Vol. 621: 95–105, 2019
https://doi.org/10.3354/meps13007

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published July 4

Nitrogen and phosphorus cycling in the digestive system and shell biofilm of the eastern oyster *Crassostrea virginica*

Nicholas E. Ray[1,*], Maria C. Henning[2], Robinson W. Fulweiler[1,2]

[1]Boston University, Department of Biology, Boston, MA 02215, USA
[2]Department of Earth and Environment, Boston University, Boston, MA 02215, USA

ABSTRACT: The shell and digestive system of bivalves provide microhabitats for an array of microbes that can mediate biogeochemical cycles. Here, we investigated nitrogen (N) and phosphorus (P) fluxes in these microhabitats in the eastern oyster *Crassostrea virginica*. From the anoxic oyster digestive system, we measured significant production of dinitrogen gas (N_2-N; mean ± SE: 0.59 ± 0.20 µmol ind.$^{-1}$ h^{-1}) and nitrous oxide (N_2O; 0.001 ± 0.0004 µmol ind.$^{-1}$ h^{-1}), indicative of denitrification. The oxic shell biofilm released N_2O (0.0003 ± 0.0001 µmol ind.$^{-1}$ h^{-1}), as well as ammonium (NH_4^+; 1.26 ± 0.20 µmol ind.$^{-1}$ h^{-1}) and nitrite (NO_2^-; 0.05 ± 0.01 µmol ind.$^{-1}$ h^{-1}), but not N_2-N, suggesting a combination of nitrification and heterotrophic activity. The biofilm released more dissolved inorganic P than the digestive system, although the rate of release from whole oysters was closer to the rate from the digestive system alone. N remineralized by oysters is released almost exclusively as NH_4^+, at a ratio of 18.4:1 with P, i.e. relatively close to the Redfield ratio (16:1). In an ecological context, this study supports the growing literature on the ability of oysters themselves to engage in denitrification activity and at rates potentially exceeding rates of sediment denitrification. The denitrification in the digestive system appears to proceed to completion and has a very small N_2O cost (<1%). Restoring oyster populations may therefore be an important method for N reduction in coastal systems.

KEY WORDS: Oyster · Nitrogen · Denitrification · Nitrous oxide · Nitrification · Shellfish

1. INTRODUCTION

Oysters provide a suite of ecosystem services, including habitat provision and regulation of coastal nitrogen (N) cycling processes (Coen et al. 2007). At an ecosystem scale, oyster reefs and aquaculture provide habitat for a range of higher trophic level organisms such as fish and invertebrates (Peterson et al. 2003), as well as microbial communities (Nocker et al. 2004). At an individual scale, the oyster itself is host to a diverse microbiome in both its digestive system and the biofilm that lives on its shell (King et al. 2012, Arfken et al. 2017). Oysters regulate N cycling at the ecosystem scale by transporting suspended particulates to the sediment during filter-feeding (Newell et al. 2005) and at the individual scale through digestion processes and provision of habitat for biofilms (Wahl et al. 2012, Lacoste & Gaertner-Mazouni 2015). Oysters are naturally evolved to live in dense populations, so while the magnitude of N-cycling processes occurring in the digestive system and in the shell biofilm of individual oysters may seem insignificant, regulation of N-cycling by large oyster populations could have important impacts at the ecosystem scale.

The 2 microhabitats in oysters differ greatly in microenvironmental conditions and thus support communities with different life strategies: the digestive system is a low-oxygen and low-light environment, while the shell is exposed to oxygen and light

*Corresponding author: nray@bu.edu

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during the day. The bivalve shell biofilm hosts microbes that can oxidize ammonium (NH_4^+) to create nitrate (NO_3^-) through nitrification (Welsh & Castadelli 2004, Caffrey et al. 2016) and also provides substrate for epiphytes including suspension-feeders, algae, and single-cell primary producers (Lacoste & Gaertner-Mazouni 2015) that can excrete or assimilate NH_4^+. For the rest of this paper, we use the term 'biofilm' to collectively describe the whole shell community. The oyster digestive system likely functions similarly as in other bivalves, supporting denitrification (the reduction of NO_3^- to dinitrogen [N_2] gas) and thus potentially provides the ecosystem service of reducing the biologically available N load in coastal ecosystems (Stief 2013).

The contrasting oxygen availabilities and associated N-cycling processes occurring in each oyster microhabitat potentially create a feedback loop, as different N-cycling processes are spatially and functionally coupled in oysters. The shell nitrifier community is 'fertilized' by NH_4^+ excreted by the bivalve (Heisterkamp et al. 2013), and the NO_3^- created during nitrification can be transported into the bivalve as it feeds, providing resources for denitrifiers living in the digestive system. In marine and aquatic systems, release of nitrous oxide (N_2O), a greenhouse gas with 298 times the global warming potential of carbon dioxide (Myhre et al. 2013), is positively correlated with the concentration of NH_4^+ and NO_3^- in the water column (Seitzinger & Nixon 1985, Beaulieu et al. 2011). Consequently, excretion of NH_4^+ by the oyster digestive system could drive N_2O production by the shell biofilm (Heisterkamp et al. 2013, Erler et al. 2017), and the NO_3^- produced by the biofilm could drive N_2O production in the digestive system (Stief et al. 2009, Heisterkamp et al. 2010, Svenningsen et al. 2012, Welsh et al. 2015, Bonaglia et al. 2017).

In addition to N, the eastern oyster *Crassostrea virginica* is known to influence estuarine phosphorus (P) cycling by excreting phosphate (PO_4^{3-}) (Satomi & Pomeroy 1965). The contribution of the digestive system and shell biofilm to PO_4^{3-} release from the whole oyster remains unclear, although we hypothesize that the majority of PO_4^{3-} release will be from the oyster digestive system, as PO_4^{3-} is created during the digestion of organic material such as phytoplankton. The amount of P released relative to N is important when considering which nutrient limits phytoplankton growth in coastal systems.

The magnitude of nitrification and denitrification in bivalves occurs at rates that are ecologically significant (Welsh & Castadelli 2004, Stief et al. 2009) and can proceed at rates higher than in the surrounding sediment (Smyth et al. 2013, Arfken et al. 2017). Large oyster reefs were once dominant features of coastal ecosystems (Beck et al. 2011, Zu Ermgassen et al. 2012), and declines in oyster populations due to overharvesting and disease have altered how many coastal ecosystems function (Newell 1988). Today, oyster populations are increasing in some areas as a result of reef restoration and aquaculture development, highlighting the importance of understanding how these microhabitats interact and regulate N biogeochemistry in coastal systems. While native oyster species are being restored in some ecosystems, non-native species have been introduced to others. Understanding how different oyster and bivalve species regulate nutrient recycling and removal in coastal ecosystems — and the mechanisms by which they do so — is an important step toward making sound decisions regarding species introductions.

In this study, we measured net fluxes of dissolved inorganic N and P, N_2, and N_2O from whole oysters, whole oysters with the shell biofilm removed, and oyster shells with an intact biofilm. These treatments allowed us to measure rates of N and P cycling processes in the oyster digestive system, the shell biofilm community, and how the N and P cycling processes in these 2 microhabitats interact. We hypothesized that denitrification would occur primarily in the oyster digestive system and nitrification would mainly occur in the shell biofilm. We also hypothesized that rates of denitrification and nitrification would be higher in whole oysters due to the coupling of N cycling processes occurring in each microhabitat. Denitrification was measured as net release of N_2-N and N_2O from the oyster digestive system. Nitrification was inferred as release of N_2O and NO_3^- and consumption of NH_4^+ by the shell biofilm. Interaction between the 2 microhabitats would be demonstrated by higher rates of N_2-N efflux and lower rates of NH_4^+ release from whole oysters relative to the digestive system alone.

2. MATERIALS AND METHODS

2.1. Oyster collection and characterization

Market-size eastern oysters *Crassostrea virginica* were purchased from East Beach Farm in Ninigret Pond, Rhode Island, USA, on 3 occasions in the summer of 2015 (23 July, 4 August, and 12 August). Ninigret Pond is a shallow coastal lagoon which is separated from Block Island Sound by a barrier spit. Water exchanges with Block Island Sound through a

small breachway that is occasionally dredged, with a residence time of approximately 10 d. Multiple oyster farms operate in the lagoon, and efforts to build restored reefs are underway. A HOBO dissolved oxygen (DO) data logger deployed adjacent to the oyster farm recorded mean temperatures of (mean ± SE) 25.2 ± 0.0°C with a range in temperature of 21.2–30.1°C over the course of the experiment. The site experiences strong diurnal cycles in DO concentration, ranging from 3.9 ± 2.7 mg l^{-1} to 15.0 ± 4.0 mg l^{-1} during the course of our study. Mean salinity was 30.6 ± 0.8 in July and 30.7 ± 0.3 in August.

We brought the oysters to Boston University in a cooler with site water, where they were moved to an environmental chamber set to 24.0°C, i.e. the water temperature at the oyster farm when we collected oysters for the first incubation. We used the same temperature for all 3 incubations in order to eliminate any temperature-associated variation in fluxes. The oysters were kept overnight in aerated site water, and incubations to measure gas fluxes were conducted the following day. Prior to treating the oysters and beginning the incubations, we weighed them and measured the length, width, and depth of their shell. We then labeled each oyster by loosely wrapping it with a rubber band with a waterproof tape tag. Each incubation included 36 oysters. Following each incubation, we shucked the oysters, dried the tissue at 60.0°C, and recorded the dry tissue mass once the weight changed less than 1% over a period of 24 h.

2.2. Oyster treatments

We measured fluxes from whole oysters, the shell biofilm, and whole oysters with the shell biofilm removed. The role of the shell biofilm was isolated by carefully shucking oysters, removing the animal tissue, and then putting the shells back together (Erler et al. 2017). We inferred microbial activity in the digestive system using whole oysters with the shell biofilm removed. Any processes occurring in the mantle cavity are also captured in our shell biofilm treatment.

We removed the shell biofilm using a combined chemical and mechanical approach presented by Tamburri et al. (1992) that is quick, minimally invasive, and removes all of the external biofilm. First, the oysters were vigorously scrubbed with a soft plastic bristle brush for 3 min, then rinsed 3 times, and soaked in artificial sea water (ASW) with a salinity of 31 for 5 min. Next, the oysters were bathed in a 2.5% household bleach solution for 5 min. Following this bath, the oysters were again rinsed 3 times with ASW and then kept in ASW until the start of the incubation (at least 2 h). All oysters were closed during the course of chemical and mechanical biofilm removal, and we visually ensured all oysters were open and pumping following the biofilm removal before beginning the incubation. We also tested the ASW in which the oysters were soaked following the bleach treatment and the chambers they were incubated in for the presence of chlorine using a Hach Model CN-66T chlorine test kit. In all cases, there was no chlorine present in the water.

2.3. Incubation procedure

We filled gas-tight chambers (28 cm long clear PVC tubes, 10 cm diameter, total volume 2.15 l) with unfiltered site water that had been bubbled overnight, and then added 4 whole oysters, scrubbed oysters, pairs of shells, or water only (as control) depending on the treatment. Each treatment was replicated in triplicate, for a total of 12 chambers per incubation (3 replicates of each of the 4 treatments). The cores were capped with gas-tight acrylic lids with an inflow port connected to a 20 l carboy of site water, an outflow port from which samples were collected, and a magnetic stir bar to keep water in the chamber gently mixed (~40 revolutions min^{-1}; Foster & Fulweiler 2016).

Water samples for analysis of DO, dissolved inorganic N (NH_4^+, NO_3^-, NO_2^-), and dissolved inorganic P (PO_4^{3-}) concentrations were collected at the beginning and end of the experiment. DO was measured using an optical sensor (LDO101; Hach). Inorganic nutrient samples were filtered through a Whatman GF/F filter (0.7 micron) into 2 acid-washed polyethylene bottles, which were immediately frozen until analysis.

We collected duplicate water samples for N_2-N and N_2O concentrations at 5 time points during the incubation. At each of the 5 time points, we filled 12 ml glass exetainers (Labco; n = 4) from the bottom with sample water, allowing the exetainer to overflow 3 times (Foster & Fulweiler 2016). We then added 25 µl of saturated zinc chloride solution to preserve the sample prior to capping the exetainer with a gas-tight cap. During sample collection, both the inflow and outflow ports were opened so that water from the carboy would replace the collected sample water. At all times throughout the incubations, there were no bubbles or headspace in any of the chambers (Foster & Fulweiler 2016). Sample time points for dissolved

gas analysis were spaced to allow for at least a 2.0 mg l^{-1} drop in DO in the chamber water between the first and last sample while maintaining DO concentration above the hypoxic threshold of 2.0 mg l^{-1} (Foster & Fulweiler 2016). Incubations ranged from 2–3.5 h.

Accounting for gas sample collection and occasional DO checks, replacement of water within the chambers was less than 15% throughout the course of the incubation. Lights in the environmental chamber were left on during the course of the incubation.

2.4. Sample analysis

Dissolved nutrient samples were analyzed using high-resolution digital colorimetry on a SEAL Autoanalyzer 3 with standard techniques (SEAL methods G171-96 for NH_4^+, G173-96 for NO_2^-, G172-96 for NO_x, and G175-96 for PO_4^{3-}). The detection limits during these analyses were 0.08 µM NH_4^+, 0.013 µM NO_x (NO_2^- + NO_3^-), 0.006 µM NO_2^-, and 0.01 µM PO_4^{3-}. NO_3^- concentrations were calculated as [NO_x] – [NO_2^-]. In cases where [NO_2^-] was greater than [NO_x], we considered [NO_3^-] to equal 0. We used the following equation to estimate fluxes of DO and dissolved nutrients:

$$\text{Flux} = \frac{[\,]_{\text{final}} - [\,]_{\text{initial}} \times \text{Volume}}{\text{Time} \times \text{Number of oysters}} \quad (1)$$

For dissolved nutrients, the initial and final concentrations were the average value of the 2 replicate samples collected at each time point.

We determined N_2-N concentrations using a quadrupole membrane inlet mass spectrometer (MIMS) according to the N_2/Ar method (Kana et al. 1994). This method has a high precision (± 0.03% N_2/Ar), and N_2 concentration is estimated by multiplying the N_2/Ar ratio of the sample determined by MIMS by the theoretical Ar concentration of the sample given its temperature and salinity in order to estimate the sample N_2-N concentration (Weiss 1970, Colt 1984). While there is some potential for oxygen in the water samples to influence N_2 measurements on the MIMS, this interference is small and within the range of precision of the instrument (Kana & Weiss 2004).

Concentrations of dissolved N_2O were measured using a headspace equilibration technique followed by analysis on a gas chromatograph (GC; Foster & Fulweiler 2016). A gas headspace in the sample exetainer was created by simultaneously adding 5 ml of ultra-high purity helium and removing 5 ml of water sample through the septa, using gas-tight glass syringes. Following creation of the headspace, vials were shaken vigorously for 10 s and left upright to equilibrate for at least 1 h. After equilibration, 4 ml of sample head-space were removed and injected into a Shimadzu 2014 GC equipped with an electron capture detector with ^{63}Ni source for analysis of N_2O. The GC columns were packed with HayeSep® and Shimalite®. We used N_2 gas as the carrier gas and P5 (5% CH_4 and 95% Ar) as the electron capture detector make-up gas. We estimated the concentration of N_2O in our samples by comparing the area under the peaks generated for our samples against a standard curve of peak areas of different concentrations of an externally mixed standard made up of 500 ppb N_2O in N_2 (Airgas). All standard curves had $R^2 \geq 0.995$ for the 6 time points. The detection limit for N_2O during sample analysis was 0.386 µM.

Flux rates for N_2-N and N_2O in each incubation chamber were estimated by first calculating the rate of change in concentration of the gas over time using a linear regression and then calculating a gas flux rate per oyster using the slope calculated in the regression analysis. We only considered fluxes to be significant when $R^2 \geq 0.65$ and $p \leq 0.10$ (Prairie 1996, Foster & Fulweiler 2014, 2016). When the fluxes did not meet these criteria, we considered there to be no change in gas concentration over time, and thus the flux was 0. All fluxes measured in this study were linear. Finally, to remove the effect of any gas flux occurring in the water used during the incubations, we subtracted the average flux of the 3 water-only chambers from the individual treatment chambers from that incubation (Table S1 in the Supplement at www.int-res.com/articles/suppl/m621p095_supp.pdf).

2.5. Statistical analyses

We used R statistical software v. 3.3.2 (R Core Team 2014) to perform all statistical analyses and considered the results of all statistical tests to be significant when $p \leq 0.05$. We compared the physical characteristics (whole oyster wet weight, shell length, shell width, and shell height) of oysters used in different incubations, between treatments pooled among incubations using Kruskal-Wallis tests, followed by Dunn's test when the Kruskal-Wallis test was significant.

The flux data generated in this study were of a relatively small sample size (n = 9 for each treatment), were not normally distributed, and there were differences in starting water chemistry, so we used a non-parametric statistical approach. We tested whether fluxes of dissolved gases and nutrients for each treatment (whole oyster, shell biofilm only, digestive sys-

Table 1. Whole oyster weight and shell size dimensions of eastern oysters used in the 3 incubations (mean ± SE) in 2015. Within a column, different letters indicate significant differences ($p \leq 0.05$) in the weight or size metric between incubations using a Kruskal-Wallis test and Dunn post hoc analysis

Incubation date	Wet weight (g)	Length (cm)	Width (cm)	Height (cm)
24 July	44.90 ± 1.47^{a}	7.66 ± 0.14^{a}	4.81 ± 0.10^{a}	1.90 ± 0.04^{a}
5 August	47.10 ± 1.41^{a}	7.68 ± 0.10^{a}	5.03 ± 0.15^{b}	1.62 ± 0.04^{b}
13 August	50.92 ± 1.41^{b}	7.21 ± 0.13^{b}	5.16 ± 0.10^{b}	2.06 ± 0.06^{c}

tem only) were significantly greater than 0 (or less than 0 for O_2 fluxes) by pooling fluxes from the 3 incubations and using a 1-sample Wilcoxon signed rank test. We compared fluxes between the 3 treatments using a Kruskal-Wallis test followed by Dunn's test if the Kruskal-Wallis test was significant.

3. RESULTS

3.1. Oyster sizes and incubation conditions

The mean ± SE whole oyster wet weight in the 3 incubations was 47.67 ± 0.85 g. The oysters had a shell length of 7.51 ± 0.07 cm, shell width of 5.00 ± 0.07 cm, and shell height of 1.86 ± 0.03 cm. Oysters purchased for the third incubation were heavier than those used for the first 2 incubations, as well as slightly shorter in length, wider, and with a deeper cup (Table 1). Despite differences in the sizes of oysters purchased on different dates, oyster wet weight ($p = 0.65$), shell length ($p = 0.56$), shell width ($p = 0.28$), and shell height ($p = 0.84$) were not different between treatments when the 3 incubations were pooled. The average dry tissue mass of oysters used in this study was 2.93 ± 0.05 g. Starting dissolved inorganic N and P concentrations were similar between incubations, although starting concentrations of NH_4^+ and PO_4^{3-} were slightly higher during the second of the 3 incubations (Table S2).

3.2. Comparison of fluxes from the shell biofilm and digestive system

The oyster digestive system and shell biofilm both consumed significant quantities of O_2 (Table 2). O_2 consumption in whole oysters (12.36 ± 1.48 µmol ind.$^{-1}$ h^{-1}) was nearly twice the sum of consumption in the 2 microhabitats (Fig. 1).

Fluxes of dissolved nutrients were generally higher from the shell biofilm than the oyster digestive system, significantly so for NH_4^+, NO_2^-, and PO_4^{3-} (Fig. 2). Compared to the digestive system, the shell

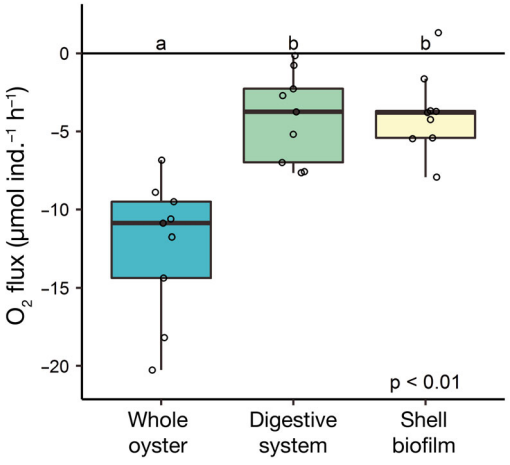

Fig. 1. Fluxes of O_2 from whole eastern oysters, the oyster digestive system, and the shell biofilm. The solid line in the middle of each box is the median, the lower and upper edges of the boxes indicate the 25th and 75th percentiles, and the end of each whisker indicates the smallest and largest value within 1.5 times the interquartile range. Each point represents an individual flux measurement. p-values show the result of Kruskal-Wallis tests comparing treatments, with different letters indicating groups that are significantly different following pairwise comparison. Points below the 0 line show net consumption, while points above 0 indicate net production

Table 2. Fluxes from whole eastern oysters, their digestive system, and their shell biofilm. All values indicate mean (± SE). p-values in parentheses indicate whether the flux was significantly different from 0 following a 1-sample Wilcoxon test. Significant values ($p \leq 0.05$) are in **bold**. All fluxes are in µmol ind.$^{-1}$ h^{-1}

Treatment	O_2 flux	NH_4^+ flux	NO_3^- flux	NO_2^- flux	PO_4^{3-} flux	N_2-N flux	N_2O flux
Whole oyster	−12.36 ± 1.48 **(0.002)**	1.42 ± 0.27 **(0.002)**	0.80 ± 1.46 (0.752)	0.05 ± 0.02 **(0.002)**	0.08 ± 0.02 **(0.002)**	0.41 ± 0.22 **(0.049)**	0.0004 ± 0.0001 **(0.005)**
Digestive system	−4.11 ± 0.96 **(0.002)**	0.62 ± 0.17 **(0.006)**	0.03 ± 0.19 (0.410)	0.01 ± 0.00 **(0.004)**	0.05 ± 0.02 **(0.006)**	0.59 ± 0.20 **(0.014)**	0.001 ± 0.0004 **(0.007)**
Shell biofilm	−3.84 ± 0.86 **(0.004)**	1.26 ± 0.20 **(0.002)**	2.17 ± 1.55 (0.285)	0.05 ± 0.01 **(0.002)**	0.46 ± 0.12 **(0.002)**	0.00 ± 0.17 (0.500)	0.0003 ± 0.0001 **(0.010)**

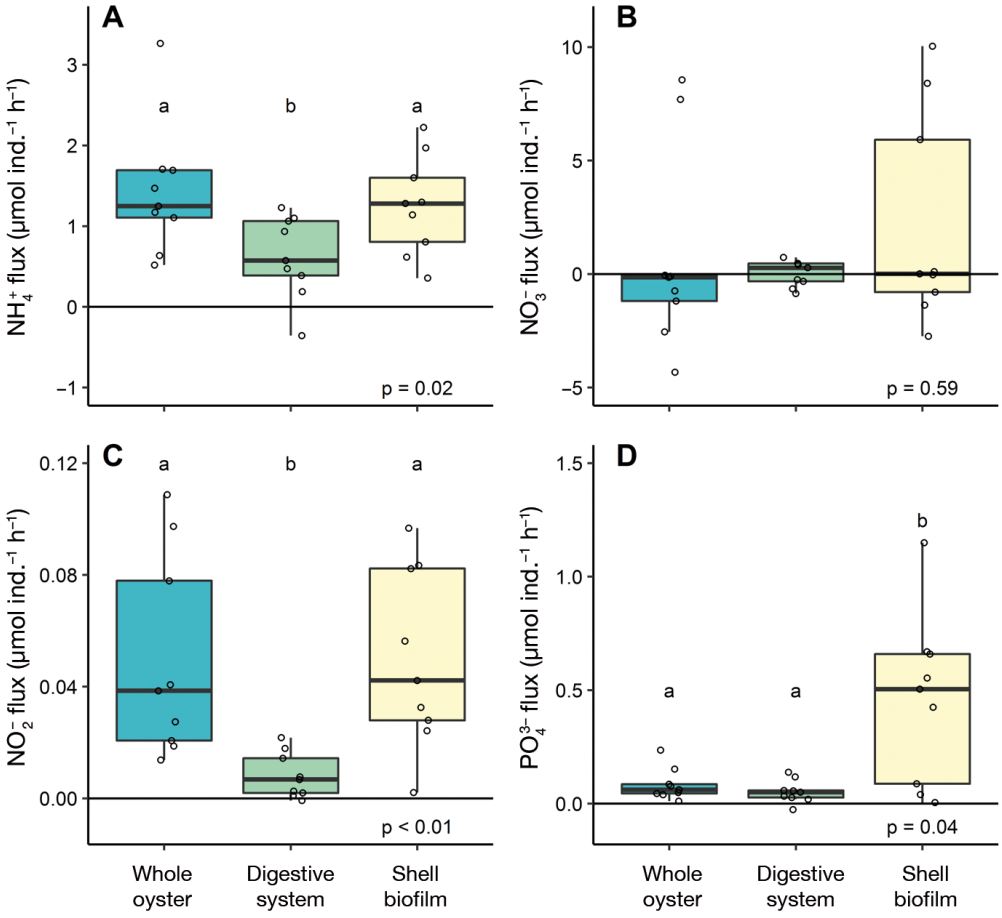

Fig. 2. Fluxes of (A), NH_4^+, (B) NO_3^-, (C) NO_2^-, and (D) PO_4^{3-} from whole oysters, the oyster digestive system, and the shell biofilm. Other details as in Fig. 1

biofilm released twice as much NH_4^+, 5 times as much NO_2^-, and 9 times as much PO_4^{3-} (Table 2). We did not record significant fluxes of NO_3^- for any treatment.

There was no statistical difference in N_2-N production between the digestive system and the shell biofilm ($p = 0.12$; Fig. 3). However, N_2-N fluxes from the shell biofilm were not significantly different from 0, while the digestive system released N_2-N at a rate of 0.59 ± 0.20 µmol ind.$^{-1}$ h^{-1}, approximately equal to the rate of N_2-N production for whole oysters (0.41 ± 0.22 µmol ind.$^{-1}$ h^{-1}; Table 2). N_2O was released by both the shell biofilm (0.0003 ± 0.0001 µmol ind.$^{-1}$ h^{-1}) and digestive system (0.0010 ± 0.0004 µmol ind.$^{-1}$ h^{-1}), although the rate of release from whole oysters (0.0004 ± 0.0001 µmol ind.$^{-1}$ h^{-1}) was not different from the 2 microhabitats (Fig. 3).

We found no significant differences when fluxes for each treatment were compared between incubation dates.

4. DISCUSSION

4.1. N and P cycling processes in the oyster digestive system and shell biofilm

We recorded net release of N_2-N and N_2O from scrubbed oysters and measured no flux of N_2-N from the shell biofilm, indicating that all denitrification occurred in the oyster digestive system. Despite differences in methods, our measured rate of denitrification in whole oysters is comparable, albeit at the lower end of rates reported in other studies (Table 3). Unlike our study, which recorded no denitrification in the oyster shell biofilm, both Caffrey et al. (2016) and Arfken et al. (2017) recorded significant denitrification in empty oyster shells, although the contribution of the oyster shell to whole oyster denitrification varied from 27.5 to 97.5% between the studies. This variance is likely due to differences in the biofilm community inhabiting the oyster shell in different estuaries, or methodologi-

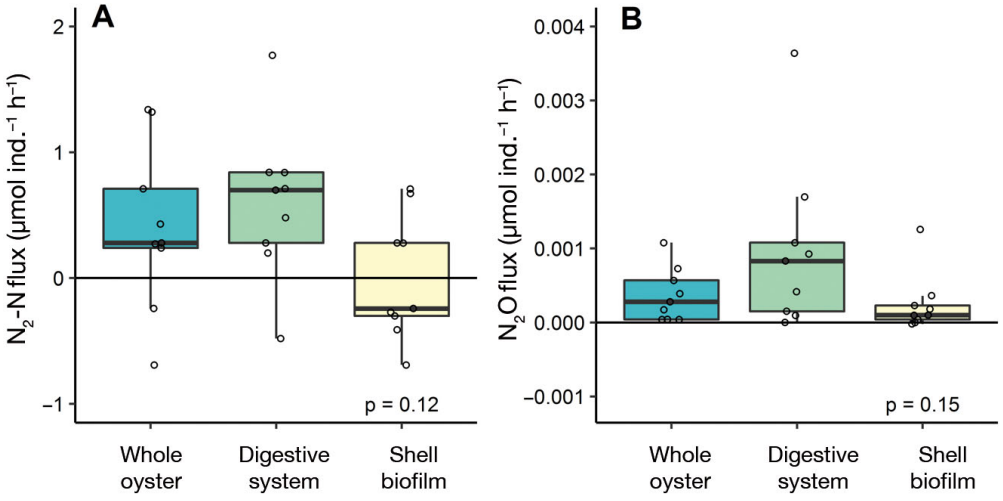

Fig. 3. Fluxes of (A) N_2-N and (B) N_2O from whole oysters, the oyster digestive system, and the shell biofilm. Other details as in Fig. 1

cal differences between studies. Smyth et al. (2013) and Arfken et al. (2017) both used flow-through incubations and the N_2/Ar method to analyze samples, while Caffrey et al. (2016) used both batch and flow-through experiments, but measured denitrification via the isotope pairing technique (IPT). Oysters in flow-through chambers have exposure to 'new' NO_3^- from inflow water, and IPT requires enriching the water with $^{15}NO_3^-$ in order to acquire a strong signal. In our batch incubations, the only NO_3^- added to the incubation chamber came from nitrification or from untreated site water that replaced volume lost in the chamber during sample collection.

We predicted that nitrification in the shell biofilm would be exhibited by release of N_2O and NO_3^-, and consumption of NH_4^+. Despite not recording any significant production of NO_3^- or consumption of NH_4^+ by the biofilm-only treatment, we did find evidence of nitrification. We measured significant release of N_2O and NO_2^-, which are a byproduct and intermediate of nitrification, respectively. Additionally, there was high variability in our NO_3^- flux data (Fig. 2), with some instances of NO_3^- release, and others of NO_3^- consumption. This pattern suggests that nitrification was occurring, though at varying rates, and to varying degrees of completion. We expected nitrification to proceed more rapidly in the shell biofilm of whole oysters compared to the shell alone due to NH_4^+ fertilization from the oyster digestive system, yet rates of N_2O and NO_2^- release

Table 3. Previously reported rates of denitrification from eastern oysters *Crassostrea virginica*, and rates recorded in this study

Study	Oyster treatment	Location (USA)	Incubation temp. (°C)	Reported denitrification rate	Converted denitrification rate (µmol ind.$^{-1}$ h^{-1})
Smyth et al. (2013)[a,b]	Biofilm removed	Calico Creek, NC	24.7	~500 µmol m^{-2} h^{-1}	1.6
Caffrey et al. (2016)[c]	No treatment	Pensacola Bay, FL	23	269 nmol cm^{-2} d^{-1}	0.82
Caffrey et al. (2016)[c]	Shell only	Pensacola Bay, FL	23	74 nmol cm^{-2} d^{-1}	0.23
Arfken et al. (2017)[a]	No treatment	Atlantic Beach, NC	30	364.4 µmol m^{-2} h^{-1}	1.17
Arfken et al. (2017)[a]	Shell only	Atlantic Beach, NC	30	355.3 µmol m^{-2} h^{-1}	1.14
This study	No treatment	Ninigret Pond, RI	24	—	0.41
This study	Biofilm removed	Ninigret Pond, RI	24	—	0.59
This study	Shell only	Ninigret Pond, RI	24	—	0[d]

[a]Smyth et al. (2013) and Arfken et al. (2017) both reported denitrification as a function of incubation chamber cross sectional area (0.0032 m^2). We converted to a rate per oyster by multiplying by this value
[b]The denitrification rate for Smyth et al. (2013) is estimated from Fig. 1 in that paper, as the actual value is not reported
[c]Caffrey et al. (2016) reported rates of denitrification per cm^2 oyster shell area. We converted this to denitrification per individual by multiplying their reported value by the average oyster shell area in that study (73.52 cm^2)
[d]The N_2-N flux for the shell biofilm was not significantly different from 0 ($p = 0.50$) and is therefore reported as 0 here

were not different between whole oysters and the shell biofilm alone, indicating that shell nitrification processes may act in autonomy from digestive processes in *Crassostrea virginica*. Our results match those reported by Caffrey et al. (2016), who also did not find significant differences in inferred rates of nitrification between whole oysters and empty oyster shells, reporting rates from 0–112 nmol NO_3^- cm^{-2} d^{-1}. Their reported rate can be converted to 0–0.34 µmol NO_3^- $ind.^{-1}$ h^{-1}, comparable to, but at the lower end of, some rates of NO_3^- production observed in our study.

Our measured rate of NH_4^+ excretion from whole oysters (12.17 µmol g^{-1} d^{-1}, dry tissue weight) falls in the lower range of previously reported rates (11.7–67.39 µmol g^{-1} d^{-1}; Hammen 1968, 1969, Pietros & Rice 2003, Ray et al. 2015). We were surprised that the magnitude of NH_4^+ release from the shell biofilm alone was greater than from the digestive system alone. This NH_4^+ efflux suggests that the oyster shell supports a substantial heterotrophic community. It is unlikely that any significant portion of NH_4^+ production was due to dissimilatory nitrate reduction to ammonium (DNRA). Nizzoli et al. (2006) found evidence of DNRA occurring in mussel culture, but were unable to estimate a rate due to 'equivocal' data. Additionally, there are likely low carbon inputs to the shell biofilm, favoring denitrification processes over DNRA (Burgin & Hamilton 2007).

We measured no difference in N_2-N production between the oyster digestive system and whole oysters, and greater NH_4^+ from whole oysters compared to just the oyster digestive system. The oyster digestive system and shell biofilm are therefore not stimulating N-cycling processes in each other. Instead, N-cycling processes in each of the 2 oyster microhabitats appear to be uncoupled. On the other hand, whole oysters consumed O_2 twice as quickly as the sum of the shell biofilm and digestive system. We consider there to be 2 possible explanations for this pattern: a feedback mechanism between the 2 microhabitats, or increased respiration as a result of higher rates of filter-feeding due to competition for food.

Both the oyster digestive system and shell biofilm released significant quantities of PO_4^3. The rate we recorded for whole oysters (0.08 µmol $ind.^{-1}$ h^{-1}) and for the oyster digestive system (0.05 µmol $ind.^{-1}$ h^{-1}) were slightly less than the rates reported by Satomi & Pomeroy (1965) of 0.2 µg atoms P g^{-1} h^{-1} (which converts to 0.28–0.38 µmol PO_4^{3-} $ind.^{-1}$ h^{-1} when incorporating the spring and summer oyster weights reported in that paper) for whole oysters with the biofilm removed.

4.2. Ecological implications of N and P fluxes from oysters

The oyster digestive system released both N_2-N and NH_4^+, indicating that despite recycling nutrients to the surrounding system, oysters also remove significant quantities of N from the system. The oysters used in this study had approximately 2.93 g tissue (dry weight) and took about 2 yr to reach that size. If we assume the tissue is 7.86% N (Higgins et al. 2011), we can estimate that 0.016 moles of N per oyster are removed from the coastal ecosystem when the oyster is harvested. This value is approximately 2 times greater than the total amount of N removed through denitrification (0.007 mol N per oyster) by the oyster digestive system over the same 2 yr time period, even if we assume that the denitrification rate we recorded stays the same regardless of oyster size, and through different seasons and temperature. However, only harvested oysters can remove N through bio-extraction. In contrast, it is likely that all oysters remove N through denitrification in their digestive system (Table 3). Even oysters that will never be harvested, such as those in restored reefs, or living in polluted waters, will provide this ecosystem service.

While the rate of denitrification for an individual oyster may seem small, oysters typically live or are raised in dense populations. As an example, the farm from which we collected oysters keeps market size oysters ready for sale in densities of at least 250 ind. m^{-2}. Oysters in such a density would yield a removal of 102.5 µmol N_2-N m^{-2} h^{-1} in addition to potentially stimulating denitrification in underlying sediment (Newell et al. 2005, Kellogg et al. 2014). In fact, if oyster aquaculture is to be used as a method to remove excess N from coastal systems, denitrification occurring in the oyster may be more easily estimated than denitrification in the sediment beneath oyster aquaculture gear. Studies to measure stimulation of sediment denitrification associated with oyster habitats have reported conflicting results, with some studies indicating significant increases in N removal when oysters are present (Hoellein & Zarnoch 2015, Smyth et al. 2015, Humphries et al. 2016, Lunstrum et al. 2018) — including a study reporting an average annual increase as high as 460 µmol N_2-N m^{-2} h^{-1} (Kellogg et al. 2013), while others reported no change (Higgins et al. 2013, Mortazavi et al. 2015, Smyth et al. 2015, Erler et al. 2017). Conversely, each study that has attempted to measure denitrification occurring in or on *C. virginica* has measured the occurrence of denitrification in a similar range (Table 3). Similarly, Erler et al. (2017)

demonstrated high rates of denitrification and anammox associated with the Sydney rock oyster *Saccostrea glomerata*, and no difference in the rate of either pathway between sediment beneath aquaculture and a control site. A possible explanation for the difference in consistency of measurement of denitrification associated with oysters and the sediment they influence has to do with the high variance in conditions that control sediment denitrification between estuarine systems in which oyster aquaculture is practiced, such as NO_3^- availability and organic matter loading, while oysters constantly filter particulates from the water and excrete NH_4^+, providing a steady supply of C and N for microbial denitrifiers.

The N removed from the ecosystem by denitrification in the oyster comes at a cost — the release of N_2O and NH_4^+. We estimate that for whole oysters, N_2O release is approximately 0.1% of the release of N_2-N, similar to the percentage reported for Manila clams *Ruditapes philippinarum* (<1%; Welsh et al. 2015), suggesting that denitrification processes in bivalve digestive systems generally proceed to completion. While N_2O in the digestive system is likely produced through denitrification, N_2O generated in the shell biofilm is likely from nitrification, or fermentation in the digestive system of animal biofilms (Svenningsen et al. 2012). N_2O release by oysters in the previously described scenario (250 ind. m^{-2}) would equal approximately 0.1 µmol N_2O m^{-2} h^{-1}. This low release of N_2O relative to denitrification is not due to anomalously low rates of N_2O emission — in fact, the rate of N_2O release by whole *C. virginica* found in this study falls within the range of previously reported rates of bivalve N_2O release (Table 4) — but is driven by high rates of N_2 production. These findings show that at an ecosystem level, N removal via denitrification in *C. virginica* comes at a low N_2O cost, and are in direct contrast to the previous suggestion that benthic epifauna will release significant quantities of N_2O with little removal of fixed N (Stief 2013).

Whole oyster excretion of NH_4^+ (1.42 µmol ind.$^{-1}$ h^{-1}) proceeds at about 3 times the rate of N_2 removal through denitrification (0.41 µmol N_2-N ind.$^{-1}$ h^{-1}). The NH_4^+ excreted is not new N in the system, as it was previously bound in phytoplankton before passing through the oyster digestive system and being returned to the water column. The shell biofilm drives this pattern, as it releases twice as much NH_4^+ as the digestive system, and does not release N_2-N. To put the amount of N removed through denitrification relative to all N cycled through the oyster in a broader context, we estimated the denitrification efficiency. Using the mean flux for N compounds released at significant rates from untreated oysters, denitrification efficiency was calculated as:

$$\text{Denitrification efficiency (\%)} = \frac{N_2\text{-N}}{\sum N} \times 100 \quad (2)$$

The efficiency of denitrification in the oyster digestive system is 22%, a rate similar to that previously reported for sediment beneath a restored oyster reef (Kellogg et al. 2013). If denitrification occurring in the oyster digestive system is to be used in nutrient management planning, the trade-off of more NH_4^+ in the water column as a result of oyster excretion must also be considered if the management plan involves the reduction of NH_4^+, although it is important to note that this NH_4^+ is simply N being recycled within the system and is not new N.

Table 4. Previously reported rates of nitrous oxide (N_2O) emission from bivalve mollusks. DW: dry weight; WW: wet weight

Study	Species	Reported N_2O emission rate
Stief et al. (2009)	*Mytilus edulis*	12.2 pmol mg DW^{-1} h^{-1}
Stief et al. (2009)	*Dreissena polymorpha*	15.0 pmol mg DW^{-1} h^{-1}
Stief et al. (2009)	*Pisidium* sp.	1.4 pmol mg DW^{-1} h^{-1}
Heisterkamp et al. (2010)	*Mytilus edulis*	0.269 nmol g WW^{-1} h^{-1}
Heisterkamp et al. (2010)	*Scrobicularia plana*	0.302 nmol g WW^{-1} h^{-1}
Heisterkamp et al. (2010)	*Cerastoderma edule*	0.126 nmol g WW^{-1} h^{-1}
Heisterkamp et al. (2010)	*Limecola balthica*[a]	1.098 nmol g WW^{-1} h^{-1}
Heisterkamp et al. (2013)	*Mytilus edulis*	~1.9 nmol ind.$^{-1}$ h^{-1}[b]
Welsh et al. (2015)	*Ruditapes philippinarum*	11.5 µmol g DW^{-1} h^{-1}
Bonaglia et al. (2017)	*Limecola balthica*	~0.5 nmol g^{-1} h^{-1}[c]
Erler et al. (2017)	*Saccostrea glomerata*	782 pmol ind.$^{-1}$ h^{-1}
This study	*Crassostrea virginica*	0.0004 µmol ind.$^{-1}$ h^{-1}
		0.12 nmol g DW^{-1} h^{-1}[d]
		0.47 nmol g WW^{-1} h^{-1}[d]

[a]Heisterkamp et al. (2010) reported N_2O emissions for *Macoma balthica*, which was renamed to *Limeocla balthica* prior to the publication of Bonaglia et al. (2017)
[b]Heisterkamp et al. (2013) did not report a value for N_2O release rate, so the value here was estimated from Fig. 1 in that paper
[c]Bonaglia et al. (2017) did not report a value for N_2O release rate, so the value here was estimated from Fig. 2 in that paper. Additionally, it is unclear if this rate is for g wet or dry tissue, or for g whole organism
[d]These values were estimated using the average oyster dry tissue mass recorded in this study and a wet:dry tissue mass conversion factor of 4:1

In addition to releasing NH_4^+, oysters release significant quantities of PO_4^{3-} at an N:P ratio of 18.4 (using NH_4^+ and NO_2^-). Oysters do not drive one nutrient to become limiting to primary production, as the ratio of N:P excreted by oysters is near the Redfield ratio of 16N:1P. PO_4^{3-} release was greater from the shell biofilm than the oyster digestive system, although PO_4^{3-} release from untreated oysters was not statistically different from either the digestive system or the biofilm. Satomi & Pomeroy (1965) found a strong positive relationship between PO_4^{3-} release and oyster DO consumption, indicating that PO_4^{3-} release is driven by the rate at which oysters are consuming and processing food. As NH_4^+ excretion is also positively correlated with DO consumption in oysters (Boucher & Boucher-Rodoni 1988), this suggests that PO_4^{3-}:NH_4^+ in oyster excretions will remain constant regardless of food availability and respiratory rate.

5. CONCLUSIONS

In this study, we quantified the magnitude of both inorganic N and P fluxes from *Crassostrea virginica* for the first time and demonstrate how 2 distinct oyster microhabitats regulate these fluxes. The results of our study show that biogeochemical processes in the 2 microhabitats are uncoupled, despite their close physical proximity and distinct redox conditions. We show that denitrification in the oyster digestive system can remove significant quantities of N from coastal ecosystems, providing an ecosystem service in estuaries with excess anthropogenic N enrichment. This process proceeds with minimal N_2O release, contrary to past predictions. While small at the individual oyster scale, when considering large populations of oysters, denitrification occurring in the oyster digestive system proceeds at rates similar to sediment and may provide a more accurate method of estimating oyster-driven N removal from estuaries than stimulation of sediment denitrification. Additionally, N remineralized by oysters is released almost exclusively as NH_4^+, at a ratio of 18.4:1 with P, i.e. relatively close to the Redfield ratio. As oyster and bivalve populations are restored, grow via aquaculture development, or as non-native species are introduced, understanding how different species regulate biogeochemical processes is an important consideration.

Acknowledgements. Funding for this research was made possible by a Rhode Island Sea Grant award to R.W.F., a Boston University Dean's Fellowship and Teaching Fellowship to N.E.R., and a Boston University Undergraduate Research Opportunities Project award to M.C.H. We thank Jim Arnoux for access to, and information about, his farm; Seth Berger for analyzing nutrient samples on the SEAL; and Alia Al-Haj for assisting with the lab incubations. Parts of this research made up M.C.H.'s senior thesis at Boston University, and we thank the BU Marine Program and her senior thesis committee members Jennifer Bhatnagar and Nathan Stewart.

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Editorial responsibility: Romuald Lipcius, Gloucester Point, Virginia, USA

Submitted: January 2, 2019; Accepted: May 24, 2019
Proofs received from author(s): June 27, 2019