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RESEARCH ARTICLE

Assessing the performance and efficiency of environmental DNA/RNA capture methodologies under controlled experimental conditions

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Abstract

1. Growing interest and affordability of environmental DNA and RNA (eDNA and eRNA) approaches for biodiversity assessments and monitoring of complex ecosystems have led to the emergence of manifold protocols for nucleic acids (NAs) isolation and processing. Although there is no consensus on a standardized workflow, the common practice for water samples is to concentrate NAs via filtration using varying pore size membranes. Using the smallest pore is assumed to be most efficient for NAs capture from a wide range of material (including sub-cellular particles); however, a trade-off must occur between detection of a meaningful molecular signal and cost/time effort when processing samples using fine pore membranes.

2. Comparative studies involving formal efficiency assessments are lacking, which restricts informed decision-making around an optimized sampling approach for applications such as biosurveillance (i.e. detection and monitoring of target taxa—nuisance organisms, endangered and indicator taxa or other species of economic or cultural importance). Here, we present an experimental study using an easily cultured microalgal species (*Alexandrium pacificum*) to test different filter membranes for capturing NAs in the context of cost/time effort and cell fractions encountered in nature (whole cells, partially lysed and naked NAs).

3. The results showed no statistically significant difference between membrane types for capturing target eDNA signal from intact and partially lysed cell treatments. In terms of time effort and volume processed, higher efficiency ratings were obtained with the larger pore size (5 μm) cellulose membranes. Positively charged nylon demonstrated enhanced capture of naked NAs, and especially eRNA signal, across treatments.

4. Our findings support using coarse pore size filters for adequate capture of target NA signal (from both eDNA and eRNA) with less processing time. The framework presented here can provide a quick and robust feasibility check and comparative assessment of new and existing NA processing technologies, and allows sufficient control over multiple parameters, including physical–chemical water properties, temporal scales, and concentration and type of input material.

1 | INTRODUCTION

Following the recent leap in biotechnologies and particularly in high-throughput sequencing techniques (Pareek et al., 2011; Soon et al., 2013), environmental DNA and RNA (eDNA and eRNA) are increasingly being used (employing a multitude of sample collection and processing protocols) for biodiversity assessment and monitoring of complex aquatic ecosystems including lakes, streams, rivers, reservoirs, coastal and oceanic waters (Adrian-Kalchhauser & Burkhardt-Holm, 2016; Clusa et al., 2016; Eichmiller et al., 2014; Laroche et al., 2017; Stoeckle, Soboleva, et al., 2017; Wood et al., 2018). In this study, we define eDNA and eRNA as the genetic material extracted in bulk from an environmental sample such as soil, sediment, air or water and originating from living organisms present in the sample as well as dead cells and free-floating DNA/RNA molecules (Ficetola et al., 2008; Nield et al., 2001; Taberlet et al., 2012). The variability of eDNA and eRNA material and its uneven distribution in the environment may substantially impact their effective capture (Hinlo et al., 2017). To harness the full potential of nucleic acid (NA)-based methods for biomonitoring and environmental management applications, the sampling approaches, designs and strategies must be optimized and decisions on the best practice workflows should be underpinned with robust scientific evidence on origin, state and amount of genetic material captured by different techniques (Aylagas et al., 2020; Bowers et al., 2021; Turner et al., 2014).

Although there is still no consensus on a standardized workflow for processing eDNA/eRNA samples, in aquatic systems the common practice is to concentrate waterborne nucleic acids (NAs) via filtration (Carim et al., 2016; Deiner et al., 2015; Egan et al., 2013; Wood et al., 2018). The type of filter membranes and pore sizes vary widely from study to study. For eDNA applications, use of filter pore sizes ranging from 0.2 μm (Andruszkiewicz et al., 2017; Collins et al., 2018; Kelly et al., 2014; Turner et al., 2014) to ≥20 μm (Egan et al., 2013; Turner et al., 2014) have been reported. Previous research (e.g. a seminal experimental study by Turner et al., 2014) indicates that the smallest pore size helps maximizing NA capture from a wide range of genetic material (including sub-cellular particles). However, using finer pore membranes increases the likelihood of clogging and prohibits processing of larger water volumes (Andruszkiewicz et al., 2017; Goldberg et al., 2013; Turner et al., 2014), thus potentially reducing the chances of detecting rare biodiversity. Alternatively, splitting samples into multiple subsamples, which invariably increases consumable costs for filtration and downstream analyses, can potentially compromise the integrity of NAs due to increased handling times. This is especially relevant for eRNA which is more prone to degradation (Pochon et al., 2017; Wood et al., 2020). On the other hand, processing large sample volumes with larger pore membranes may not effectively capture smaller particles, and can increase the concentration of inhibitory substances (e.g. humic compounds) that may suppress target molecular signals (reviewed in Beng & Corlett, 2020).

Although it has been previously shown that larger pore filters can be as effective as finer pore filters for species detection from waterborne eDNA (Sepulveda et al., 2019; Wittwer et al., 2018), more data are needed on the selectivity of different membrane pore sizes towards specific NA types and fractions (Bowers et al., 2021). Rarely (if at all) do such comparative studies involve technical efficiency assessment to address time/cost inputs in the context of molecular signal recovery. This restricts informed decision-making around an optimized sampling approach (i.e. combination of time effort and maximized signal detection) for addressing a particular research or surveillance question, for example, the detection and monitoring of nuisance organisms, endangered and indicator taxa, and species of ecological, economical and/or cultural importance.

Among the variety of filter types used in eDNA studies, cellulose-based membranes consistently outperform other filters for eDNA capture and downstream elution (Jeunen et al., 2019; Majaneva et al., 2018). However, there is still a lack of evidence for their performance capturing different NA types (eDNA vs. eRNA) and size fractions (whole cells, cell particles and naked NAs) in seawater with varying levels of organic matter. Given that DNA and RNA are negatively charged molecules, a logical expectation would be to use positively charged membranes for enhancing capture of waterborne NAs. Yet, the literature is scarce on performance of such membranes (Bessey et al., 2021).

One possible reason for scarcity of empirical evidence on the amount and type of genetic material captured during filtering steps (Bowers et al., 2021) is that it can be cumbersome and expensive to test multiple parameters simultaneously, including filter types, pore

size, extraction kits, etc. These types of experiments become even more unwieldy when adding in environmental influences such as salinity, turbidity, pH and humic compound concentrations. It is also difficult to conduct controlled studies that include different states of genetic material in the environment (e.g. whole cells, partial cells, naked DNA and RNA). Most studies to date rely on the collection and isolation of environmental samples and the use of metabarcoding for comparative analyses of species richness among molecular methods and traditional observations (Deiner et al., 2015; Deiner et al., 2018; von Ammon et al., 2020). The use of environmental samples, however, is often restricted to inherent properties that cannot be effectively controlled such as turbidity and unknown inhibitors. Additionally, there are challenges with this approach in providing a quantitative assessment of the genetic signal from a specific target.

Here, we present an experimental study using a cultured microalgal species (*Alexandrium pacificum* [Class: Dinophyceae]) as a model for a controlled filter performance comparison combined with a formal efficiency modelling component which, to our knowledge, has not yet been applied in eDNA and eRNA research. A tiered experimental design was applied to (a) assess the suitability of different membranes in their ability to capture various fractions of target eDNA and eRNA (intact cells, broken cells, naked NA) from our model species spiked into pre-filtered, ambient environmental seawater, and (b) establish efficiency and productivity assessments of different membranes in terms of optimized performance.

2 | MATERIALS AND METHODS

2.1 | Alexandrium pacificum culture

We selected the dinoflagellate *Alexandrium pacificum* (John et al., 2014) as a model species for controlled experimental assessment of eDNA/eRNA capture for several desirable traits: (a) it is single celled, allowing for ease of standardized subsampling, quantification and amenable preparation of various fractions (e.g. whole and broken cells, naked NAs), (b) it can grow to high densities to easily create sufficient biomass for experimental studies, and (c) molecular detection is possible using published and in situ validated methods (Dai et al., 2020; Ruvindy et al., 2018). This species is a major producer of paralytic shellfish toxins which can accumulate in shellfish and cause human illness (MacKenzie, 2014), making it an important target for molecular (eDNA/eRNA based) surveillance. Due to this threat to human health, *A. pacificum* has been extensively studied utilizing semi-continuous culturing methods to investigate its biology, taxonomy and toxin production (Caruana et al., 2020; Fertouna-Bellakhal et al., 2015; Han et al., 2016). We chose this species to serve as an example that could be standardized at a fundamental level and is easily amenable to the generation of known amounts of material for whole cells, partially lysed cells and naked nucleic acids.

The founder culture cell of *A. pacificum* was isolated from Opua Bay, Marlborough Sounds (New Zealand) in 2013 and is maintained

in the Cawthron Institute's Culture Collection of Microalgae (isolate CAWD234; https://cultures.cawthron.org.nz/). For this experiment, an aliquot was obtained from a semi-continuous culture growing in modified L1 medium (Guillard & Hargraves, 1993) prepared with artificial seawater. Growth conditions were 2425 μmol m^{-2} s^{-1} photon irradiance (12:12h L:D), 18 $\pm$ 0.5°C and pH 8.6.

2.2 | Preparation of ruptured cells and nucleic acid fractions

For the experiment, *A. pacificum* cell concentrations were determined using Lugol's iodine fixed material with a Sedgwick Rafter chamber just prior to the experiment, and all experimental calculations were conservatively based on the concentration of morphologically healthy cells (Figure S1; dark pigmented cells with intact theca). An aliquot of ruptured cells (50ml) was prepared by sonication of live culture for 10s on the maximum setting of a Sonicator™ Cell Disruptor (Heat System Ultrasonics). This time period was empirically determined to be adequate for rupturing a portion of the cultured cells while leaving some cells intact (Figure S2). To generate the naked RNA and DNA fractions, intact cell culture was filtered using non-charged 0.45 μm pore size Durapore membrane filters (Millipore) and co-extracted using the Zymo Quick-DNA/RNA™ Miniprep Kit (Zymo Research).

2.3 | Experimental design

A two-tier experimental setup was applied in this study. In the first experimental round, the performance of different filter membranes for capturing various fractions of eDNA/eRNA was assessed. We selected cellulose acetate (CA) membranes (repeatedly reported performing well in eDNA studies) of three pore sizes (5, 1.2 and 0.45 μm) and a positively charged nylon (PCN) 1.2 μm pore size membrane (as a potential alternative to commonly used filter types). A crossed experimental design was applied (Figure 1) with three experimental factors manipulated:

- eDNA/eRNA fractions, four levels: (1) intact (whole) cells of microalgal culture, (2) broken (sonicated) cells, (3) naked DNA and (4) naked RNA.
- Seawater, two levels: (1) pre-filtered (0.35 μm) and (2) non-filtered *A. pacificum*-free seawater (determined via light microscopy and ddPCR assay).
- Filter type, four levels: (1) 5 μm cellulose acetate (CA) membrane, (2) 1.2 μm CA, (3) 0.45 μm CA and (4) 1.2 μm positively charged nylon (PCN) membrane.

The membranes (three replicates for each filter type) with the captured material were immediately transferred to sterile centrifuge tubes and kept frozen at −80°C until processed for dual DNA/RNA extraction.
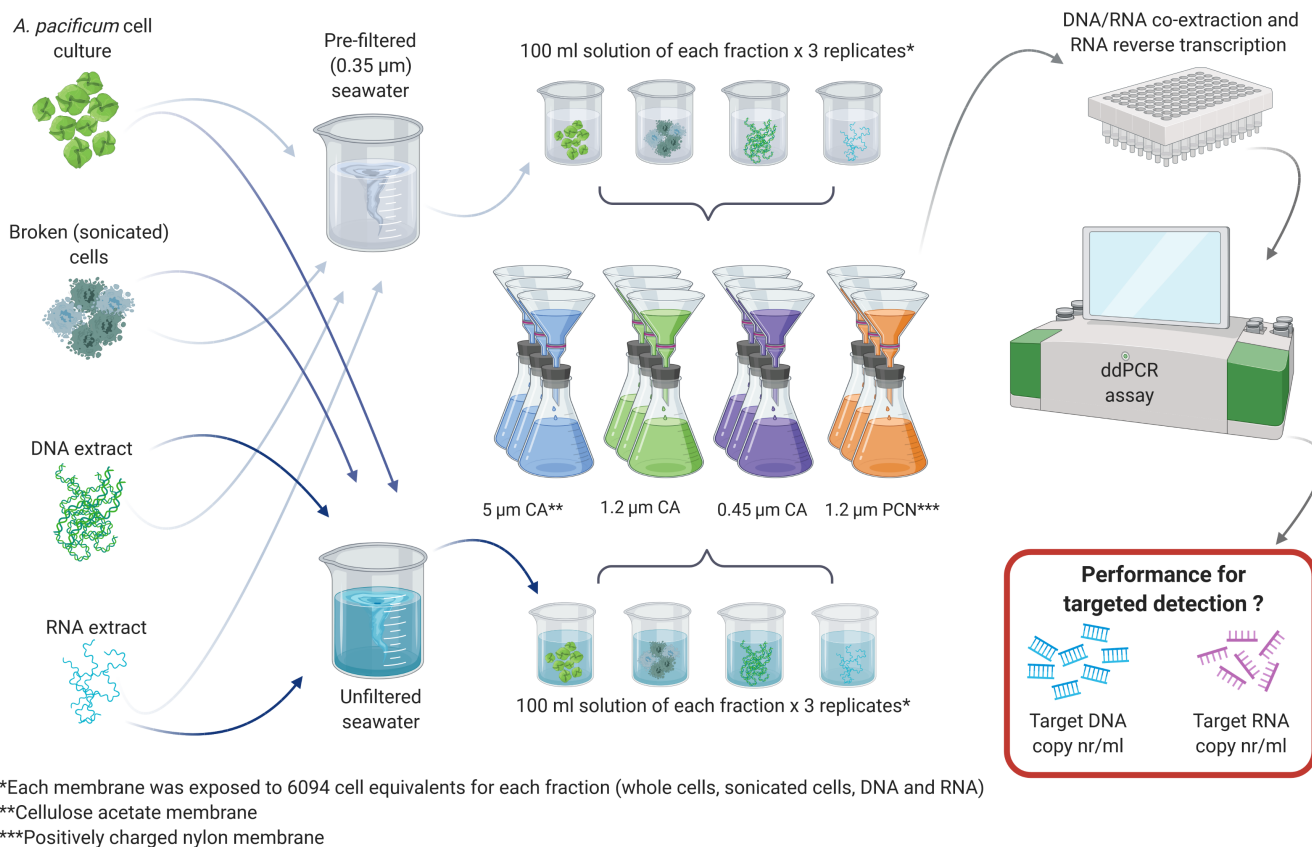
*Each membrane was exposed to 6094 cell equivalents for each fraction (whole cells, sonicated cells, DNA and RNA)
**Cellulose acetate membrane
***Positively charged nylon membrane

FIGURE 1 Schematic of the experimental design applied in the first experimental system to assess filter performance across the different cell types (whole, broken and naked DNA/RNA). Figure created in BioRender.com

The second experiment was focused on establishing input and output parameters for an efficiency model and was run with two concentrations of sonicated *A. pacificum* cells spiked into target-free (determined via light microscopy) seawater (Figure 2). The spiked water was filtered through the same four types of membrane filters as outlined above (six replicates each), while recording (a) the time required to filter 250 ml of water sample and (b) the volume filtered in 20 min (through continued filtering past the 250 ml mark in [a]). The filters from step (b) were immediately frozen at −80°C until simultaneous DNA/RNA extraction.

2.4 | Experimental Setup

We chose to use seawater as a medium in our experiment to ensure relevance of the obtained results to properties inherent in the natural environment of the target species (e.g. salinity, dissolved oxygen, pH, nutrients, trace metals, vitamins, etc.). Seawater was collected from the Tasman Bay (South Island, New Zealand). To account for potential stochastic effects of NA binding to larger suspended particles and/or presence of inhibiting substances, two seawater treatments were applied in the first experiment: filtered (0.35 μm) or unfiltered. Triplicate plastic buckets (2,050 ml Powder Pot, Pharmapac™) were prepared with 1.5 L of seawater and the cell/nucleic acid fraction being tested (whole cells, sonicated cells, naked RNA, naked DNA)

were added. Target cell addition was 100,000 total cells (or equivalent for sonicated and naked nucleic acid treatments), with the actual additions being 91,414 (filtered) or 111,896 (unfiltered) cells for a final concentration of 61 or 75 cells/ml, respectively (volume of culture added was 3 ml). This cell concentration is what we see during a natural (moderate) bloom event and is within the limits of detection for the ddPCR assay. The same volume of sonicated culture (3 ml) was added to the respective treatment buckets. Although *A. pacificum* was not observed in the study area prior to the sampling time as part of routine monitoring (data not shown), and no cells were detected in the background water (as confirmed by light microscopy screening), some 'legacy' signal could be expected as species occur occasionally in coastal waters adjacent to the sampling area (unpublished data, Cawthron Institute Micro-algae Laboratory). Therefore, to test for this potential background noise, we added field control filters to the analyses (three of each filter type in both experimental runs), which consisted of filtered seawater only, before any addition of the treatment fractions.

A homogeneous environment was maintained in the buckets via continuous mixing with a magnetic stirrer and plate. A sterile 50 ml serological pipet was used to transfer 100 ml of sample to each of the four filter types tested: CA membrane 5 μm, 1.2 μm or 0.45 μm pore size (Sartorius Biolab Products), and PCN membrane 1.2 μm pore size (Critical Process Filtration, Inc.). Filter sets were immediately transferred to −80°C and stored at that temperature until dual

FIGURE 2 Schematic of the experimental design applied in the second experiment to assess the efficiency of different membranes for eDNA and eRNA capture. Figure created in BioRender.com
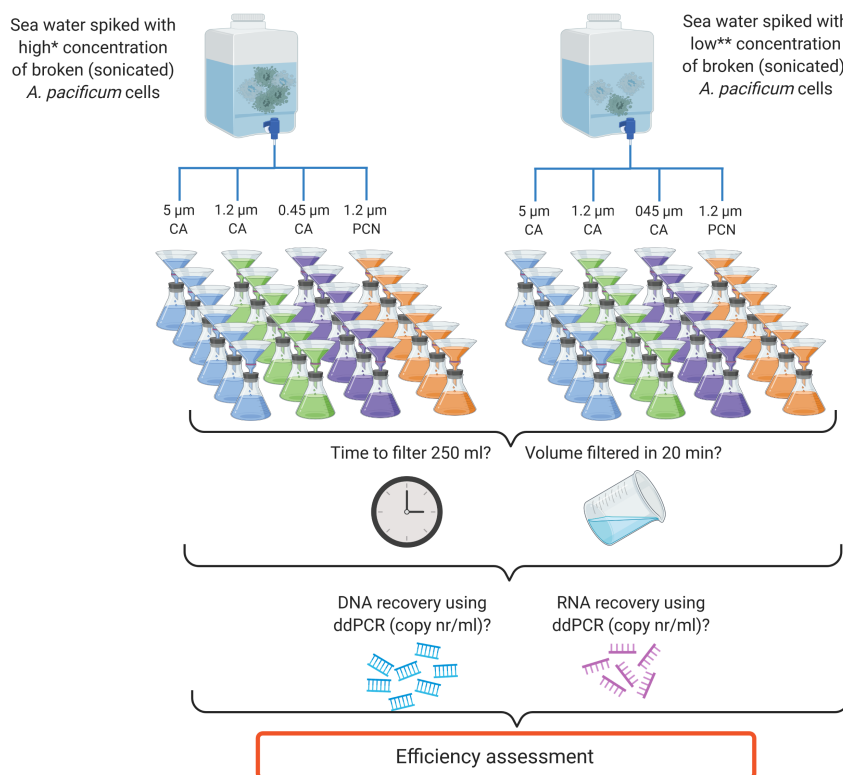
*High cell concentration corresponded to ~100 cells/ml
**Low cell concentration corresponded to ~ 10 cells/ml

DNA/RNA extractions. Filter cups were rinsed thoroughly with deionized water (prior to processing whole and sonicated treatments) or put through a 10% bleach sterilization (10 min), 0.1% sodium thiosulfate inactivation (immediate) and deionized water rinse (prior to processing DNA and RNA treatments).

For the second experiment, 40 L of coastal seawater was collected on the day of the experiment from Nelson marina (South Island, New Zealand) and immediately transported to the laboratory. Water quality parameters (measured right before the experiment commenced) were as follows: temperature 12.7°C, dissolved oxygen 8.6 mg L^{-1}, salinity 33.2 ppt, pH 5.99 as measured with a YSI (YSI, Inc.), and average turbidity 1.10 NTU as measured with a HACH (HACH Company). At the laboratory, 20 L of spiked (sonicated *A. pacificum* cells) experimental water was prepared in carboys for high (91 cells/ml) and low (9.8 cells/ml) concentration treatments. A homogeneous environment was maintained in the carboys via continuous mixing with a magnetic stirrer and plate. A graduated cylinder was used to aliquot 500 ml into six HDPE bottles, and a set of control filters was prepared with experimental water only. Filter cups, graduated cylinders and bottles were rinsed thoroughly in between filter sets.

2.5 | RNA/DNA extraction and droplet digital polymerase chain reaction (ddPCR)

RNA and DNA were extracted simultaneously from each filter using the Zymo Quick-DNA/RNA™ Miniprep Kit (Zymo Research). The filters were placed into individual ZR BashingBead.

Lysis Tubes and 0.8 ml of lysis buffer from the kit was added to each tube which was adequate volume to cover the filter. These were then homogenized by bead beating (1500 RPM, 2 min; 1600 MiniG Spex SamplePrep) such that material on all parts of the filter was exposed to the buffer and beads. After centrifugation (3000 *g*, 5 min, 20°C). DNA and RNA were co-extracted following the manufacturer's protocol.

Trace DNA in isolated RNA samples was eliminated by two sequential DNase (TURBO DNA-free™ Kit, Thermo Fisher Scientific) treatments following Langlet et al. (2013). Treated RNA was diluted to 10 ng/µl equimolar concentrations and reverse transcribed into cDNA using the SuperScript™ III reverse transcriptase (Thermo Fisher Scientific). All extracted products were stored frozen (−20°C) for DNA and cDNA, and at −80°C for RNA until analysis.

Alexandrium pacificum-specific copy numbers were quantified using ddPCR for all samples, including all negative extraction controls (DNA and RNA) on a QX200 Droplet Digital PCR System™ (Bio-Rad). For the ddPCR assays, *A. pacificum*-specific primers (ACTA-416-F and ACTA-605-R), targeting 204 bp from the large ribosomal subunit) and Taqman probe (ACTA-456-P), were used as described in Ruvindy et al. (2018). Each ddPCR reaction included 0.5, 1.0 and 0.05 µM of the forward primer, reverse primer and probe, respectively, 1 × BioRad ddPCR Supermix for probes, 1 µl pre-diluted DNA (1:25 for the first experiment and 1:50 for the second experiment) or 1 µl undiluted cDNA, and sterile water in a total reaction volume of 21 µl. The BioRad QX200 droplet generator partitioned each reaction mixture into approximately 20,000 nanodroplets by combining 20 µl of the reaction mixture with 70 µl of BioRad droplet generation oil. After processing,

this resulted in a total droplet volume of 40 μl, which was transferred to a PCR plate for amplification using the following cycling protocol: hold at 95°C for 10 min, 40 cycles of 94°C for 30 s and 54°C for 1 min, and a final enzyme deactivation step at 98°C for 10 min. Each well of the plate was then individually analysed on the QX200 instrument to establish the threshold value separating negative and positive droplets and perform absolute quantification of target DNA or cDNA. A positive control of extracted DNA from the *A. pacificum* CAWD234 culture and negative (sterile water) control was included on each plate. For the first experiment, the results were converted to copies per sample using the formula: number of copies per μl × 22 [the initial volume of the PCR reaction (μl)] × 50 [DNA elution volume] or 25 [RNA elution volume] (μl). Since various sample volumes were processed through filter membranes as part of the second experiment, the ddPCR results were standardized to number of copies per 'cell' of input material (*A. pacificum*), applying the following formula. Total volume (ml) of material that passed through the filter in 20 min multiplied by the 'cell' concentration (equivalent to 9.8 cells/ml or 91 cells/ml, see above for details) to calculate total number of 'cells' on the filter. This number was divided by 50 μl (DNA elution volume) or 25 μl (RNA elution volume) to calculate number of 'cells' per μl. Copies per μl from ddPCR results were divided by 'cells' per μl to obtain copies per 'cell'.

2.6 | Efficiency modelling

To evaluate the efficiency of the DNA/RNA capture with different membranes, we employed the Data Envelopment Analysis (DEA) approach (Land et al., 1994). DEA is a nonparametric technique based on linear programming that has been used to evaluate the efficiency of production and business units (Land et al., 1994) such as farms (Latruffe et al., 2004), banks (Hauner, 2005), factories (Tsekouras & Skuras, 2005) and utilities (von Hirschhausen et al., 2006). Under the DEA evaluation framework, the most efficient sampling method or methods receive an efficiency score of 1 indicating that they follow the best practice and are therefore in the production frontier. The rest of the methods have efficiency scores of less than 1 indicating sub-optimal status. DEA requires data on inputs and outputs. In this study, we considered time to filter 250 ml of experimental seawater and volume filtered within 20 min as input variables. The output variables were measured signals of the target eDNA and eRNA (copy numbers of *A. pacificum* DNA and RNA derived via ddPCR assay). The 20 min threshold was chosen as a practical timeframe for sample processing without compromising eDNA/eRNA integrity when multiple samples are collected in the field.

DEA is based on the generalized notion of convexity, which assumes that the functional relationship between input and output variables is continuous and differentiable. This guarantees the existence of an optimal solution for either minimizing input or maximizing output production. The general frontier surface (the area of best practice or the place where the most efficient use of resources occurs) is approximated by piecewise-linear segments with the result that observed differences in efficiency cannot be explained away as differences in

economies of scale (cost advantage achieved while increasing the scale of production). Assuming that considered inputs (time and volume parameters) are intrinsically linked to the filter membrane properties and cannot be easily adjusted for the particular membrane type, we applied the 'output-oriented' DEA, to evaluate the efficiency of capturing DNA and RNA signals. The exploratory analyses of the results from the second experimental round indicated that the 'time to filter 250 ml' and 'volume filtered in 20 min' variables were highly colinear (Spearman correlation −0.97, $p < 0.001$). Therefore, in the DEA model, only the volume parameter was used as an input variable.

2.7 | Statistical analyses

For the first experiment, nonparametric Kruskal–Wallis tests were applied to test for a significant difference in eDNA and eRNA signal yields derived from experimental treatments (types of seawater, starting material type and filter membrane used in the experiment).

Derived eDNA and eRNA signal across concentrations (low vs. high) and membrane type and water treatment levels were investigated using permutational multivariate analysis of variance (PERMANOVA) based on Bray–Curtis similarity matrices. PERMANOVA was performed using the *adonis2* function of the ᴠᴇɢᴀɴ package (Oksanen et al., 2019). To visualize the multivariate structure in signal strength of nucleic acids, principal component analysis (PCA) biplot visualization was produced using the fviz_pca_biplot function within the ꜰᴀᴄᴛᴏᴇxᴛʀᴀ package (Kassambara & Mundt, 2017).

For the second experiment, the Mann–Whitney *U* tests were applied to compare derived time and volume parameters between low and high *A. pacificum* concentration treatments and efficiency scores derived for tested filter membranes from the DEA. Where relevant, the post-hoc pairwise comparisons were performed using Dunn's test with Bonferroni alpha-correction implemented in the Pairwise Multiple Comparison of Mean Ranks package (PMCMR, Pohlert, 2018).

All analyses were performed in R v3 software (R-project, 2014).

3 | RESULTS

3.1 | Effect of membrane type on capturing different eDNA and eRNA fractions

Negligible levels of DNA and RNA signal were detected in negative control samples (ranging from 0 to 0.05 copies/ml for DNA and 0 to 0.1 copies/ml for RNA). Some legacy signal was not unexpected given that the sites where experimental waters were collected have historically contained *A. pacificum*. Overall, higher copy numbers were obtained for DNA as compared to RNA samples (Figure 3). It should be noted, however, that absolute numbers should be compared with caution, as there may be some loss biases impacting RNA quantification due to more complex sample processing, for example, reverse transcription steps (Laroche et al., 2017; Pochon et al., 2017).
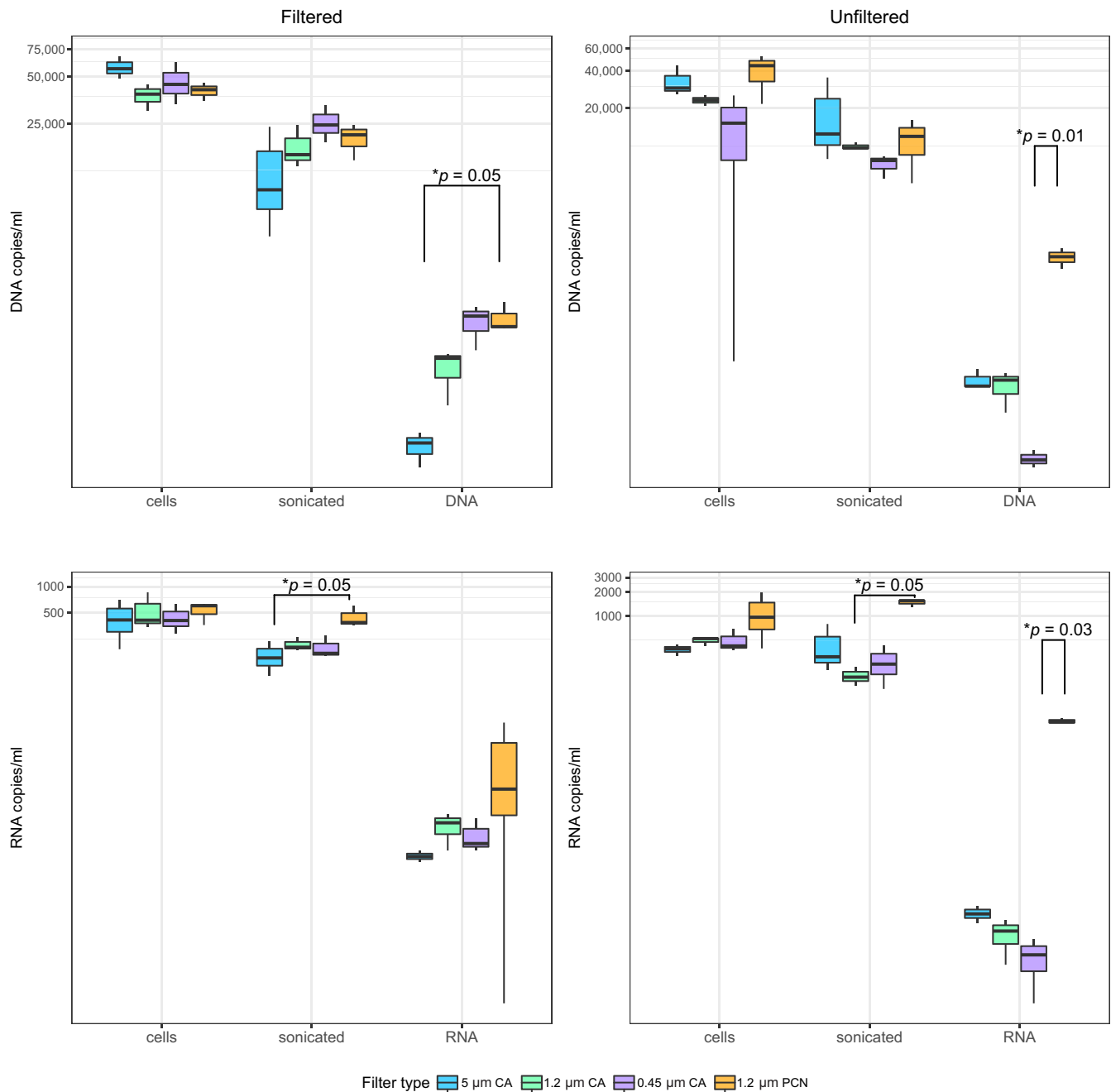
FIGURE 3 *Alexandrium pacificum* DNA and RNA copy numbers quantified using droplet digital PCR from four filter membrane types, for four types of genetic material (intact and sonicated microalgal cells, naked DNA and RNA), tested in filtered and unfiltered seawater treatments. Data range (whiskers), upper and lower quartiles (edges) and the median (horizontal line) are represented for three replicates. Statistically significant pairwise within-treatment differences are indicated with horizontal brackets and corresponding *p*-values

The difference in DNA signal from filtered water treatment was statistically significant between all three types of starting material—whole cells, sonicated cells and naked DNA (Dunn/Kruskal–Wallis multiple comparison adjusted *p* < 0.05, Figure 3). In the unfiltered water treatment, DNA yield from naked DNA material was significantly lower than whole and sonicated cells (*p* < 0.05).

For RNA, in both filtered and unfiltered water, only naked RNA treatment resulted in substantially lower RNA copy numbers,

compared to whole and ruptured cell treatments (*p* < 0.05). Overall decreasing trends of copy numbers were observed from intact cells to broken cells to naked NAs, for both DNA and RNA, filtered and unfiltered water (Figure 3).

Regarding filter effect, no statistically significant difference was observed for DNA signal from intact and ruptured cell treatments. However, the positively charged nylon membrane yielded higher copy number from naked DNA and RNA, as well as RNA from broken cells when compared to the other filter types (except filtered water
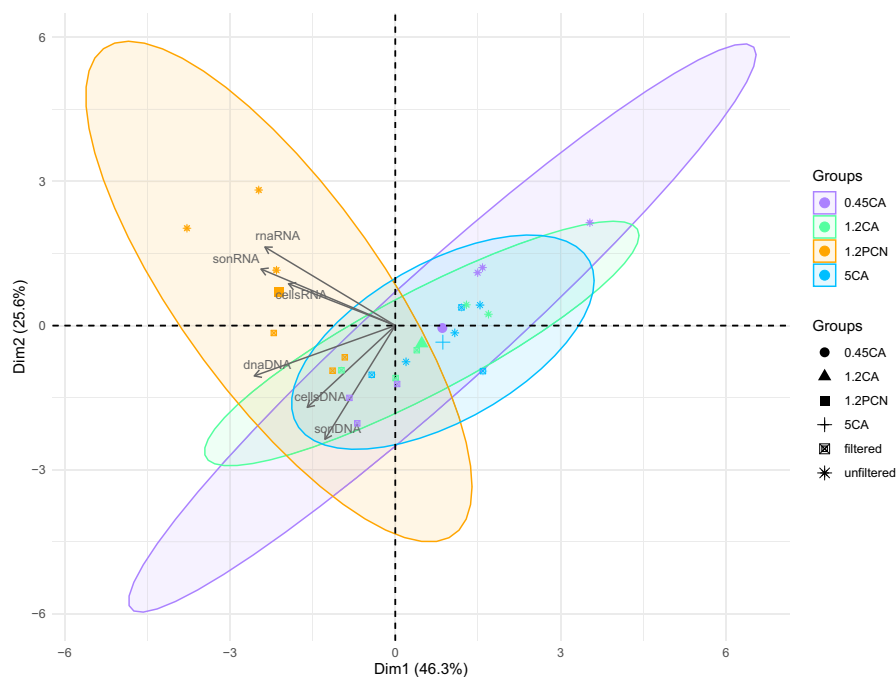
FIGURE 4 Two-dimensional principal component analysis (PCA) visualizations of eDNA and eRNA signal partitioned by membrane filter type and water treatment effect (see Figure 1 for details). The overlaid vectors show the type of starting material and their correlation with either of the two main axes: *cellsDNA*—DNA from intact cells; *cellsRNA*—RNA from intact cells; *sonDNA*—DNA from sonicated cells; *sonRNA*—RNA from sonicated cells; *dnaDNA*—Naked DNA; *rnaRNA*—Naked RNA. The concentration ellipses cover 95% confidence interval for each group of samples. Centroids for each group are represented by larger symbols

treatment, where it performed about the same as the 0.45 μm CA membrane, Figure 3).

These findings were also confirmed with the multivariate analysis (adonis) and PCA ordination (Figure 4). Overall NAs signal was captured consistently across CA filters; however, stronger signals from the naked NAs as well as cellular RNA fractions were closely associated with the positively charged membrane samples.

3.2 | Efficiency modelling of the tested membranes for target eDNA and eRNA capture

A slightly higher background *A. pacificum* signal was detected in negative control samples in the second experimental round compared to the first round (ranging from 0 to 26.3 copies/ml for DNA and 0 to 10 copies/ml for RNA), likely indicating the presence of some legacy fragments in harbour water at the sampling site (data not shown). This was 2–3 orders of magnitude lower than the molecular signal derived from experimental samples; thus, this background signal was considered negligible.

There was no significant difference (Mann–Whitney U test $p > 0.05$) between low and high *A. pacificum* cell concentration treatments with regards to the time required to filter 250 ml or the volume filtered over 20 min timeframe (Figure 5). However, there were significant differences between filter types, both in volume and time for filtration, and high and low concentration treatments. The most apparent difference was between 5 μm CA and 1.2 μm PCN filters (Figure 5).

In terms of the derived eDNA and eRNA signals resulting from the ddPCR analysis, there were statistically significant differences between high and low concentration treatments (Mann–Whitney U test, $p < 0.05$). Overall, the DNA yields on the different filters were

ca. two orders of magnitude higher than those of RNA (Figure 6). For both DNA and RNA, higher copy numbers per cell were detected in the low concentration treatment. However, DNA copy numbers decreased from 5 μm CA to 0.45 μm CA and 1.2 μm PCN filter treatment, while in the RNA treatment an opposite trend was observed both for low and high concentrations. These general trends were maintained also for the copy numbers calculated per μl of material that passed through the filter, although for DNA higher overall signal was observed in high concentration treatment (Figure S2).

The efficiency assessment of four tested filter types, based on the observed volume of sample that passes through within a reasonable timeframe (defined as 20 min for this study), indicated overall higher efficiency scores for capturing eDNA signal at low target cell concentrations (Table 1). These ranged from 0.237 to 1.0 (both values derived for 1.2 μm PCN membrane), while for RNA, low concentration treatment efficiency scores ranged from 0.026 to 1.0 (1.2 μm PCN and 5 μm CA, respectively). The high cell concentration treatment yielded efficiency scores ranging between 0.053 (1.2 μm PCN) and 1.0 (5 μm CA) for eDNA and between 0.003 (1.2 μm PCN) and 1.0 (5 μm CA) for eRNA.

The combined DEA indicated that for optimized holistic NA capture (i.e. eDNA and eRNA), when the target abundance is unknown, 5 μm CA membrane performs significantly better (Mann–Whitney U test, $p < 0.02$) than other filter membrane types (Figure 7).

4 | DISCUSSION

In this experimental study, we have showcased a multidisciplinary approach to measure the efficiency of different filter membranes for the capture of eDNA and eRNA (presented as whole cells, broken cells and naked NAs) from marine water samples. The results
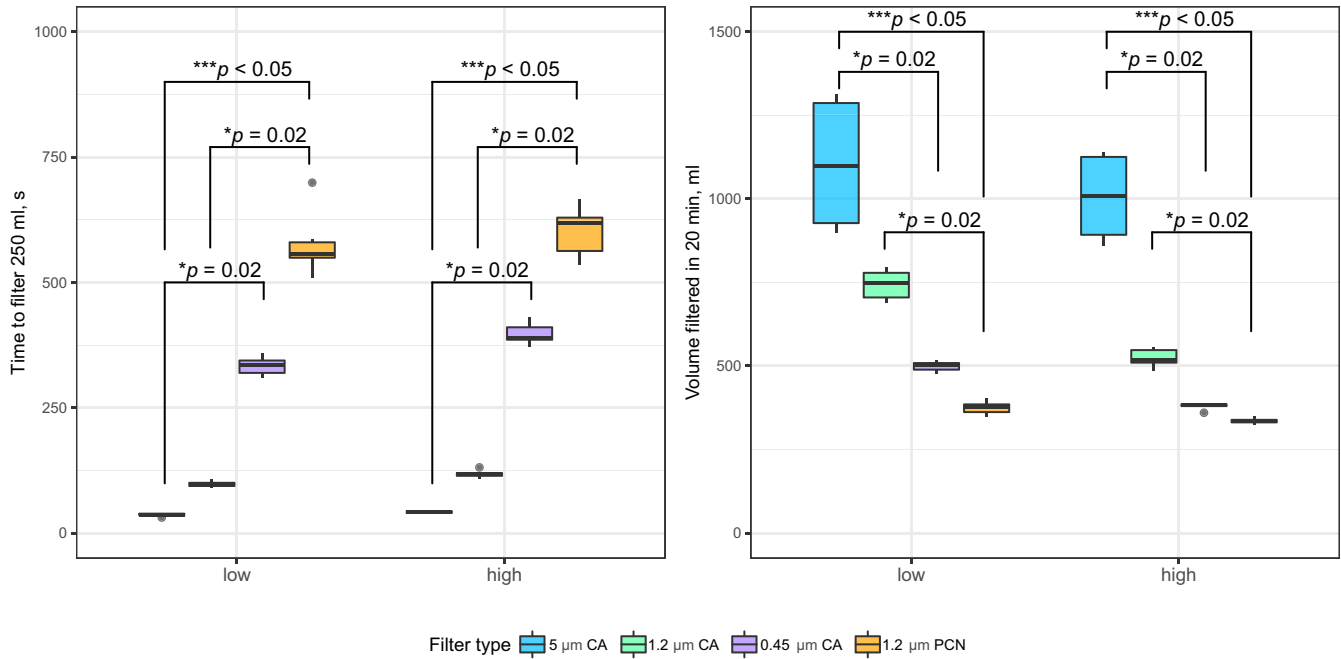
FIGURE 5 Results from the second experiment: Recorded time to filter 250 ml of sample and sample volume filtered in 20 min for four filter membrane types and two cell concentration treatments. Data range (whiskers), upper and lower quartiles (edges) and the median (horizontal line) are represented for six replicates. Statistically significant pairwise within-treatment differences are indicated with horizontal brackets and *p*-values

FIGURE 6 *Alexandrium pacificum* DNA and RNA copy numbers quantified per input cell using droplet digital PCR from four filter membrane types, low and high cell concentration treatments. Data range (whiskers), upper and lower quartiles (edges) and the median (horizontal line) are represented for six replicates. Statistically significant pairwise within-treatment differences are indicated with horizontal brackets and *p*-values
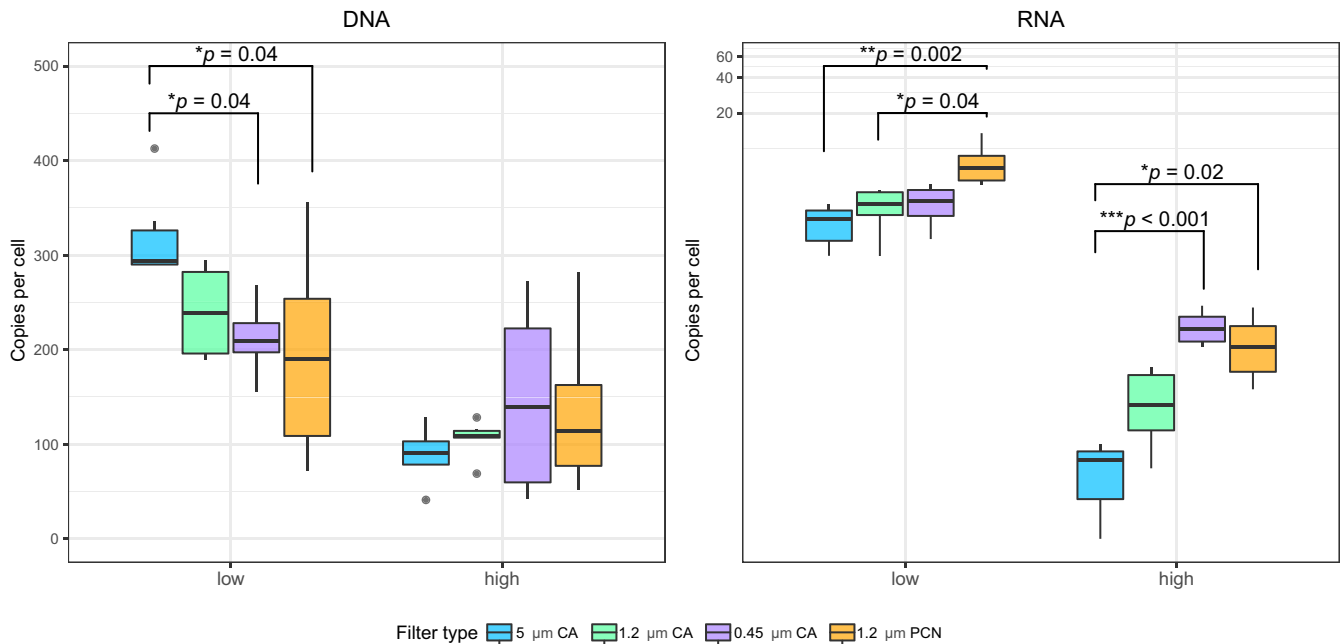
provide valuable empirical information for optimizing the approach to molecular signal detection in waterborne samples. The evidence that coarse pore-size membranes might perform as well as smaller pore-size filters for capturing different fractions of NAs from water

has important practical implications for monitoring and surveillance programmes. Efficient sampling of NAs from varied aquatic ecosystems routinely involves concentration of heterogeneously distributed genetic material across large areas or water volumes

TABLE 1 Efficiency scores derived from the data envelopment analysis (DEA) for capturing target eDNA and eRNA signal by four tested filter membrane types, and low and high cell concentration treatments

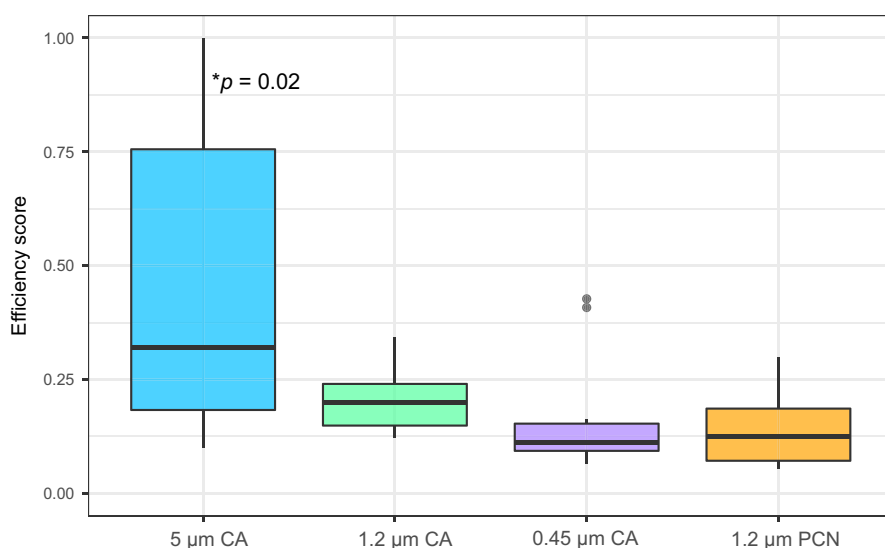| Filter type | Efficiency score (mean ± *SD*) | | | |
	Low cell concentration, DNA	Low cell concentration, RNA	High cell concentration, DNA	High cell concentration, RNA
5 µm CA	0.742 ± 0.204	0.543 ± 0.329	0.589 ± 0.243	0.397 ± 0.377
1.2 µm CA	0.662 ± 0.104	0.269 ± 0.173	0.236 ± 0.055	0.047 ± 0.038
0.45 µm CA	0.505 ± 0.098	0.156 ± 0.072	0.206 ± 0.168	0.006 ± 0.002
1.2 µm PCN	0.535 ± 0.306	0.052 ± 0.019	0.158 ± 0.093	0.009 ± 0.005

FIGURE 7 Overall efficiency scores derived from the Data Envelopment Analysis (DEA) for four tested filter membrane types, combined eDNA and eRNA capture, independent of the target cell concentration. Data range (whiskers), upper and lower quartiles (edges) and the median (thick horizontal line) are represented for 12 replicates

(Verdier et al., 2021; von Ammon et al., 2020; Wood et al., 2019). This crucial aspect of the workflow is quite challenging, entailing tedious, time and resource-intensive human effort. By using larger pore-size membranes for sample filtration, the recurring problem of rapid clogging can be mitigated, allowing for larger volumes of water to be processed and hence—better recovery of waterborne NAs. Research aimed at identifying optimal parameter values for in situ systems concluded that customized filter packs equipped with 5 µm filter membranes captured significantly more target eDNA than those with 1 µm membranes (Thomas et al., 2018). Our findings support using coarse pore size filters for adequate capture of NA signal (from both eDNA and eRNA) in less processing time, thereby minimizing risk of NA degradation at the pre-extraction step (Albers et al., 2013; Hunter et al., 2019). This is particularly relevant for waterborne eRNA, advocated as a better proxy for detecting the living fraction of biodiversity (Pochon et al., 2017), which is prone to much faster degradation due to its single-stranded structure (Laroche et al., 2017).

To our knowledge, this study is the first to test positively charged membranes for direct active filtration of eDNA material. Our findings demonstrated enhanced yields of naked NAs are obtained using this filter type, possibly due to electrostatic attraction of negatively charged particles. Yet, these results should be treated with caution as a non-charged nylon membrane was not tested for comparison in our study. In fact, for our study, we had difficulty locating and

procuring pre-cut nylon filters. Several earlier studies have reported the use of nylon membranes (Adrian-Kalchhauser & Burkhardt-Holm, 2016; Gargan et al., 2017); however, there was no charge attributed to the filters, and no indication from further examination of manufacturer's websites. Bessey et al. (2021) compared positively charged nylon filters to non-charged cellulose ester filters in a field deployment setting, whereby membranes were suspended for a fixed amount of time (4–24 h) to passively collect eDNA. At one site, they detected three times more fish species with the non-charged cellulose ester filters; however, at the second site, there was no difference between the two filter types. The limited data from the current and Bessey et al. (2021) studies warrant further investigation under various scenarios—for example, comparison of different combinations of charged and uncharged membranes (including pore sizes), different environmental settings (freshwater vs. marine, varying turbidity ranges) and capturing different NA fractions (whole/partial cells, naked NAs).

The Data Envelopment Analysis (DEA), applied in this study, aimed to evaluate the overall efficiency of the different filter membranes for capturing eDNA and eRNA and inform decision-making around optimized sampling design and resource allocation for addressing a particular research or surveillance question. This efficiency assessment approach maximizes the use of the available data and allows the calculation of a generalized efficiency score for the multiple outputs (RNA and DNA copy number yields in our study) and a corresponding set of

inputs (volume processed and timeframe in our study), across different scales of considered variables. DEA is sensitive to the presence of outliers (Ondrich & Ruggiero, 2002; Tran et al., 2010), and it does not account for the stochastic nature of the data (which may be due to measurement errors and omitted variables). However, compared to financial data and production data where DEA has been extensively applied, datasets from DNA/RNA capture are produced under a 'controlled' environment so the expected effect of outliers is minimal. To the best of our knowledge, this is the first application of DEA in the context of eDNA/eRNA sampling methodology assessment.

The results derived from DEA indicated that among four tested filter membranes, the 5 μm cellulose acetate membrane proved to be the best practice option for optimized capture of waterborne NAs. However, there may be other stochastic factors that influence the efficiency of the filtration process. For example, as revealed from our results, the water quality and target concentration may substantially affect the NA signal detection from water. This is consistent with results from previous studies, where the influence of dissolved substances in the water column on DNA detection rates has been reported, and explained by PCR inhibition effects (Albers et al., 2013; Hunter et al., 2019) or nucleic acids binding to suspended particles (Stoeckle, Beggel, et al., 2017). The stronger ddPCR signal reported from the low concentration cell treatments can be attributed to better discrete cloud clustering and therefore higher amplitude level readout in diluted samples (Kokkoris et al., 2021). The negative effect of high target concentration was especially evident in RNA dataset; however, the pattern of membrane performance remained consistent across treatments, suggesting no or limiting impact of that factor on efficiency assessment results.

Using a quantifiable culture of a unicellular algae as a target allowed us to better control the input of the starting material for the NA fractions considered in this case study, which is difficult to achieve when utilizing a sample collected from nature and applying a particle size fractionation technique on a sample as a whole (e.g. sequential filtration; Turner et al., 2014). This approach could mask the performance of a given test parameter (e.g. filter type and pore size) if naked nucleic acids become bound to particles and become part of the 'particulate fraction' (Zaiko et al., 2020). Results presented here could be applicable to other targets (including multicellular organisms); however, other factors might come into play affecting particular species detectability from environmental samples. These include, for example, differential NA shedding rates, types and origin of genetic material released by different taxa (Sassoubre et al., 2016; Turner et al., 2014; Wood et al., 2020), water chemistry and hydrodynamics (Barnes et al., 2014; Strickler et al., 2015), turbidity and/or presence of specific contaminants and inhibitors (Kumar et al., 2022; Lance & Guan, 2020; Sidstedt et al., 2015). In the future, for comparative efficiency assessments of NA capture and processing workflows, it might be of interest to adjust the experimental setup for tests with other target species (including mixed communities of species), while controlling a combination of input parameters (physical–chemical water properties, temporal scales, and concentration and type of input material). For such complex setups, efficiency assessment models accounting for the inherent stochasticity, such as, for example, the stochastic frontier regression with error decomposition (O'Donnell, 2018), might be considered.

It is important to emphasize that in this case study we assessed the performance of filter membranes for recovering the molecular signal of a target species. If assessing efficiency for other NA applications, for example, capturing legacy NAs of mobile macrofauna (e.g. fish, marine mammals), or NAs aimed for genomic or transcriptomic analyses or community composition assessments, the approach and outcomes might be different. It is also noteworthy that our findings are conditional to using commercial spin-column extraction kits that are not fully optimized for isolating extracellular NA from waterborne samples. This might have affected lower yields of amplifiable NA in naked DNA and RNA treatment. Still, the presented experimental model can be easily revised and adapted to optimize molecular workflows and assess the comparative performances of different sampling and processing tools, including different NA isolation protocols.

Appreciation of the current methodological limitations has impelled revision of sampling approaches and development of novel NA capture techniques from aquatic environments, including passive accumulation by functionalized substrates or natural samplers (Mariani et al., 2019; Verdier et al., 2021), molecular-biological sensing (McQuillan & Robidart, 2017) and fit-for-purpose in situ sampling systems (Thomas et al., 2018; Thomas et al., 2019). The framework presented here can provide a cheap and easy way to get a first pass on the success of these methods as well as quick feasibility check of new filter types and technologies coming onto the market or emerging in adjacent fields (e.g. medical applications).

The experimental setup can also be adjusted for addressing other practical questions relevant for interpreting NA-derived biodiversity information, for example, establishing linkage between eDNA concentrations and organism abundance (Andruszkiewicz et al., 2017), decay rates of different NA fractions in the environment (Moushomi et al., 2019; Zhao et al., 2021) and corresponding detectability of their signal over time (Holman et al., 2021; Wood et al., 2020). Implemented at comparatively small scales, such experimental comparisons would enable shorter timeframes from sample collection to interpreted results, improving communication pathways between researchers and environmental managers and, where relevant, help streamline operational feedbacks to manufacturers. This, in turn, will foster further technological developments and fit-for-purpose solutions to the topical challenges associated with environmental genomics.

AUTHORS' CONTRIBUTIONS

A.Z., K.F.S., X.P. and H.A.B. conceived the study; A.Z., J.S., K.F.S. and H.A.B. defined the design and conducted the experiments; J.S., U.v.A. and H.A.B. performed the sample processing and molecular analyses; R.Y. and M.W. performed the efficiency modelling; A.Z. performed the statistical analyses and produced the first draft of the manuscript; U.v.A., K.F.S., R.Y., X.P. and H.A.B. revised the early versions of the manuscript and contributed to writing. All authors contributed to revision of later versions and final proof-reading.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

All data collected and analysed during this study are deposited in the Dryad repository (Zaiko et al., 2022) https://doi.org/10.5061/dryad.vhhmgqnw8. No custom computer code or algorithm was used to generate results. The software environment R was used for all data exploration, statistical analyses and model development described in this manuscript using freely available R packages.

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