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Release and degradation of environmental DNA and RNA in a marine system

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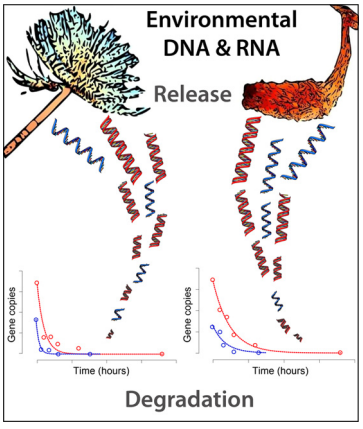
HIGHLIGHTS

- The efficacies of eDNA/RNA based approaches depend on the release and degradation of these molecules.
- eDNA/RNA release and degradation was investigated for two marine invertebrates using ddPCR.
- eRNA persisted for longer than expected (13 h) and decay rate constants for eDNA/RNA were similar.
- There was no evidence that the decay rates constants for eDNA and eRNA were different.
- Using eRNA may provide new opportunities for improved biodiversity surveys and transcriptomics.

GRAPHICAL ABSTRACT



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ABSTRACT

Over the last decade, there has been growing interest in the analysis of environmental DNA (eDNA) to infer the presence of organisms in aquatic environments. The efficacy of eDNA/eRNA based tools are highly depend on the turnover rate of the molecule (their release and degradation). Environmental DNA has been shown to persist for days, weeks or years in environmental samples. Environmental RNA (eRNA) is thought to degrade faster than eDNA, however to our knowledge, no experimental studies have explored this. Here we present an aquarium study to investigate eDNA and eRNA shedding rates and degradation for two sessile marine invertebrates. The copy numbers for eDNA and eRNA were assessed using droplet digital PCR targeting the mitochondrial *Cytochrome c Oxidase* subunit 1 (COI) gene. Environmental RNA persisted after organism removal for much longer than expected with detections for up to 13 h. In contrast, eDNA was detected is samples collected up to 94 h after organism removal. There was no evidence that the decay rates constants for eDNA and eRNA were different (p = 0.6, Kruskal-Wallis tests). Both eDNA and eRNA was detected in biofilms collected at the end of the experiment (day 21). This suggests binding with organic or inorganic compounds or stabilization of these molecules in the biofilm matrix. The finding of the prolonged persistence of eRNA may provide new

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opportunities for improved biodiversity surveys through reducing false positives caused by legacy DNA and could also facilitate new research on environmental transcriptomics.

1. Introduction

Molecular techniques applied to environmental DNA (eDNA) provide a powerful non-invasive method to identify specific species or characterize biological communities in environmental samples. Data generated from the analysis of eDNA is now used in conservation and invasion biology, and for a range of applications in biomonitoring and biodiversity surveys (Cristescu and Hebert, 2018; Foote et al., 2012; Keeley et al., 2018; Mächler et al., 2014; Valentini et al., 2016). The number of published eDNA studies is increasing exponentially and management agencies worldwide are now integrating eDNA-based techniques into their monitoring programs (Harper et al., 2019; Hering et al., 2018; Pawlowski et al., 2018; Simmons et al., 2015). Unlike DNA, which can persist for variable lengths of time in the water column, RNA is thought to break down rapidly after cell death (Eigner et al., 1961; Mengoni et al., 2005). A more rapid turnover of eRNA (relative to DNA) would provide a more accurate insight into the presence of living species in an ecosystem, but this assumption remains untested. Several lines of evidence suggest that eRNA could persist for long periods of time under special conditions (Cristescu, 2019). In this study we refer to eDNA/eRNA which has been released by organisms into the surrounding environment and is in cellular, vesicular, or free form in the absence of progenitor organisms. DNA associated with very small organisms captured in bulk samples is not considered eDNA for the purposes of this study.

Although eDNA analysis has undoubtedly facilitated significant advancements in many fields, there is still limited knowledge on the factors that influence eDNA release and degradation and how this varies between species and environments (Barnes et al., 2014; Deiner and Altermatt, 2014; Dejean et al., 2012; Lacoursière-Roussel et al., 2016; Pilliod et al., 2014; Seymour et al., 2018). Environmental DNA may persist long enough to be transported in the water column, and consequently be detected at locations where the organism has not actually been present (Carraro et al., 2018; Shogren et al., 2017). Additionally, its long persistence may increase the rates of false positives due to detection of locally extinct species and/or past community assemblages. Recent research has estimated eDNA decay rate constants (the rate at which the concentration of DNA decreases per time unit) of marine and freshwater organisms by quantifying the presence of eDNA in experimental microcosms and mesocosms over time. Such experiments report values ranging between 0.015 and 0.701 hr^{-1} (Collins et al., 2018; Eichmiller et al., 2016; Lance et al., 2017; Maruyama et al., 2014; Sassoubre et al., 2016; Strickler et al., 2015; Thomsen et al., 2012). Recent studies concur that eDNA persistence in water rarely exceeds 4 weeks, with most degradation occurring within the first few days following removal of the organisms (Collins et al., 2018; Lance et al., 2017; Strickler et al., 2015; Tsuji et al., 2017; Weltz et al., 2017). These findings support a model of exponential decay of DNA first proposed by Willerslev et al. (2004). In contrast, eDNA in sediment and biofilms is known to persist for much longer time periods (Corinaldesi et al., 2008; Dell'Anno and Danovaro, 2005; Domaizon et al., 2017). Knowledge on eDNA degradation rates has advanced rapidly over the last few years, but research into organism and habitat-specific eDNA degradation rates is essential, especially when monitoring for rare or invasive species.

To date, research on the applicability of using RNA for biomonitoring has largely focused on bulk environmental samples (i.e. sediments) where RNA is primarily extracted directly from whole or parts of an organism (Dowle et al., 2015; Keeley et al., 2018; Pawlowski et al., 2014; Pochon et al., 2015), as opposed to extraorganismal RNA which could be in cellular, vesicular or free form (Cristescu, 2019). To our knowledge, the only study that has at least partially explored extraorganismal RNA is from Pochon et al. (2017) who investigated eRNA signals in bilge water samples. Although most of the eRNA signal measured in this study was arguably of extraorganismal RNA origin, whole organisms were undoubtedly also included in their data. There is a pressing need for a clearer understanding of the mechanisms of RNA release, transport, molecular state, and degradation rates to allow its utility for environmental monitoring to be evaluated. If the assumption that eRNA is less stable than eDNA is correct, this may provide an avenue to overcome some of the issues related to detection of eDNA at locations were an organism is not currently present, as long as its short persistence allows detection. A molecule with a faster turnover rate could prove invaluable in instances when differentiating between deceased and living organisms is crucial, for example, monitoring populations of a highly endangered species, or determining whether eradication of invasive species has been successful.

In the present study, we evaluated whether there was a difference in decay rate between eDNA and eRNA molecules in aquarium experiments and explored the effects of time and species. Two morphologically distinct marine invasive species were assessed: *Sabella spallanzanii*, a large fanworm native to the Mediterranean Sea and Atlantic coast of Europe (Patti and Gambi, 2001), and *Styela clava* (Herdman, 1881; Ascidiacea: Styelidae), a club tunicate characterized by its leathery cylindrical body (Clarke and Therriault, 2007). Once established, both species can form dense populations (100 s to 1000 s per m^2) covering a variety of marine habitats. Study organisms were placed into individual aquaria, removed after 36 h and samples collected for 21 days. The presence of eDNA and eRNA from the study organisms was determined in water and biofilm samples using droplet digital PCR assays targeting the mitochondrial *Cytochrome c Oxidase* subunit 1 (COI) gene. In our study, we use the term biofilm to refer to the assemblage of marine bacteria, diatoms, protozoa, fungi and other adsorbed organic matter that grew embedded within a slimy extracellular matrix on the surfaces of the aquariums.

We tested the following hypotheses: (1) eRNA degrades faster than eDNA; (2) eRNA will only be detected during the shedding/release phase due to its rapid decay, (3) concentration of nucleic acid released into the water will depends on the biology of organism (e.g. the surface area), and (4) eDNA, but not eRNA, will accumulate in the biofilms that form on the aquarium bottom over the experiment.

2. Material and methods

2.1. Experimental set-up and collection of study organisms

Fifteen aquaria (15 L) were thoroughly cleaned with detergent and 10% bleach, followed by multiple rinses with hot tap water.

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The aquaria were filled with filtered (0.22 μm) and UV-treated seawater (14.5 L), and maintained at 19 ± 1 °C with a 14:10 h light:-dark cycle and continuously aerated. *Sabella spallanzanii* (n = 10) were collected by hand from the side of pontoons in Westhaven Marina (Auckland, New Zealand; 36°84′S, 174°75′E; 23 January 2018), and immediately transported to the laboratory wrapped between seawater-soaked cloths on trays (c. 4 h). *Styela clava* (n = 10) were collected by Scuba divers from the Nelson Marina (New Zealand, 41°26′S, 173°28′E; 23 January 2018), and immediately transported back to the laboratory (c. 1 h) in large plastic jars with seawater. In the laboratory, organisms were carefully rinsed with pre-filtered and UV-treated seawater to remove sedimented material from their surface. The experimental set up was as follows: five *S. spallanzanii* were individually attached to a small rock using a plastic cable tie to ensure they stood upright, and placed into five separated aquaria (i.e. one organism per aquarium), and five *S. clava* were individually attached by their stem to a string that was suspended subsurface in each of five separated aquarium to allow them to hang vertically down into the aquarium (Fig. 1). Additionally, five aquaria contained a combination of one *S. spallanzanii* and one *S. clava* using the set up described above. The rational for the dual organism set up was to provide some initial data as to whether eDNA/eRNA degraded faster when organism density, and likely microbial density, was greater, thus providing some initial data for future studies. The organisms were maintained in the aquaria without feeding for 36 h before removal. Three *S. spallanzanii* individuals (in aquariums with *S. spallanzanii* only) did not survive the 36-hour period -and these data were removed from further analysis.

2.2. Sample collection and processing

Aerator pipes were gently swirled once around each aquarium to ensure mixing prior to sampling. Water samples (50 mL) were collected 4 cm below the surface using sterile 60 mL syringes. A control water sample was collected from each tank 4 h before the addition of the organisms. Water samples were then collected 12 and 20 h after the addition of the organisms to investigate eDNA/eRNA shedding rates. After removal of the organisms (36 h) water samples (50 mL) were collected immediately after removal and then at: 4, 8, 12, 24, 72, 120 (5 days), 168 (7 days), 336 (14 days) and 504 (21 days) h.

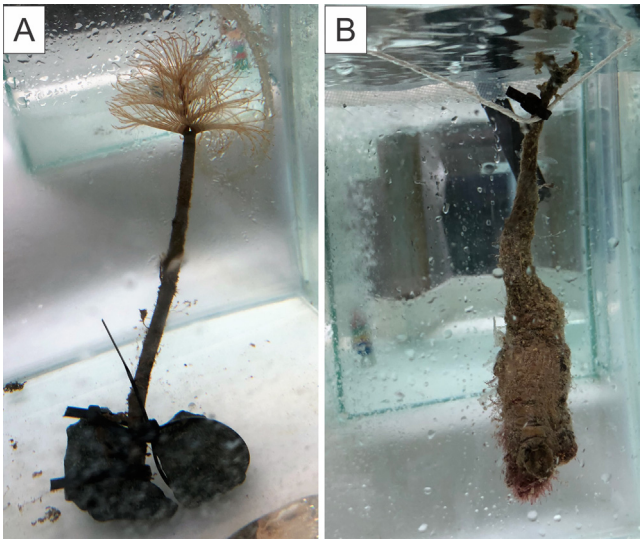

Fig. 1. *Sabella spallanzanii* (**A**) and *Styela clava* (**B**) during the aquarium experiments.

Immediately after collection, the water samples were filtered (polycarbonate, 3 μm, 47 mm dia.; Whatman, UK). Between each sample, tweezers and filter holders were soaked in 10% bleach and 0.1% sodium thiosulfate (5 min each), followed by rinsing in MQ-water and drying. A single Milli-Q water (50 mL) sample was also processed at the same time as a control on each sampling date. The filter papers were transferred into bead tubes (ZR BashingBead Lysis Tubes; 2.0 mm Zymo Research, CA, USA), and stored at −80 °C until DNA and RNA extractions. At the end of the 21-day sampling period, the water from all aquariums was emptied and the biofilm accumulated on the bottom of each aquarium was collected using sterile blades and placed in 15 mL tubes. The total weight of the biofilm in each sample was recorded and tubes were stored at −80 °C until DNA and RNA extractions.

Physico-chemical parameters (salinity, dissolved oxygen and pH) were measured at every sampling event using a hand-held YSI multi-probe. Temperature data loggers (HOBO® Pendant temperature loggers, Onset, USA) were also placed in three aquariums (one of each treatment), and temperature was recorded every 15 min. The probe and loggers were wiped with bleach and rinsed in Milli-Q water prior to immersion in the aquariums.

2.3. DNA and RNA extraction and cDNA synthesis

Each step of the molecular analysis (i.e. DNA and RNA extractions, PCR setup, template addition and ddPCR analysis) was conducted in a separate sterile laboratory dedicated to that step with sequential workflow to ensure no cross-contamination. Each room was equipped with UV sterilization that was switched on for a minimum of 15 min before and after each use. The PCR set-up and template addition were undertaken in laminar flow cabinets with HEPA filtration. Aerosol barrier tips (Axygen BioScience, CA, USA) were used throughout.

Lysis Buffer (1 mL) from the ZR-Duet™ DNA/RNA MiniPrep Kit Plus (Zymo Research, CA, USA) was added to the ZR BashingBead Lysis Tubes containing the filters. These were then homogenized by bead beating (1,500 RPM, 2 min; 1600 MiniG Spex SamplePrep NJ, USA) and centrifuged (3,000×*g*, 5 min, 20 °C). DNA and RNA were then co-extracted using the ZR-Duet™ DNA/RNA MiniPrep Kit Plus (Zymo Research, CA, USA), following the manufacturer's protocol. The quality and purity of isolated DNA and RNA in all samples were checked using a spectrophotometer (Eppendorf, Leipzig, Germany). DNA and RNA were co-extracted from biofilm subsamples (0.2 g) using the same protocol.

Trace DNA molecules carried over in RNA extracts were eliminated by two sequential DNase treatments as described in Langlet et al. (2013). To confirm the absence of DNA in RNA eluents each of the ddPCR assays (*S. spallanzanii* and *S. clava*) described below were run on each RNA sample after DNase treatment. The DNAse-treated RNA was transcribed into cDNA, using the SuperScript® III reverse transcriptase (Life Technologies, CA, USA). The various extract products (DNA, RNA, and cDNA) were separated into aliquots and stored frozen (−20 °C for eDNA/cDNA and −80 °C for pure RNA and DNAse-treated RNA samples) until further analysis. The cDNA products are hereafter referred to as eRNA. When a cDNA sample produced a positive result, the original RNA samples was rechecked using the appropriate ddPCR assay to further ensure that the result was not due to any DNA contamination.

2.4. Droplet digital polymerase chain reaction

Droplet digital PCR (ddPCR) was conducted in an automated droplet generator (QX200 Droplet Digital PCR System™, BioRad). Copy numbers (per μL) of the COI gene were measured in all samples using primers and probes specific to *S. spallanzanii* and *S. clava*,

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as described in previous studies, respectively Wood et al. (2018); Sab3-QPCR-F, Sab3-QPCR-R primers and Sab3-QPCR-Probe, and Gillum et al. (2014); SC1F, SC1R primers and SC1-QPCR probe. The hydrolysis probes were dual-labelled with a 5′ 6-carboxyfluorescein (6-FAM) fluorescent tag and a 3′ Black Hole Quencher. Each ddPCR reaction included 1 μL of 450 nM of each primer and probe, 10 μL BioRad ddPCR Supermix for probes, 1 μL DNA, and sterile water for a total reaction volume of 22 μL. The BioRad QX200 droplet generator partitioned each reaction mixture into nanodroplets by combining 20 μL of the reaction mixture with 70 μL of BioRad droplet oil. After processing, this resulted in a total nanodroplet 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, 60 °C 1 min, and a final enzyme deactivation step at 98 °C for 10 min. The plate was then analyzed on the QX200 instrument. For each ddPCR plate run, at least one negative control (RNA/DNA-free water Life Technologies), and one positive control (genomic DNA extracted from *S. clava* or *S. spallanzanii*) were included. Results were then converted to copies mL^{-1} using the following formula; number of copies per μL × 22 μL (the initial volume of the PCR reaction) × 60 μL (the volume used to elute the DNA during extraction, and assuming 100% extraction efficiency which we acknowledge is unlikely), divided by 50 (the amount of water filtered). The concentrations of eDNA and eRNA present in biofilm samples were converted into copies/tank using the following formula: [total weight of biofilm sample/0.2 (weight of subsample used for eDNA/eRNA extraction)] × [number of copies per μL × 22 μL × 60 μL]. Based on dilution experiments undertaken with serial dilutions of *S. spallanzanii* and *S. clava* (data not shown) and our experience and observations of noise (e.g., proportions of fluorescing droplets in water blanks), the detection of both assays was set to 0.1 copies/μL. DNA and cDNA samples were run on separate plates, and on different days.

2.5. Data analysis

All statistical analyses were conducted in R version 3.4.1 software (R-project, 2013). Decay rate constants were established by fitting the exponential decay model $N(t) = N_0 e^{-\lambda t}$ to all raw data, where $N(t)$ is the concentration of *S. spallanzanii* or *S. clava* eDNA/eRNA at time t, N_0 is the concentration of *S. spallanzanii* or *S. clava* eDNA/eRNA at time 0 (measured immediately after removal of the organisms from experimental aquaria), and λ is the decay rate constant. Decay rate models were fitted using 'easynls' package implemented in R software (Kaps and Lamberson, 2009; R-project, 2013). The time taken for *S. spallanzanii* and *S. clava* eDNA/eRNA to degrade to undetectable levels was calculated using the estimated decay rate constants (λ), as in Strickler et al. (2015). A Kruskal-Wallis test was performed to determine whether the decay rate constants were significantly different among treatments (both species in isolation and when they were combined), and Wilcoxon rank sum test and Wilcoxon signed rank test for paired samples were used to determine whether decay rate constants were significant difference between eDNA/eRNA. A Kruskal-Wallis test was also used to determine whether there was statistical significance between the starting concentrations of eDNA/eRNA of each treatment, and the concentration of eDNA/eRNA present in biofilm samples among treatments.

3. Results

3.1. Environmental DNA/RNA release and shedding prior to organism removal

Following DNase treatments, RNA samples were assessed for DNA contamination using the ddPCR assays (*S. spallanzanii* and *S. clava*). All samples were negative. Prior to the removal of organisms, eDNA and eRNA copy numbers were highest at 12 h for *S. spallanzanii* in isolation, and 20 and 36 h for *S. spallanzanii* kept with *S. clava* (Fig. 2). *Styela clava* in isolation shed/released the least amount of eDNA and eRNA. The Kruskal-Wallis tests showed a statistically significant difference at the time of organism removal (36 h) between treatments for eDNA ($p = 0.036$), but not for eRNA ($p = 0.245$). There was no amplification in any of the aquaria control (collected prior to the addition of organisms) or filtration controls.

3.2. Environmental DNA/RNA decay rates

Physico-chemical parameters (dissolved oxygen, salinity, conductivity and water temperature) remained relatively constant throughout the experiment (Table S1 and Fig. S1).

The eDNA and eRNA of both species exhibited an exponential decay (Fig. 3, Fig. S2). Decay rate constants ranged between 0.104 and 0.338 h^{-1} for eDNA, and 0.156 to 0.682 h^{-1} for eRNA (Table 1). Kruskal-Wallis tests showed no significant difference in decay rate constants among treatments for eDNA or eRNA ($p = 0.183$ and $p = 0.196$ respectively). The statistical test also indicated a non-significant difference between decay rate constants of eDNA and eRNA ($p = 0.66$ and 0.63, overall and paired test).

Sabella spallanzanii eDNA signals dropped below the ddPCR detection limit after 42 h in isolation, and 35 h when combined with *S. clava* (Table 1). *Styela clava* eDNA signals were undetectable after 94 h in isolation, and 87 h when combined with *S. spallanzanii*. Environmental RNA signals were below the ddPCR detection limit in less than 13 h for all treatments (Table 1).

3.3. Environmental DNA/RNA in biofilms

Environmental DNA was detected in all biofilm samples and eRNA of *S. spallanzanii* in four samples (in three aquaria containing *S. spallanzanii* only and in one aquarium containing both *S. spallanzanii* and *S. clava*; Table 2). For the eDNA and eRNA from *S. spallanzanii,* the copy numbers were highest in aquaria where this organism was in isolation. The opposite pattern was observed for *S. clava* eDNA with the highest copy numbers measured in aquaria where both species were combined and lowest in aquariums where *S. clava* was isolated (Table 2). No eRNA from *S. clava* was detected in the biofilms. The concentrations of eDNA were highly variable and there was no significant difference (Kruskal-Wallis test) between treatments ($p = 0.369$), however difference between eRNA treatment concentrations were significant ($p = 0.048$).

4. Discussion

The analysis of eDNA is rapidly becoming a powerful tool for characterizing the Earths' aquatic biomes, undertaking biomonitoring studies, and detecting rare and invasive species (e.g. Jerde et al., 2011; Thomsen and Willerslev, 2015; Valentini et al., 2016). While most studies show relatively rapid eDNA decay (Collins et al., 2018), some report detections for up to 58 days (Strickler et al., 2015). There is no information on the decay rate of eRNA in the environment (Cristescu, 2019; Pochon et al., 2017; Wood et al., 2018). In the present study, we investigated DNA and RNA shedding and degradation rates from two sessile marine invertebrates.

4.1. Environmental DNA/RNA degradation

RNA is widely accepted to be less stable than DNA, although to our knowledge no studies have experimentally investigated degra-

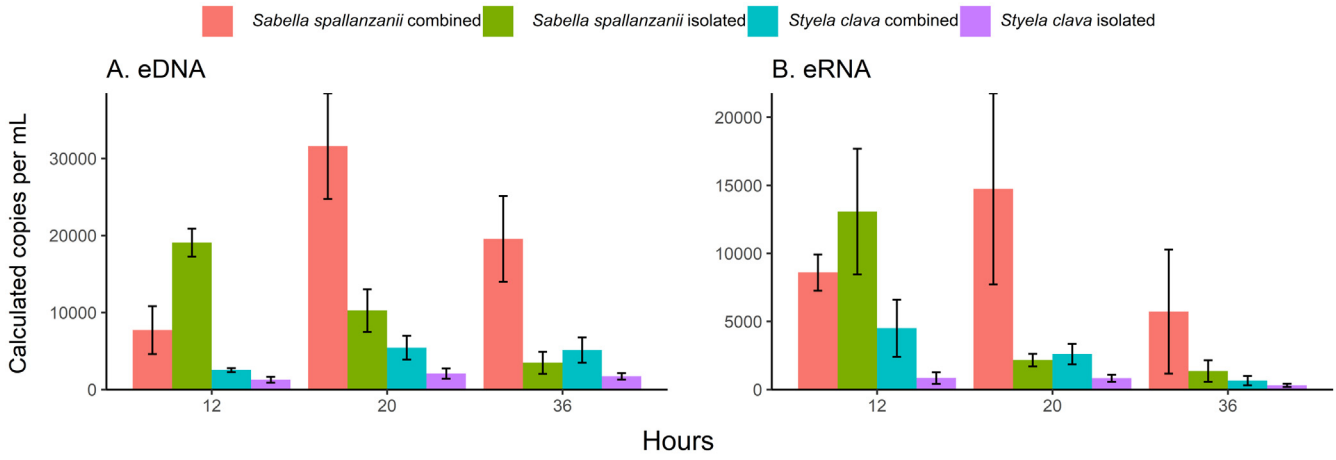

Fig. 2. Average copies per mL of the *Cytochrome c oxidase* subunit 1 (COI) gene present in: (A) eDNA and (B) eRNA samples for both *Sabella spallanzanii* and *Styela clava* in isolation and combined with each other. Vertical lines represent standard errors. Note different y-axis scales.

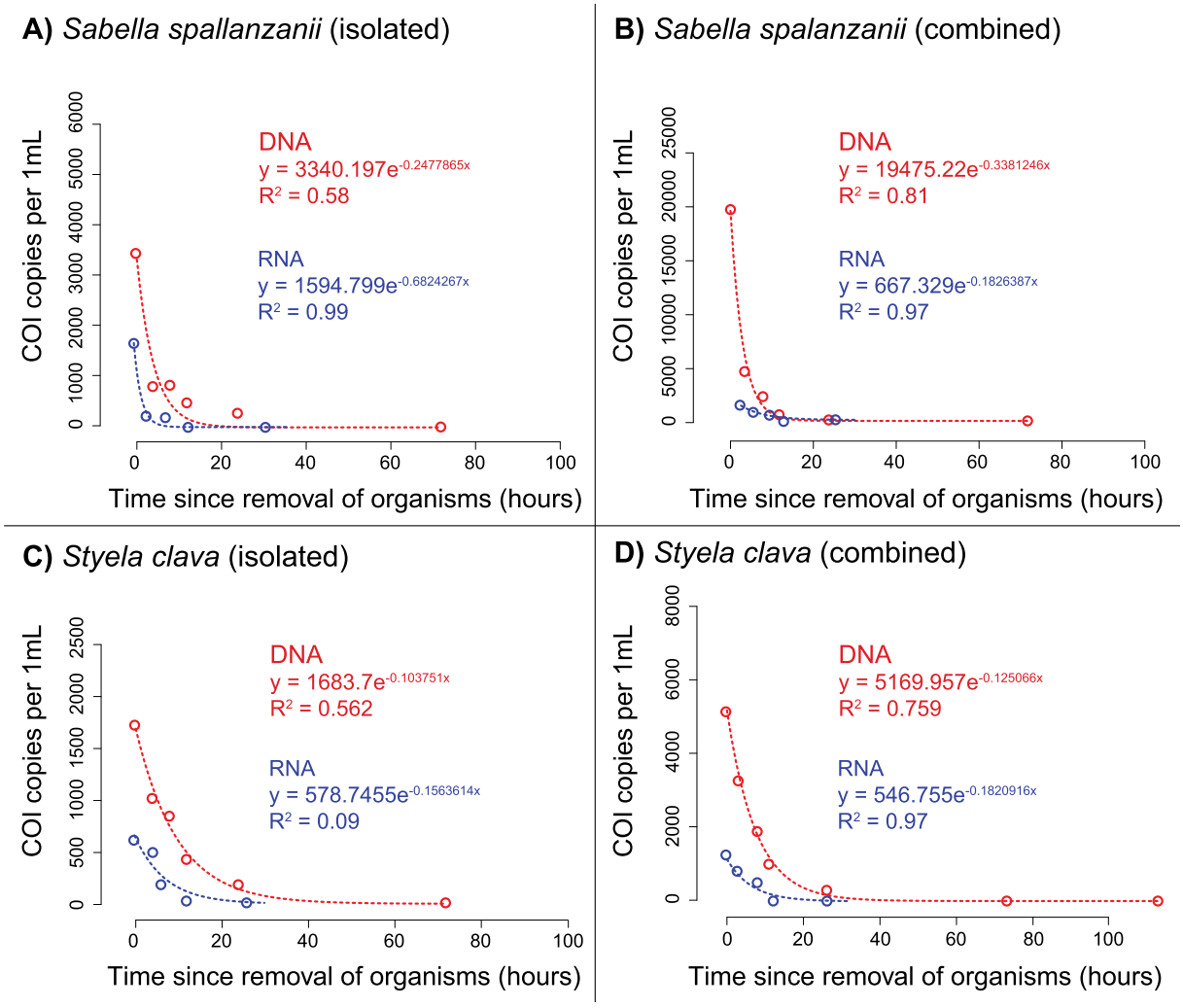

Fig. 3. Time-dependent changes in average environmental DNA and RNA concentration (based on detection of the *Cytochrome c Oxidase* subunit 1 [COI] gene) for: (A) *Sabella spallanzanii* in isolation, (B) *S. spallanzanii* when combined with *Styela clava*, (C) *S. clava* in isolation, and (D) *S. clava* when combined with *S. spallanzanii*. Equations show the rate of exponential decay after applying the decay model $N(t) = N_0 e^{-\lambda t}$ to all raw data. R^2 values indicate the closeness of fit of raw data to the fitted decay model. Individual curves for DNA and RNA are shown in Fig. S1.

dation rates of eRNA (Cristescu, 2019). The instability of RNA is often linked to its conformation. The single-stranded structure of RNA degrades more rapidly than double-stranded DNA, as it is more prone to auto-hydrolysis (Voet and Voet, 2011). Moreover, the presence of hydroxyl groups within RNA make it particularly susceptible to chemical break down through the process of base-

Table 1
Decay rate constants for *Sabella spallanzanii* and *Styela clava* environmental DNA/RNA based on detection of the *Cytochrome c Oxidase* subunit 1 (COI) gene, in isolation and combined with each other. These values were estimated by fitting the exponential decay rate model $N(t) = N_0 e^{-\lambda t}$ to the raw data. The droplet digital PCR detection limit is defined as 0.1 copies μL^{-1} in this study.

	Species	Treatment	N_0 (COI copies per mL)	Average model-derived decay Rate Constant (λ)	Estimated hours until degradation of eDNA/eRNA below detection limits
eDNA	*Sabella spallanzanii*	Isolated	3,460	0.248	42
		Combined	19,550	0.338	35
	Styela clava	Isolated	1,720	0.104	94
		Combined	5,140	0.125	87
eRNA	*Sabella spallanzanii*	Isolated	1,735	0.682	9
		Combined	5,770	0.182	8
	Styela clava	Isolated	310	0.156	13
		Combined	660	0.182	7

Table 2
Average copies of the *Cytochrome c Oxidase* subunit 1 (COI) gene present in biofilm samples for *Sabella spallanzanii* and *Styela clava*, in isolation and combined with each other. *nd* = not detected. * signal detected in three aquaria for isolated and in one for combined.

	Species	Treatment	Average calculated copies per tank	Range of calculated copies
eDNA	*Sabella spallanzanii*	Isolated	625,130	34,485–1,085,960
		Combined	336,830	50,860–643,770
	Styela clava	Isolated	273,480	35,755–502,850
		Combined	741,270	459,720–1,331,590
eRNA	*Sabella spallanzanii*	Isolated*	376	0–750
		Combined*	129	n/a
	Styela clava	Isolated	*nd*	–
		Combined	*nd*	–

catalyzed hydrolysis (Fontaine and Guillot, 2003). Because RNA has an exposed hydroxyl group in its sugar-phosphate backbone that is not found in structure of DNA, RNA is more susceptible to abiotic breakdown by base-catalyzed hydrolysis (Li and Breaker, 1999). Although still in its infancy, the analysis of eRNA is now gaining interest with researchers suggesting that it could provide a better indication of whether taxa detected in environmental samples are alive (Corinaldesi et al., 2008; Cristescu, 2019; Dell'Anno and Danovaro, 2005; Orsi et al., 2013; Pochon et al., 2017; Wood et al., 2018). The results of the present study demonstrate that eRNA is more stable than previously thought and appears to have a similar decay rate to eDNA and not 50-fold higher as indicated in the literature (Eigner et al., 1961). It is worth noting that in the present study the starting concentrations of eRNA were lower than eDNA. It is possible that a considerable amount of RNA was lost at the extraction stage when RNA is particularly exposed and labile, but confirmation of this requires further investigation. While this may reduce sensitivity, it may also be advantageous as there is concern that some of the DNA signal detected in water samples is due to organisms that are no longer present in the waterbody.

The experiments in this study were conducted in water that was sterile prior to the addition of organisms. Bacterial extracellular nuclease activity has been shown to be an important factor in eDNA degradation (Lance et al. 2017), but this was not considered in this experiment. The experimental conditions in this study were relatively depurated of microorganisms. In the environment, water is likely to contain many other bacteria and organisms that would lead to faster eDNA/eRNA degradation. Although not statistically significant, the eDNA decay rate constants of *S. spallanzanii* and *S. clava* were higher when both species were combined. One plausible explanation is that greater density of organisms supports a higher or more diverse microbial community. As noted above, previous research showed that increased microbial activity stimulates the breakdown of DNA through the production of exogenous nucleases (Strickler, et al. 2015).

In aquatic environments, in addition to degradation, diffusion of eDNA/eRNA is the other major component that impacts the likelihood of its detection (Ficetola et al., 2008). This is particularly relevant in marine systems where the large water-volume biomass ratio combined with the motion of sea-currents and wave action rapidly dilute and transport eDNA/eRNA from its source location (Port et al., 2016; Thomsen et al., 2012). Based on the decay rates observed in this study, the eDNA/eRNA from *S. spallanzanii* and *S. clava* could be transported to locations remote from its source before complete degradation, potentially complicating the interpretation of the detected signal. Future research that incorporates eDNA/eRNA degradation factor into oceanographic and particle transport models may ultimately assist in developing well-informed predictive probability maps of source populations similar to those being developed for river systems (Sansom and Sassoubre, 2017).

4.2. Environmental DNA and RNA release/shedding

One of the most influential factors which affects the likelihood of species detection with molecular methods is the concentration of its genetic material in the environment, which is directly linked to the rate of eDNA/eRNA shedding and consequently the type, size, life stage and abundance of the organisms (Sassoubre et al., 2016). In this study, we hypothesized that there would be differential DNA/RNA released between the two study organisms due to the biology of the organism, i.e., surface area. When the organisms were in the aquaria and immediately following removal, concentrations of *S. spallanzanii* eDNA and eRNA were generally higher than those of *S. clava*. This is likely to be attributed to their anatomical differences. The body shape of *S. spallanzanii* with a high surface-area to biomass ratio and its fragile protruding crown of feeding tentacles likely facilitates cell and tissue shedding in this species (Fig. 1A). Conversely, *S. clava* has a leathery tunic and smaller surface-area to biomass ratio and considerably robust siphons and filtering apertures, less prone to fragmentation and thus

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releasing cellular genetic material (Fig. 1B). The effect of morphology and life form was also demonstrated by Sassoubre et al. (2016), where variable eDNA shedding and decay rates were reported in three marine fish. The authors attributed this variation was attributed to different eDNA sources, i.e. scales versus mucus, as well as fish size and possibly physiologies, metabolic rates and feeding activities.

4.3. Persistence of eDNA/eRNA in the biofilms

Once released from an organism, the fate of eDNA and eRNA is largely unknown. In addition to chemical and physical breakdown of free-floating nucleic acids and release of eDNA/eRNA from cells, there is speculation that the these molecules may bind to inorganic or organic particles or become trapped in biofilms or sediments, where it might provide a food source for microbial organisms (Barnes and Turner, 2016). In the first experimental study of eDNA accumulation in biofilms, Seymour et al. (2018) found little evidence for eDNA accumulation with only low-level detection of one of their study species during stream mesocosm experiments. In contrast, in the present study, eDNA (both study organisms) and eRNA (*S. spallanzani*) was detected in the biofilm 21 days after removal of the organisms. This was surprising considering that eDNA signals in the water column of all aquariums were undetectable after 5 days for DNA and after 13 h for RNA. This result potentially indicates that eDNA/eRNA is more stable once bound to inorganic or organic particles, provided it has sufficient time to settle and be incorporated into these matrices. There is evidence to support this notion in the study of Shogren et al. (2017) who found that finer substrate on riverbeds allows for greater *Cyprinus carpio* eDNA uptake. An alternative hypothesis is supported by the study of Barnes et al. (2014) who explored *C. carpio* eDNA degradation and demonstrated an increase under lower aerobic activity and chlorophyll levels. It suggests that biological activity, such as that occurring in biofilms, may counterintuitively assist eDNA preservation. Early research suggested that the polysaccharides matrix of biofilms can absorb eDNA (Pavoni et al., 1972). The matrix including the eDNA can be enzymatically degraded but most of the time the matrix is preserved since it has important roles such as structural, protection, nutrient absorption, cell communication, and horizontal gene transfer (Decho and Gutierrez, 2017).

5. Conclusion

This study is the first to report shedding and degradation rates for both eDNA and eRNA. Environmental RNA persisted for longer than expected with detections of up to 13 h after organism removal, and the decay rate constants for eRNA and eDNA were not significantly different. One of the most surprising finding was the detection of both eDNA, and eRNA in the biofilms at the conclusion of the experiment despite the absence of detection in water samples. This suggests binding with organic or inorganic compounds, or stabilization of these molecules in the extracellular matrix.

6. Authors' contributions

SAW, XP, MC and AZ designed the research. SAW, XP, JL, FA, UA, LB performed the experiments and gathered the data. JL, AZ and LB analyzed the data and all authors contributed to the drafts of the manuscript.

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Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.135314.

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