *2*(4), 601–613.

Medema, G., Heijnen, L., Elsinga, G., Italiaander, R., & Brouwer, A. (2020). Presence of SARS-Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in The Netherlands. *Environmental Science & Technology Letters*, *7*(7), 511–516.

Merou, N., et al. (2020). An eDNA/eRNA-based approach to investigate the life cycle of non-cultivable shellfish micro-parasites: The case of *Bonamia ostreae*, a parasite of the European flat oyster *Ostrea edulis*. *Microbial Biotechnology*, *13*(6), 1807–1818.

Minot, S. S., Krumm, N., & Greenfield, N. B. (2015). One codex: A sensitive and accurate data platform for genomic microbial identification. *bioRxiv*. https://doi.org/10.1101/027607

Mirna Lorena, S., Cynthia, P. C., Maria Isabela, A. R., Pamela, V. M., Gabriela, R. H., Jorge, B., & Mariano, G. (2023). Nucleic acids isolation for molecular diagnostics: Present and future of the silica-based DNA/RNA purification technologies. *Separation and Purification Reviews*, *52*(3), 193–204.

Moen, A. E. F., et al. (2016). Simultaneous purification of DNA and RNA from microbiota in a single colonic mucosal biopsy. *BMC Research Notes*, *9*(1), 328.

Muha, T. P., Robinson, C. V., Garcia de Leaniz, C., & Consuegra, S. (2019). An optimised eDNA protocol for detecting fish in lentic and lotic freshwaters using a small water volume. *PLoS One*, *14*(7), e0219218.

Nieuwenhuijse, D. F., Oude Munnink, B. B., Phan, M. V. T., The Global Sewage Surveillance Project Consortium, Hendriksen, R. S., Bego, A., Rees, C., Neilson, E. H., Coventry, K., Collignon, P., Allerberger, F., Rahube, T. O., Oliveira, G., Ivanov, I., Sopheak, T., Vuthy, Y., Yost, C. K., Tabo, D. A., Cuadros-Orellana, S., … Koopmans, M. P. G. (2020). Setting a baseline for global urban virome surveillance in sewage. *Scientific Reports*, *10*(1), 13748.

Okazaki, Y., Nguyen, T. T., Nishihara, A., Endo, H., Ogata, H., Nakano, S. I., & Tamaki, H. (2023). A fast and easy method to co-extract DNA and RNA from an environmental microbial sample. *Microbes and Environments*, *38*(1), ME22102.

Olds, B. P., Jerde, C. L., Renshaw, M. A., Li, Y., Evans, N. T., Turner, C. R., Deiner, K., Mahon, A. R., Brueseke, M. A., Shirey, P. D., Pfrender, M. E., Lodge, D. M., & Lamberti, G. A. (2016). Estimating species richness using environmental DNA. *Ecology and Evolution*, *6*(12), 4214–4226.

Peters, L., Spatharis, S., Dario, M. A., Dwyer, T., Roca, I. J. T., Kintner, A., Kanstad-Hanssen, Ø., Llewellyn, M. S., & Praebel, K. (2018). Environmental DNA: A new low-cost monitoring tool for pathogens in salmonid aquaculture. *Frontiers in Microbiology*, *9*, 3009.

Petralia, S., & Conoci, S. (2017). PCR Technologies for point of care testing: Progress and perspectives. *ACS Sensors*, *2*(7), 876–891.

Philo, S. E., Keim, E. K., Swanstrom, R., Ong, A. Q. W., Burnor, E. A., Kossik, A. L., Harrison, J. C., Demeke, B. A., Zhou, N. A., Beck, N. K., Shirai, J. H., & Meschke, J. S. (2021). A comparison of SARS-CoV-2 wastewater concentration methods for environmental surveillance. *Science of the Total Environment*, *760*, 144215.

Pollock, J., Glendinning, L., Wisedchanwet, T., & Watson, M. (2018). The madness of microbiome: Attempting to find consensus "best practice" for 16S microbiome studies. *Applied and Environmental Microbiology*, *84*(7), e02627-17.

Pont, D., Meulenbroek, P., Bammer, V., Dejean, T., Erős, T., Jean, P., Lenhardt, M., Nagel, C., Pekarik, L., Schabuss, M., Stoeckle, B. C., Stoica, E., Zornig, H., Weigand, A., & Valentini, A. (2023). Quantitative monitoring of diverse fish communities on a large scale combining eDNA metabarcoding and qPCR. *Molecular Ecology Resources*, *23*(2), 396–409.

Raja, S., Ching, J., Xi, L., Hughes, S. J., Chang, R., Wong, W., McMillan, W., Gooding, W. E., McCarty, K. S., Jr., Chestney, M., Luketich, J. D., & Godfrey, T. E. (2005). Technology for automated, rapid, and quantitative PCR or reverse transcription-PCR clinical testing. *Clinical Chemistry*, *51*(5), 882–890.

Ruppert, K. M., Kline, R. J., & Rahman, M. S. (2019). Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. *Global Ecology and Conservation*, *17*, e00547.

Saah, A. J., & Hoover, D. R. (1997). "Sensitivity" and "specificity" reconsidered: The meaning of these terms in analytical and diagnostic settings. *Annals of Internal Medicine*, *126*(1), 91–94.

Sanchez, I., Remm, M., Frasquilho, S., Betsou, F., & Mathieson, W. (2015). How severely is DNA quantification hampered by RNA co-extraction? *Biopreservation and Biobanking*, *13*(5), 320–324.

Sarathkumara, Y. D., Browne, D. J., Kelly, A. M., Pattinson, D. J., Rush, C. M., Warner, J., Proietti, C., & Doolan, D. L. (2022). The effect of tropical temperatures on the quality of RNA extracted from stabilized whole-blood samples. *International Journal of Molecular Sciences*, *23*(18), 10609.

Sato, Y., Mizuyama, M., Sato, M., Minamoto, T., Kimura, R., & Toma, C. (2019). Environmental DNA metabarcoding to detect pathogenic Leptospira and associated organisms in leptospirosis-endemic areas of Japan. *Scientific Reports*, *9*, 6575.

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 of the United States of America*, *116*(18), 8931–8940.

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*, 4.

Sharkey, F. H., Banat, I. M., & Marchant, R. (2004). Detection and quantification of gene expression in environmental bacteriology. *Applied and Environmental Microbiology*, *70*(7), 3795–3806.

Shea, D., et al. (1937). Environmental DNA from multiple pathogens is elevated near active Atlantic salmon farms. *Proceedings of the Royal Society B: Biological Sciences*, *2020*(287), 20202010.

Spens, J., Evans, A. R., Halfmaerten, D., Knudsen, S. W., Sengupta, M. E., Mak, S. S. T., Sigsgaard, E. E., & Hellström, M. (2017). Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: Advantage of enclosed filter. *Methods in Ecology and Evolution*, *8*(5), 635–645.

Tillotson, M. D., Kelly, R. P., Duda, J. J., Hoy, M., Kralj, J., & Quinn, T. P. (2018). Concentrations of environmental DNA (eDNA) reflect spawning salmon abundance at fine spatial and temporal scales. *Biological Conservation*, *220*, 1–11.

Tsuji, S., Inui, R., Nakao, R., Miyazono, S., Saito, M., Kono, T., & Akamatsu, Y. (2022). Quantitative environmental DNA metabarcoding shows high potential as a novel approach to quantitatively assess fish community. *Scientific Reports*, *12*(1), 21524.

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.

Valledor, L., Escandón, M., Meijón, M., Nukarinen, E., Cañal, M. J., & Weckwerth, W. (2014). A universal protocol for the combined isolation of metabolites, DNA, long RNAs, small RNAs, and proteins from plants and microorganisms. *The Plant Journal*, *79*(1), 173–180.

van de Groep, K., et al. (2019). Moderate positive predictive value of a multiplex real-time PCR on whole blood for pathogen detection in critically ill patients with sepsis. *European Journal of Clinical Microbiology & Infectious Diseases*, *38*(10), 1829–1836.

Williams, K. E., Huyvaert, K. P., & Piaggio, A. J. (2017). Clearing muddied waters: Capture of environmental DNA from turbid waters. *PLoS One*, *12*(7), e0179282.

Wilson, V. L., Tatford, B. C., Yin, X., Rajki, S. C., Walsh, M. M., & LaRock, P. (1999). Species-specific detection of hydrocarbon-utilizing bacteria. *Journal of Microbiological Methods*, *39*(1), 59–78.

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.

Xie, Y., Zhang, X., Yang, J., Kim, S., Hong, S., Giesy, J. P., Yim, U. H., Shim, W. J., Yu, H., & Khim, J. S. (2018). eDNA-based bioassessment of coastal sediments impacted by an oil spill. *Environmental Pollution*, *238*, 739–748.

Xing, Y., Gao, W., Shen, Z., Zhang, Y., Bai, J., Cai, X., Ouyang, J., & Zhao, Y. (2022). A review of environmental DNA field and laboratory protocols applied in fish ecology and environmental health. *Frontiers in Environmental Science*, *10*, 725360.

Xu, C., Houck, J. R., Fan, W., Wang, P., Chen, Y., Upton, M., Futran, N. D., Schwartz, S. M., Zhao, L. P., Chen, C., & Mendez, E. (2008). Simultaneous isolation of DNA and RNA from the same cell population obtained by laser capture microdissection for genome and transcriptome profiling. *The Journal of Molecular Diagnostics*, *10*(2), 129–134.

Yamanaka, H., Minamoto, T., Matsuura, J., Sakurai, S., Tsuji, S., Motozawa, H., Hongo, M., Sogo, Y., Kakimi, N., Teramura, I., Sugita, M., Baba, M., & Kondo, A. (2017). A simple method for preserving environmental DNA in water samples at ambient temperature by addition of cationic surfactant. *Limnology*, *18*(2), 233–241.

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.

Yu, Z., Ito, S. I., Wong, M. K. S., Yoshizawa, S., Inoue, J., Itoh, S., Yukami, R., Ishikawa, K., Guo, C., Ijichi, M., & Hyodo, S. (2022). Comparison of species-specific qPCR and metabarcoding methods to detect small pelagic fish distribution from open ocean environmental DNA. *PLoS One*, *17*(9), e0273670.

Yuan, S., Cohen, D. B., Ravel, J., Abdo, Z., & Forney, L. J. (2012). Evaluation of methods for the extraction and purification of DNA from the human microbiome. *PLoS One*, *7*(3), e33865.

Zaiko, A., Martinez, J. L., Schmidt-Petersen, J., Ribicic, D., Samuiloviene, A., & Garcia-Vazquez, E. (2015). Metabarcoding approach for the ballast water surveillance – An advantageous solution or an awkward challenge? *Marine Pollution Bulletin*, *92*(1), 25–34.

Zaiko, A., Pochon, X., Garcia-Vazquez, E., Olenin, S., & Wood, S. A. (2018). Advantages and limitations of environmental DNA/RNA tools for marine biosecurity: Management and surveillance of non-indigenous species. *Frontiers in Marine Science*, *5*, 322.

Zhang, J. X., Lau, E., Paik, D. T., Zhuge, Y., & Wu, J. C. (2020). High-throughput preparation of DNA, RNA, and protein from cryopreserved human iPSCs for multi-omics analysis. *Current Protocols in Stem Cell Biology*, *54*(1), e114.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Browne, D. J., Miller, C. M., O'Hara, E. P., Courtney, R., Seymour, J., Doolan, D. L., & Orr, R. (2024). Optimization and application of bacterial environmental DNA and RNA isolation for qualitative and quantitative studies. *Environmental DNA*, *6*, e589. https://doi.org/10.1002/edn3.589