

# Differential Effects of Bivalves on Sediment Nitrogen Cycling in a Shallow Coastal Bay

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**Abstract** In coastal ecosystems, suspension-feeding bivalves can remove nitrogen through uptake and assimilation or enhanced denitrification. Bivalves may also retain nitrogen through increased mineralization and dissimilatory nitrate reduction to ammonium (DNRA). This study investigated the effects of oyster reefs and clam aquaculture on denitrification, DNRA, and nutrient fluxes ( $\text{NO}_x$ ,  $\text{NH}_4^+$ ,  $\text{O}_2$ ). Core incubations were conducted seasonally on sediments adjacent to restored oyster reefs (*Crassostrea virginica*), clam aquaculture beds (*Mercenaria mercenaria*) which contained live clams, and bare sediments from Smith Island Bay, Virginia, USA. Denitrification was significantly higher at oyster reef sediments and clam aquaculture site than bare sediment in the summer; however, DNRA was not enhanced. The clam aquaculture site had the highest ammonium production due to clam excretion. While oyster reef and bare sediments exhibited seasonal differences in rate processes, there was no effect of season on denitrification, or dissimilatory nitrate reduction to ammonium (DNRA) or ammonium flux at the clam aquaculture site. This suggests that farm management practices or bivalve metabolism and excretion may override seasonal

differences. When water column nitrate concentration was elevated, denitrification increased in clam aquaculture site and oyster reef sediments but not in bare sediment; DNRA was only stimulated at the clam aquaculture site. This, along with a significant and positive relationship between denitrification and sediment organic matter, suggests that labile carbon limited nitrate reduction at the bare sediment site. Bivalve systems can serve as either net sinks or sources of nitrogen to coastal ecosystems, depending mainly on the type of bivalve, location, and management practices.

**Keywords** Denitrification · DNRA · *Mercenaria mercenaria* · *Crassostrea virginica* · Nitrogen · Eutrophication

## Introduction

Eutrophication, frequently caused by excess nitrogen (N) inputs, affects coastal systems worldwide (Diaz and Rosenberg 2008). Excess N can fuel primary production leading to algal blooms, dead zones, and habitat loss (Paerl 1997; Hauxwell et al. 2001). N can be removed from ecosystems by sediment denitrification, a stepwise reduction pathway of nitrate to dinitrogen gas ( $\text{N}_2$ ). Modification to the timing and rate of carbon delivery, concentration of nitrate, the terminal electron acceptor, and accumulation of sulfide may promote dissimilatory nitrate reduction to ammonium (DNRA) instead of denitrification, resulting in N retention rather than removal (Burgin and Hamilton 2007; Hardison et al. 2015). DNRA and denitrification compete for nitrate produced via nitrification, or the oxidation of ammonium to nitrate/nitrite, or by supplying directly from the water column. The potential for coastal systems to remove N and combat the negative

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consequences of eutrophication relies in part on the competition between these two nitrate reducing processes.

There is growing interest in using shellfish to mitigate the effects of eutrophication and manage N pollution (Bricker et al. 2014; Kellogg et al. 2014; Rose et al. 2014). Suspension feeding bivalves such as oysters and clams provide top-down control of phytoplankton biomass and enhance sediment N cycling through benthic-pelagic coupling (Dame et al. 1984; Newell 2004; Smyth et al. 2013a; Murphy et al. 2016a). These organisms can also serve as habitat for nitrifying and denitrifying bacteria (Welsh and Castadelli 2004; Stief 2013; Welsh et al. 2015). As bivalves feed, particulate N contained in phytoplankton and other organic matter is removed from the water column. A portion of this N is assimilated, of which a fraction is excreted as dissolved N directly to the water column; the remainder is egested as biodeposits and transferred to the sediments. N in the biodeposits can be buried or utilized by the microbial community (e.g., remineralized, assimilated, nitrified) and may enhance denitrification (Newell et al. 2002; Kellogg et al. 2013; Smyth et al. 2013a). Additionally, the delivery of organic carbon to the sediments in biodeposits may stimulate DNRA, which is favored over denitrification in high carbon and low nitrate conditions (Tiedje 1988; Burgin and Hamilton 2007; Hardison et al. 2015).

To date, evidence of the effectiveness for bivalves to control N availability is equivocal (Table 1). The amount of N that is recycled rather than removed seems to depend on species, environmental characteristics, and grow-out practices. For example, commercial hard clam (*Mercenaria mercenaria*) aquaculture in a shallow polyhaline tributary of Chesapeake Bay contributed to reduced conditions in the sediments and accumulation of sulfide, which resulted in N regeneration via DNRA rather than removal via denitrification (Murphy et al. 2016a). However, in the highly eutrophied Po River Delta (Italy) sediments from clam (*Tapes philippinarum*), aquaculture sites had higher rates of denitrification than DNRA (Nizzoli et al. 2006). Oyster (*Crassostrea virginica*) reefs tended to increase denitrification relative to bare sites (Piehler and Smyth 2011; Smyth et al. 2013b; Kellogg et al. 2013; Humphries et al. 2016) but had a minimal influence on sediment N cycling in eutrophic systems (Hoellein and Zarnoch 2014). The majority of studies on oyster reefs have focused on denitrification, and estimates of DNRA are limited (Table 1). Based on the few studies which are available, denitrification is favored over DNRA in oyster reef sediments and sediments affected by oysters, either adjacent to oyster reefs or floating aquaculture cages, tended to have higher rates of DNRA than bare sediment (Smyth et al. 2013b; Erler et al. 2017).

Within the same ecosystem, the effects of bivalves on sediment biogeochemistry will likely differ based on the type of bivalve, growing conditions, and physical substrates that can be colonized by denitrifying and nitrifying bacteria. For example, in Chesapeake Bay, the largest estuary in the US, clams (*Mercenaria mercenaria*) and oysters (*Crassostrea virginica*) are the predominate bivalve species. Clams may enhance

nitrification by supplying ammonium and increasing O<sub>2</sub> penetration depth through bioturbation, leading to more coupled nitrification-denitrification (Pelegri and Blackburn 1994; Welsh 2003; Nizzoli et al. 2006). Additionally, the shell, soft tissue, and digestive system can be colonized by nitrifying and denitrifying bacteria and these areas exhibited high rates of these processes (Welsh and Castadelli 2004; Stief 2013; Welsh et al. 2015). However, in an aquaculture context, some of the natural function of clams may be impacted by the method of cultivation. In the Chesapeake Bay region, the high density of clams and use of predator exclusion nets, which restrict clam movement, modify water flow, and serve as habitat for macroalgae, may affect exchanges at the sediment-water interface (Secrist 2013). The predator exclusion net is a plastic netting placed flush to the sediment surface (over the clams) and held in place with reinforcing bars and sandbags. Macroalgae grow rapidly using the NH<sub>4</sub><sup>+</sup> generated from the clams which requires periodic removal (Murphy et al. 2015). The predator exclusion net and the high density may contribute to greater organic enrichment of sediment (Newell 2004), resulting in reduced and sulfidic conditions that promote DNRA (Murphy et al. 2016a). While natural clam beds still exist in the USA, clam aquaculture is becoming a more common feature in the coastal landscape (Murphy et al. 2016b; Emery 2015).

Oyster reefs have dramatically declined in areas in coastal ecosystems due to factors such as disease and over-fishing (Beck et al. 2011). Oysters are sessile, epifaunal suspension-feeding bivalves that form biogenic reefs. The three-dimensional reef structure on top of the sediment helps concentrate organic matter on the sediment surface. The reef structure increases biogeochemical cycling compared to unstructured sediment (Smyth et al. 2016) and provides habitat and refugia for diverse infaunal and epifaunal communities, which include many bioturbating organisms (Kellogg et al. 2013). While the reef structure alone can increase biogeochemical cycling, the oysters can have a direct impact on nitrogen cycling. Filtration and associated biodeposition deliver organic C and N to the sediments, leading to elevated rates of nitrogen cycling compared to bare sediment (Newell et al. 2002; Kellogg et al. 2013; Smyth et al. 2013a). At the same time, the oysters add ammonium directly through excretion and consume oxygen through respiration (Kellogg et al. 2013; Smyth et al. 2013a). Additionally, the shell and gut of oysters can serve as habitat for nitrifying and denitrifying bacteria (Caffrey et al. 2016). Clam aquaculture and oyster reefs have been shown to affect both DNRA and denitrification relative to bare sediments in a variety of coastal systems (Table 1); however, few studies have measured both processes simultaneously. Given the different ecological features between clam aquaculture and oyster reefs, it is expected that the effect of these organisms on benthic metabolism and nitrogen cycling would be different.

As aquaculture continues to expand, there is competition for available space with oyster reef restoration efforts, since the range of conditions tolerated by clams and oysters is similar. In order to

**Table 1** Reported rates of denitrification and DNRA measured from the eastern oyster (*Crassostrea virginica*) or hard clam (*Mercentaria mercenaria*) ecosystems using either the N<sub>2</sub>:Ar method or isotope pairing technique (IPT)

Bivalve species	Context	Denitrification (μmol m <sup>-2</sup> h <sup>-1</sup> )	DNRA (μmol m <sup>-2</sup> h <sup>-1</sup> )	Denitrification increased above bare sediment site?	DNRA increased above bare sediment site?	Location	Methods	Reference
<i>Mercentaria mercenaria</i> (hard clam)	Aquaculture	0.77–2.9	2.7–14.2	Only in the fall	Yes, all seasons	Shallow polyhaline sediments (Cherrystone Inlet, VA)	Whole core batch incubation; isotope pairing technique	Murphy et al. 2016a
<i>Mercentaria mercenaria</i> (hard clam)	Aquaculture	4.9–6.5	0.32–2.36	Yes	No	Shallow coastal bay (Smith Island Bay, VA)	Whole core continuous-flow incubation; isotope pairing technique	This study
<i>Crassostrea virginica</i> (eastern oyster)	Aquaculture	0–65	–	No	–	Shallow mesohaline sediments below floats (St. Jerome Creek and Spencer Creek, VA)	Whole core continuous-flow incubation; N <sub>2</sub> :Ar technique	Higgins et al. 2013
<i>Crassostrea virginica</i> (eastern oyster)	Aquaculture	0–1097	–	Yes	–	Back barrier lagoon (Ninigret Pond, RI)	Whole chamber batch incubations; N <sub>2</sub> :Ar technique	Humphries et al. 2016
<i>Crassostrea virginica</i> (eastern oyster)	Aquaculture	–4.7–12.1	–	No	–	Shallow subtropical estuary below floats (Mobile Bay, AL)	Whole core continuous-flow incubation; N <sub>2</sub> :Ar	Mortazavi et al. 2015
<i>Crassostrea virginica</i> (eastern oyster)	Aquaculture	< 1–19.2	< 1 to 40.3	Yes	Yes	Shallow polyhaline sediments (Cherrystone Inlet, VA)	Whole core batch incubation; isotope pairing technique	Lunstrum et al. 2017
<i>Crassostrea virginica</i> (eastern oyster)	Restored reef	250–1590	–	Yes	–	Mesohaline subtidal reef (Choptank River, MD)	Whole chamber batch incubations; N <sub>2</sub> :Ar technique	Kellogg et al. 2013
<i>Crassostrea virginica</i> (eastern oyster)	Restored reef	0–332	0.8–104	Yes	Yes, in the summer	Intertidal sediments adjacent to reef (Bogue Sound, NC)	Whole core continuous-flow incubation; N <sub>2</sub> :Ar; then <sup>15</sup> N <sub>2</sub> O <sub>3</sub> for potential DNRA measurements	Smyth et al. 2013b
<i>Crassostrea virginica</i> (eastern oyster)	Restored reef	0–1803	–	Yes	–	Back barrier lagoon (Ninigret Pond, RI)	Whole chamber batch incubations; N <sub>2</sub> :Ar technique	Humphries et al. 2016
<i>Crassostrea virginica</i> (eastern oyster)	Restored reef	1.1–16.0	0.01–0.55	Yes	No	Shallow coastal bay (Smith Island Bay, VA)	Whole core continuous-flow incubation; isotope pairing technique	This study
<i>Crassostrea virginica</i> (eastern oyster)	Natural reefs	10–30	–	Yes, enriched location only	–	Intertidal sediments adjacent to reef in an enriched and reference streams (Great Bay Estuary, NH)	Whole core continuous-flow incubation with <sup>15</sup> N <sub>2</sub> O <sub>3</sub> addition	Hoellein et al. 2015

aid in the evaluation of ecosystem services provided by clam aquaculture and oyster reef restoration, we assessed sediment N dynamics at a restored oyster reef (*C. virginica*) and clam aquaculture site (*M. mercenaria*). Specifically, we investigated the relative importance of DNRA and denitrification in sediments adjacent to a restored oyster reef and from a clam aquaculture bed as well as a reference bare subtidal flat (bare site) sediment, seasonally in Smith Island Bay, Virginia, USA. We hypothesized that sediments from oyster reefs and clam aquaculture would enhance microbial N cycling activity compared to the bare site due to increased C and N delivery to the sediments through bivalve biodeposition. We expected restored oyster reef sediments to have higher rates of denitrification than sediments from clam aquaculture sites, but that clam aquaculture sites would have greater DNRA than oyster reef sediments because of the high clam density and the use of predator exclusion nets that enhance organic matter accumulation. Additionally, we expected seasonal differences in rate processes associated with temperature and food availability for the bivalves.

## Materials and Methods

### Study Site and Field Sample Collection

To determine how clam aquaculture and oyster reefs affect sediment N cycling, sediment samples were collected from a clam aquaculture site which contained littleneck clams (3.5 to 5.2 cm long) and from sediment adjacent to a restored oyster reef in Smith Island Bay, VA (37° 08' 57.08" N, 75° 53' 06.81" W). Smith Island is one of the southern barrier islands of the Delmarva Peninsula and part of the Virginia Coastal Reserve Long-Term Ecological Research site (VCR LTER; Fig. 1). Smith Island Bay has an average water depth of 0.4 m, semi-diurnal tides that range about 1.2 m, and a residence time from 4 to 12 days (Safak et al. 2015). Sampling sites included (1) a restored oyster reef located on an intertidal flat, (2) a clam aquaculture bed, which is part of a commercial aquaculture lease with predator exclusion nets, and (3) a bare subtidal flat (bare site) located approximately 50 m from the reef and aquaculture operation. Smith Island Bay was selected for this study because both clam aquaculture and restored oyster reefs are found within close proximity to each other. Samples were taken for sediment biogeochemical flux experiments and sediment physico-chemical properties (sediment organic matter, benthic microalgal biomass, and pore-water nutrients) seasonally in April, July, and November 2014.

### Continuous Flow Incubations

Continuous flow core incubations were used to examine rates of N exchanges at the sediment-water interface. Triplicate sediment cores (9.5 cm inner diameter × 10 cm sediment

depth) were collected by hand from each of the three sampling sites. Sediment samples from the oyster reef were collected adjacent to the reef and did not contain live oysters. For samples from the clam aquaculture site, the predator exclusion net was removed prior to sample collection. Associated infauna were left undisturbed for all samples; therefore, if present, live clams were not removed from the sediment cores collected at the clam aquaculture site. Water chemistry was assessed at the site with a YSI 6600 data sonde (YSI, Inc., Yellow Springs, OH, USA). Approximately 170 L of water was collected from Smith Island Bay for use in the continuous flow core incubations and for dissolved nutrient analyses.

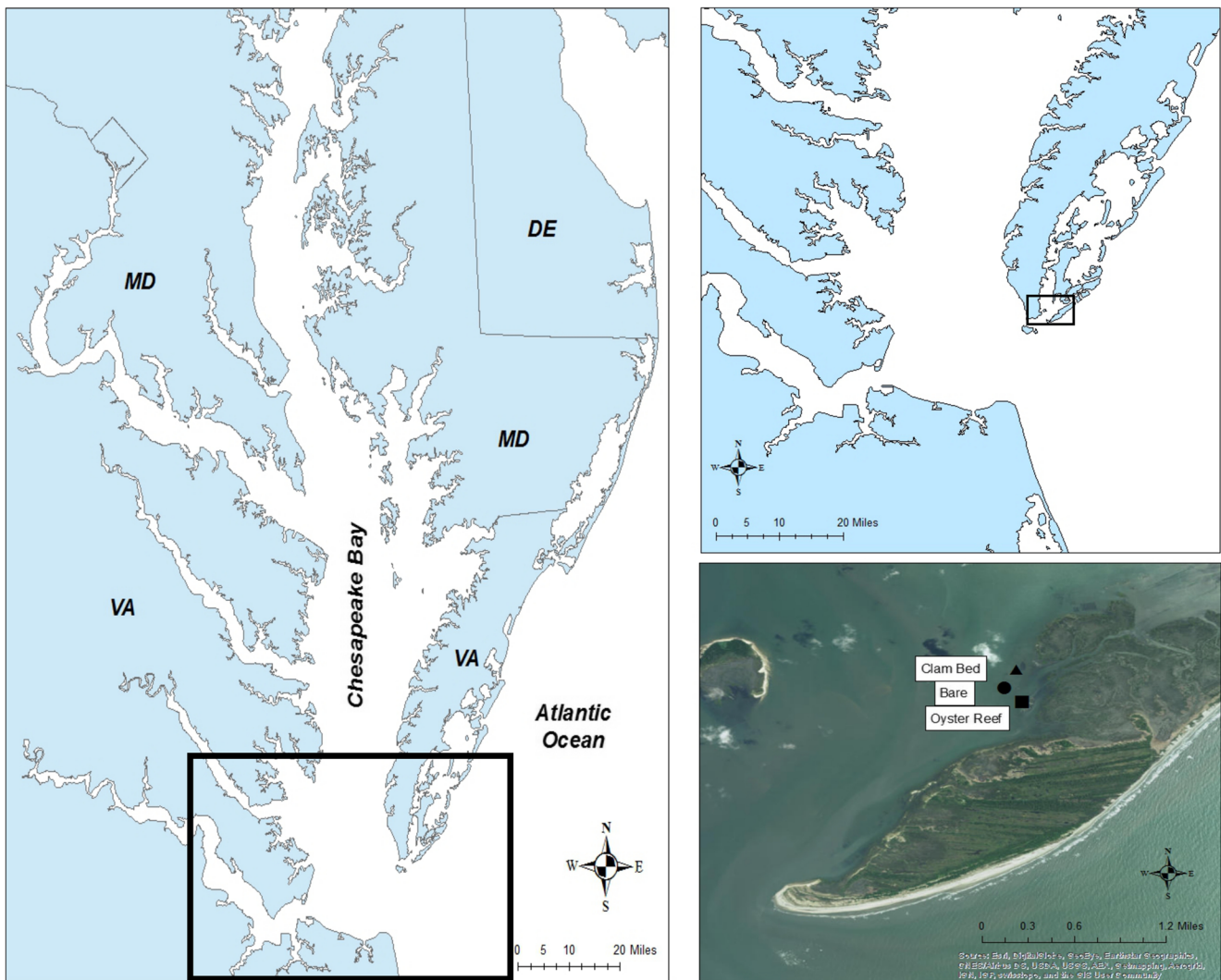
Upon collection, sediment cores and water were transported to an environmental chamber set to in situ temperature at the Virginia Institute of Marine Science (VIMS) in Gloucester Point, Virginia, USA. At VIMS, cores were submerged in aerated site water and held in the dark for 12–16 h. The following day each core was sealed with a gas tight lid equipped with inflow and outflow ports and incubated in a continuous flow system (Gardner and McCarthy 2009). Unfiltered, aerated site water was passed over the cores at a flow rate of 3 ml per minute. Dark conditions were maintained throughout the course of the incubation to reduce the effects of photosynthetic algae (An and Joye 2001) and to prevent the formation of bubbles that affect dissolved gas concentrations (Reeburgh 1969).

Cores were acclimated for 24 h before sampling to allow the system to reach steady state. Samples for dissolved nutrients (combined nitrate/nitrite ( $\text{NO}_x^-$ ) and ammonium ( $\text{NH}_4^+$ )) and gasses ( $\text{O}_2$  and Ar) were collected three times over the course of 24 h after the initial pre-incubation period. A bypass line that flowed from the replacement water tank directly into the sample vial and not through a core tube was used to determine the concentration of dissolved constituents entering the cores. After the initial 24-h sampling period, replacement water was enriched with  $^{15}\text{N}$ - $\text{NaNO}_3$  (98 atm%) to a final concentration of  $\sim 100 \mu\text{mol L}^{-1}$  for isotopic pairing experiments (Nielsen 1992; Risgaard-Petersen et al. 1995; Yin et al. 2014). After a 24-h equilibration period, samples were collected three times over the following 24 h to measure DNRA and denitrification. At the end of the 4-day experiment, average outflow oxygen concentration ranged from  $4.8 \pm 0.15 \text{ mg/L O}_2$  (69% of saturation) in the summer to  $7.8 \pm 0.9 \text{ mg/L O}_2$  (91% of saturation) in the fall.

### Sediment Physico-Chemical Properties

Upon completion of the flux incubations, clams were removed and counted. The upper 2 cm of sediment was homogenized and analyzed for sediment organic matter (SOM), determined by loss on ignition ( $n = 3$ ). Sediments were dried for 24 h at 60 °C then combusted at 525 °C for 4 h. The difference between the weights of dried and combusted samples constituted SOM, expressed as a percentage of the total sediment mass.





**Fig. 1** Image of Smith Island Bay, Chesapeake Bay, USA. Locations of clam beds (triangle), oyster reefs (square), and bare sediment (circle) are identified

Pore-water ( $n = 3$ ) was collected in the field from the clam aquaculture site, bare sediment, and oyster reef sediment using a stainless steel push-point sampler (2 cm screen; MHE Products, East Tawas, MI, USA), inserted 5–7 cm into the sediment. Pore-water nutrient samples were immediately filtered (0.45  $\mu\text{m}$  Whatman polyethersulfone) and frozen until analysis for dissolved inorganic nitrogen (DIN). Pore-water sulfide samples were immediately fixed with zinc acetate and analyzed on a spectrophotometer within a week of collection (Cline 1969). Sediment samples for benthic chlorophyll  $a$ , a proxy for algal biomass ( $n = 3$ ), were collected at the clam aquaculture site, bare sediment, and oyster reef adjacent sediment from the upper 0.3 cm of sediment using a cutoff syringe (1.1 cm ID) and stored frozen until analysis. Samples for sediment chlorophyll  $a$  were extracted with 10 ml of 90% ethanol, sonicated for 30 s, and extracted at  $-15\text{ }^{\circ}\text{C}$  for 24 h. Benthic algal biomass was determined using spectrophotometry (Lorenzen 1967), modified to include the extraction

of the sediment in 10 ml of solvent (Pinckney et al. 1994). Samples for pore-water and benthic chlorophyll  $a$  were collected prior to removing the predator exclusion net at the clam aquaculture site.

### Analytical Methods and Calculations

Samples for nutrient analysis were immediately filtered through a 0.45- $\mu\text{m}$  Whatman polyethersulfone (PES) filter and frozen until analysis. Filtrate was analyzed with a Lachat QuickChem 8000 (Lachat Instruments, Milwaukee, WI, USA) automated ion analyzer for  $\text{NO}_x$  (combined  $\text{NO}_3^-$  and  $\text{NO}_2^-$ ) and  $\text{NH}_4^+$ . Detection limits for  $\text{NO}_x$  and  $\text{NH}_4^+$  were 0.20 and 0.36  $\mu\text{M}$ , respectively.

Samples for dissolved gasses were collected by filling 12-ml Exetainer vials from the bottom up. Vials were allowed to overflow by several volumes before being preserved with 100  $\mu\text{l}$  of saturated  $\text{ZnCl}_2$ . Exetainers were capped and stored

underwater below collection temperature until analysis for dissolved gasses ( $O_2$ ,  $^{29}N_2$ ,  $^{30}N_2$ , Ar) on a membrane inlet mass spectrometer (MIMS) (Kana et al. 1994; An et al. 2001). An inline furnace was added to the MIMS for  $^{29}N_2$  and  $^{30}N_2$  samples to increase precision and remove  $O_2$  which can lead to overestimation of denitrification (Lunstrum and Aoki 2016). Samples for DNRA ( $^{15}NH_4^+$ ) were filtered through a 0.45- $\mu m$  Whatman polyethersulfone (PES) filter and frozen until analysis using the OX/MIMS method (Yin et al. 2014). The OX/MIMS method uses hypobromite iodine solution to oxidize  $^{15}NH_4^+$  to  $^{29}N_2$  or  $^{30}N_2$ , and concentrations of both isotopic species were determined on the MIMS with an inline furnace (Yin et al. 2014). The production of  $^{29}N_2$  and  $^{30}N_2$  associated with the hypobromite oxidation of  $^{15}NH_4^+$  was calculated by the difference between paired oxidized and unoxidized samples, including those from the bypass line.

Fluxes were calculated as

$$J = ([i_{outflow}] - [i_{inflow}]) \times \frac{F}{A}$$

where  $[i_{outflow}]$  and  $[i_{inflow}]$  are the concentrations ( $\mu M$ ) of dissolved constituents leaving and entering the core, respectively;  $F$  is the peristaltic pump flow rate ( $3 \text{ ml min}^{-1}$ ); and  $A$  is the surface area of the core ( $m^2$ ). A positive flux indicates release from the sediment to the water column while a negative flux represents uptake from the water column by the sediment. Negative  $O_2$  fluxes are expressed as sediment oxygen demand (SOD). Denitrification of ambient  $^{14}NO_3^-$  ( $D_{14}$ ) and added  $^{15}NO_3^-$  ( $D_{15}$ ) was calculated using the isotope pairing technique equations (Nielsen 1992):

$$D_{15} = p29 + 2p30$$

$$D_{14} = D_{15} \times (p29/2p30)$$

where  $p29$  and  $p30$  are production rates of  $^{29}N_2$  and  $^{30}N_2$ , respectively. In an ecosystem such as Smith Island Bay, where ambient water column  $NO_x^-$  is low (less than  $1 \mu M$ ) relative to the experimentally added  $^{15}N$ - $NaNO_3$  ( $\sim 100 \mu M$ ),  $D_{15}$  is considered the potential denitrification rate or the capacity of the sediments to denitrify when  $NO_x^-$  is provided in excess. Rates of denitrification utilizing nitrate in the water ( $D_w$ ) were calculated based on the concentration ( $\mu M$ ) of  $^{15}NO_3^-$  relative to  $^{14}NO_3^-$  in the inflow water, times  $D_{15}$  using the following equation:

$$D_w = ([^{14}NO_3^-]/[^{15}NO_3^-])^* D_{15}$$

Denitrification supported by nitrate produced through nitrification in the sediments ( $D_n$ ) was calculated by the difference between  $D_{14}$  and  $D_w$  (Nielsen 1992). We did not account for incomplete denitrification resulting in  $N_2O$  production or  $N_2$  production from anammox. Potential rates of DNRA ( $DNRA_{15}$ ) were determined as  $^{15}NH_4^+$  production (An and Gardner 2002). The concentration of  $^{15}NH_4^+$  was determined

from the total  $^{15}N_2$  produced after hypobromite oxidation as described by Yin et al. (2014). Ambient DNRA ( $DNRA_{14}$ ) was calculated based on the assumption that the relative rates of DNRA utilizing  $^{15}NO_3^-$  and  $^{14}NO_3^-$  occur at the same ratio as denitrification (Risgaard-Petersen et al. 1995):

$$DNRA_{14} = DNRA_{15}^* (D_{14}/D_{15})$$

DNRA, based on  $NO_3^-$  from the water column ( $DNRA_w$ ), was calculated using the ratio of  $^{14}NO_3^-$  to  $^{15}NO_3^-$  concentration. Rates of DNRA coupled to sediment nitrification ( $DNRA_n$ ) were estimated from  $DNRA_w$  and the ratio between  $D_n$  and  $D_w$  (Risgaard-Petersen et al. 1995).

Nitrification rates were calculated as the sum of ambient denitrification ( $D_{14}$ ), ambient DNRA ( $DNRA_{14}$ ), and  $NO_x^-$  effluxes:

$$Nitrification = \text{Positive } NO_x \text{ flux} + D_{14} + DNRA_{14}$$

DNRA measurements are considered conservative because we did not extract  $NH_4^+$  from the sediment and only calculated for ammonium fluxing to the water column (i.e., DNRA is in the sediment not the overlying water) (Bruesewitz et al. 2013). Since nitrification rates are calculated from DNRA, these are also conservative.

## Statistical Analysis

Statistical analyses were performed in R 2.13.1 (R Development Core Team 2015). Mixed effects models were used to examine the interactive effects of site (oyster reef sediment, clam aquaculture sediment, bare sediment) and season for nutrient fluxes,  $D_{14}$ ,  $D_{15}$ ,  $DNRA_{14}$ ,  $DNRA_{15}$ , and SOM. The mixed effects model (*lme* function from the 'nlme' package) consisted of a random effect of number of bivalves in sediment cores, to account for the fact that a random number of clams were included in samples from the clam aquaculture site and no oysters were included in oyster reef samples, and fixed effect of site and season. Benthic chlorophyll *a*, pore-water DIN, and  $H_2S$ , which were collected from the field, were also analyzed with a linear model but did not include number of clams (*gls* function from the 'nlme' package). Tukey HSD post hoc analysis was used to compare means when an effect was significant. A mixed effects model was also used to compare ambient and potential rates (i.e.,  $D_{15}$  to  $D_{14}$  as well as  $DNRA_{15}$  to  $DNRA_{14}$ ) measured from the same core in each season, followed by Tukey HSD post hoc analysis. Linear regressions were used to assess the relationship between sediment oxygen demand (SOD) or sediment organic matter (SOM) and nitrate reduction rates, calculated nitrification, and nutrient fluxes across all sites and seasons. Pearson correlation coefficients were also calculated. Assumptions of normality and homogeneity were tested using Shapiro-Wilkes and Levene's tests, respectively. Logarithmic or Box-Cox transformations were performed if necessary to meet assumptions of analyses. All analyses were considered significant at the  $p < 0.05$  level.

## Results

### Site Characteristics

Water temperatures, salinity, and dissolved nutrient concentrations varied between seasons at Smith Island Bay (Table 2). Over the course of the study, water temperature ranged from 14 to 25 °C, with the highest temperature in summer and lowest in fall. Salinity was relatively consistent, varying by 3 units over the course of the study.  $\text{NH}_4^+$  concentration ranged from 0.45 to 3.06  $\mu\text{M}$ , with the highest concentration in the spring.  $\text{NO}_x$  concentration was less than  $\text{NH}_4^+$  for all seasons and only detectable in the fall when the concentration was 0.72  $\mu\text{M}$ . Dissolved oxygen (DO), measured around mid-day, was always above 90% sat. and ranged from 6.36 mg/l (91.5% sat.) in the summer to 8.05 mg/l (98.1% sat.) in the spring for Smith Island Bay (Table 2).

Pore-water  $\text{NO}_x$  was similar across seasons (Table 3;  $p = 0.424$ ) and between sites (Table 3;  $p = 0.419$ ). There was no seasonal effect on pore-water  $\text{NH}_4^+$  (Table 3;  $p = 0.098$ ), but there were site differences (Table 3;  $p = 0.001$ ). Pore-water  $\text{NH}_4^+$  concentration was higher at the clam aquaculture site compared to bare sediment (Tukey HSD;  $p = 0.001$ ) and oyster reef sediment (Tukey HSD;  $p = 0.002$ ). Pore-water  $\text{H}_2\text{S}$  varied seasonally (Table 3;  $p = 0.012$ ), with the highest concentration in the summer, but there were no differences between sites ( $p = 0.092$ ). SOM varied between the sites (Table 3;  $p = 0.005$ ) and was significantly higher at the oyster reef and clam aquaculture site compared to the bare sediment (Tukey HSD;  $p = 0.001$ ,  $p = 0.001$ , respectively). The interaction between site and season affected benthic chlorophyll *a* ( $p \leq 0.001$ ). Sediment chlorophyll *a* was higher in the fall compared to the spring and summer for each site (Table 3). The sediment cores from the clam aquaculture site contained different numbers of clams, ranging from 0 to 4. There were no clams present in cores collected from the clam aquaculture site in the summer, four clams in each core were collected during the fall, and in the spring two cores contained three clams and one core contained four clams (Supplemental Table 1).

### Nitrate Reduction Rates

Denitrification ( $D_{14}$ ) rates showed a significant interaction between site and season ( $p < 0.001$ ). Oyster reef sediments

had higher  $D_{14}$  in the summer compared to spring and fall, clam bed sediments showed no difference among seasons, and the bare sediment exhibited a sequential decline from spring, to summer, to fall (Fig. 2). There were also differences between the sites within each season. During the summer,  $D_{14}$  was highest in sediments from oyster reef sediment and lowest at the bare sediment with clam aquaculture site having an intermediate rate. In the fall,  $D_{14}$  at the clam aquaculture site was higher than the bare sediment and oyster reef sediment, but in the spring there were no differences between the sites.

In contrast to  $D_{14}$ ,  $\text{DNRA}_{14}$  showed a significant difference between sites ( $p = 0.045$ ) and season ( $p = 0.026$ ; Fig. 2), with no significant interaction ( $p = 0.830$ ).  $\text{DNRA}_{14}$  was similar in the spring and fall (Tukey HSD,  $p = 0.47$ ) but higher in the fall compared to the summer (Tukey HSD,  $p = 0.021$ ). Overall,  $\text{DNRA}_{14}$  was not significantly enhanced at the clam aquaculture site or oyster reef sediment compared to the bare sediment (Tukey HSD,  $p = 0.219$ , 0.601, respectively), but  $\text{DNRA}_{14}$  at the clam aquaculture site was significantly higher than the oyster reef sediment (Tukey HSD,  $p = 0.038$ ). For all sites,  $D_{14}$  was at least three times greater than  $\text{DNRA}_{14}$  (Fig. 2a). The majority of  $D_{14}$  and  $\text{DNRA}_{14}$  was coupled to nitrification (Fig. 2);  $D_n$  ranged from 94.7% of total nitrate reduction at the oyster reef sediment in the fall to 99.8% of total nitrate reduction from the bare sediment in the spring.  $\text{DNRA}$  followed a similar pattern, with nitrification accounting for more than 95% of the nitrate used for  $\text{DNRA}$ .

Potential nitrate reduction rates ( $D_{15}$  plus  $\text{DNRA}_{15}$ ) (i.e., when nitrate was experimentally added) exhibited similar patterns to ambient nitrate reduction ( $D_{14}$  plus  $\text{DNRA}_{14}$ ) rates. Potential denitrification ( $D_{15}$ ) showed a significant interaction between site and season ( $p < 0.001$ , Fig. 3). For the oyster reef sediment,  $D_{15}$  was significantly higher in the summer than in the spring or fall. This was also true for the bare sediment. The clam aquaculture site,  $D_{15}$ , was highest in the summer but not significantly different compared to the other seasons. During the summer, the oyster reef sediment had the highest  $D_{15}$  ( $75.23 \pm 18.51 \mu\text{mol N m}^{-2} \text{h}^{-1}$ ). This was also the overall highest  $D_{15}$ . In the other seasons, the clam aquaculture site had the highest  $D_{15}$ .  $D_{15}$  was higher than  $\text{DNRA}_{15}$  for all sites and seasons (Fig. 3).  $\text{DNRA}_{15}$  was affected by both site ( $p = 0.042$ ) and season ( $p = 0.034$ ).  $\text{DNRA}_{15}$  was highest in the fall and lowest in the

**Table 2** In situ water properties at each sampling date. Mean and standard error ( $n = 3$ ) are presented for water column nutrients. *BD* below detection

Season	Date	Temp. (°C)	Salinity	Dissolved oxygen (mg/l) [ $\text{O}_2\%$ ]	$\text{NO}_x$ ( $\mu\text{M}$ )	$\text{NH}_4^+$ ( $\mu\text{M}$ )
Spring	24 Apr. 14	15.6	33	8.05 [98.1%]	BD	$3.06 \pm 0.04$
Summer	23 Jun. 14	25	30.5	6.36 [91.5%]	BD	$0.46 \pm 0.01$
Fall	5 Nov. 15	14	31.7	8.28 [96.8%]	$0.72 \pm 0.01$	$1.76 \pm 0.04$

**Table 3** Seasonal sediment characteristics for each site and sampling date for all parameters. H<sub>2</sub>S in the clam bed in spring, bare sediment in summer, and oyster reef in fall only had one sample above the detection limit. Data are mean ± standard error (SE) for all parameters. NA for SE indicates that only one sample was above the detection limit

Season	Site	SOM (%)	Benthic Chl (μg/cm <sup>2</sup> )	H <sub>2</sub> S (μM)	Pore-water NO <sub>x</sub> (μM)	Pore-water NH <sub>4</sub> <sup>+</sup> (μM)
Spring	Bare	0.69 ± 0.05	0.41 ± 0.03	25.74 ± 5.55	0.62 ± 0.18	70.73 ± 12.67
	Clam bed	0.82 ± 0.04	0.80 ± 0.08	2.20 ± NA	0.39 ± 0.07	187.89 ± 25.82
	Oyster reef	0.88 ± 0.13	0.74 ± 0.07	33.97 ± 11.04	0.36 ± 0.02	24.34 ± 10.12
Summer	Bare	0.63 ± 0.11	2.11 ± 0.19	10.89 ± NA	0.56 ± 0.04	51.40 ± 11.29
	Clam bed	0.88 ± 0.06	1.47 ± 0.09	265.81 ± 99.27	0.92 ± 0.22	184.76 ± 28.30
	Oyster reef	1.07 ± 0.08	1.30 ± 0.52	228.20 ± 115.04	0.65 ± 0.06	121.59 ± 67.31
Fall	Bare	0.58 ± 0.01	5.54 ± 0.45	11.11 ± 3.59	0.49 ± 0.09	52.34 ± 14.48
	Clam bed	1.09 ± 0.09	7.37 ± 0.43	53.28 ± 17.68	0.96 ± 0.60	102.98 ± 12.98
	Oyster reef	0.97 ± 0.02	3.69 ± 0.19	4.72 ± NA	0.50 ± 0.03	42.63 ± 13.96
Mean	Bare	0.63 ± 0.04	2.69 ± 0.77	15.95 ± 3.90	0.56 ± 0.06	58.16 ± 7.17
	Clam bed	0.93 ± 0.05	3.22 ± 1.05	137.07 ± 59.76	0.76 ± 0.21	158.5 ± 18.15
	Oyster reef	0.97 ± 0.05	1.91 ± 0.48	93.84 ± 56.34	0.52 ± 0.05	67.67 ± 26.13

summer, with spring having an intermediate rate. Mean DNRA<sub>15</sub> was higher (3.8 times) at the clam aquaculture site compared to the oyster reef sediment and 3.7 times higher than bare sediment, but the increase was only significant for the clam aquaculture site compared to the oyster reef sediment (Tukey HSD,  $p = 0.04$ ). DNRA<sub>15</sub> was not different at the clam aquaculture site (Tukey HSD,  $p = 0.191$ ) or oyster reef sediment (Tukey HSD,  $p = 0.640$ ) compared to the bare sediment. The highest D<sub>15</sub> did not correspond to the highest DNRA<sub>15</sub>.

D<sub>15</sub> was higher than D<sub>14</sub> ( $p < 0.001$ ), and DNRA<sub>15</sub> was higher than DNRA<sub>14</sub> ( $p < 0.001$ ). This indicates that nitrate reduction increased with water column nitrate; however, the magnitude of this increase was site dependent. D<sub>15</sub> significantly increased compared to D<sub>14</sub> at the oyster reef sediment (Tukey HSD,  $p < 0.001$ ) and clam aquaculture site (Tukey HSD;  $p = 0.005$ ), while D<sub>14</sub> and D<sub>15</sub> at the bare site were not significantly different from each other (Tukey HSD,  $p = 0.173$ ). This indicates that denitrification was limited by nitrate at the bivalve sites but not at the bare site. The response of DNRA to nitrate addition was less than denitrification, with the largest increase observed at the clam aquaculture site. The clam aquaculture site was the only site where DNRA<sub>15</sub> was significantly higher than DNRA<sub>14</sub> (Tukey HSD,  $p < 0.001$ ).

### Nutrient Fluxes

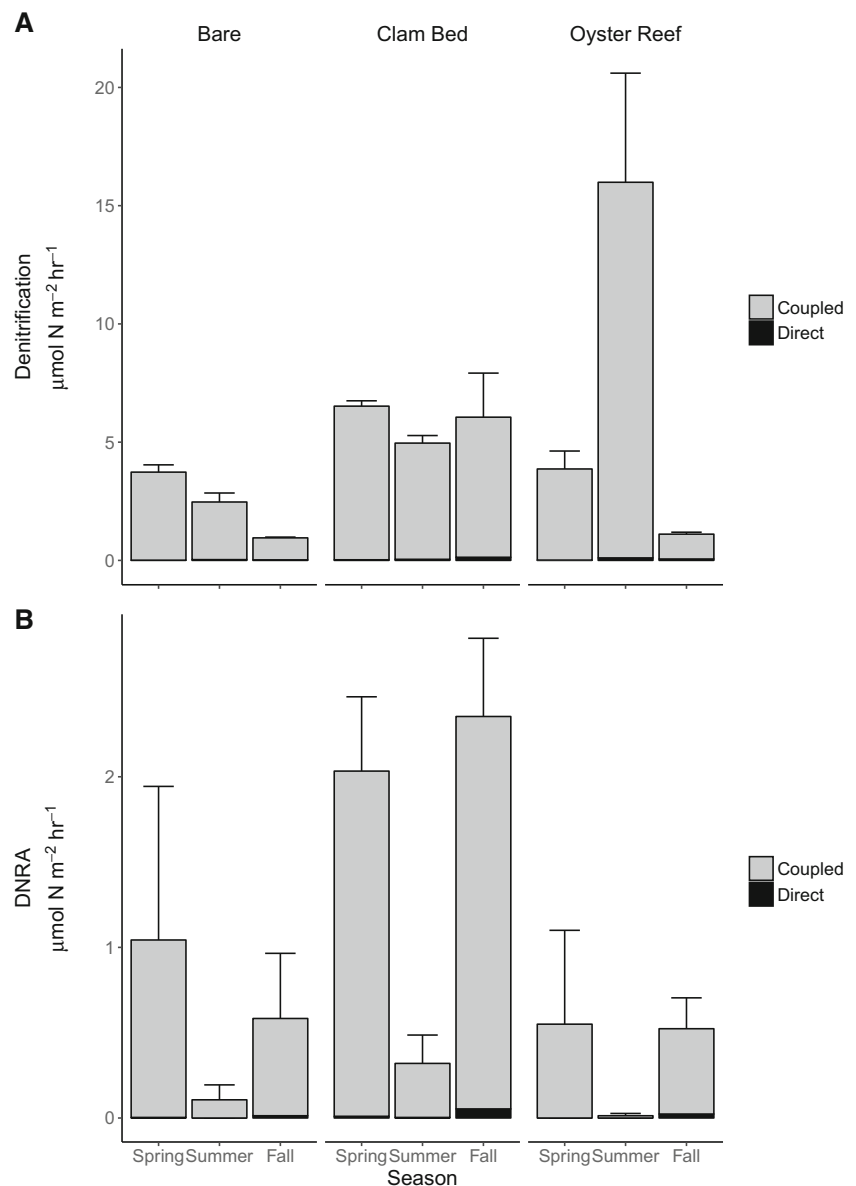
The largest efflux of both NH<sub>4</sub><sup>+</sup> and NO<sub>x</sub> was observed from the clam aquaculture site, in the spring and in the summer, respectively (Table 4). NO<sub>x</sub> fluxes were affected by site ( $p = 0.006$ ) and season ( $p = 0.001$ ), and the interaction was not significant ( $p = 0.050$ ). All sites had a positive NO<sub>x</sub> flux during the summer, resulting in summer fluxes being different from those in spring (Tukey HSD;  $p =$

0.009) and fall (Tukey HSD;  $p = 0.009$ ). NO<sub>x</sub> fluxes were higher at the clam aquaculture site compared to the bare sediment (Tukey HSD;  $p = 0.005$ ), but the NO<sub>x</sub> flux from the oyster reef sediment was not significantly different compared to the bare sediment (Tukey HSD;  $p = 0.072$ ) or the clam aquaculture site (Tukey HSD;  $p = 0.42$ ).

The interaction between site and season was significant for NH<sub>4</sub><sup>+</sup> fluxes ( $p < 0.001$ ). The clam aquaculture site was the only site to have an efflux of NH<sub>4</sub><sup>+</sup> during each season, and rates were not significantly different between seasons (Tukey HSD;  $p = 0.081$  (fall-spring),  $p = 0.795$  (fall-summer),  $p = 0.247$  (spring-summer)). Oyster reef sediments had the highest NH<sub>4</sub><sup>+</sup> flux in the summer compared to those in spring (Tukey HSD;  $p < 0.001$ ) and fall (Tukey HSD;  $p < 0.001$ ). At the bare site, NH<sub>4</sub><sup>+</sup> fluxes were positive in the summer and spring and significantly different from the negative fluxes observed in the fall (Tukey HSD;  $p < 0.001$  and  $p < 0.001$ , respectively). During the summer, NH<sub>4</sub><sup>+</sup> fluxes were similar between all the sites, but during the spring and fall, NH<sub>4</sub><sup>+</sup> flux from the clam aquaculture site was significantly higher than either the oyster reef sediment (Tukey HSD;  $p < 0.001$  (spring),  $p = 0.001$  (fall)) or bare sediment (Tukey HSD;  $p = 0.007$  (spring),  $p < 0.001$  (fall)). Calculated nitrification rates ranged from  $1.54 ± 0.42 μmol N m^{-2} h^{-1}$  at the bare site during the fall to  $18.25 ± 3.68 μmol N m^{-2} h^{-1}$  in the oyster reef during the summer (Table 4). The interaction between site and season was significant for nitrification rates ( $p = 0.03$ ). Nitrification rates were similar across all seasons for the clam aquaculture site and bare sediment but higher in the summer compared to the spring (Tukey HSD;  $p = 0.004$ ) or fall (Tukey HSD;  $p = 0.002$ ) for the oyster reef sediment. Nitrification was similar between the sites in the spring, but the clam aquaculture site was higher than the bare sediment



**Fig. 2** Actual denitrification ( $D_{14}$ ) (a) and DNRA ( $DNRA_{14}$ ) (b) rates for each site and season. Direct denitrification ( $D_w$ ) or DNRA ( $DNRA_w$ ) are in black and denitrification or DNRA coupled to nitrification ( $D_n$  or  $DNRA_n$ ) is presented in gray. Error bars are the standard error of the mean ( $n = 3$ )



(Tukey HSD;  $p = 0.019$ ) and slightly higher than the oyster reef (Tukey HSD;  $p = 0.048$ ) in the fall, while nitrification at the oyster reef was slightly higher than the bare sediment (Tukey HSD;  $p = 0.005$ ) and similar to the clam aquaculture site (Tukey HSD;  $p = 0.130$ ) in the summer.

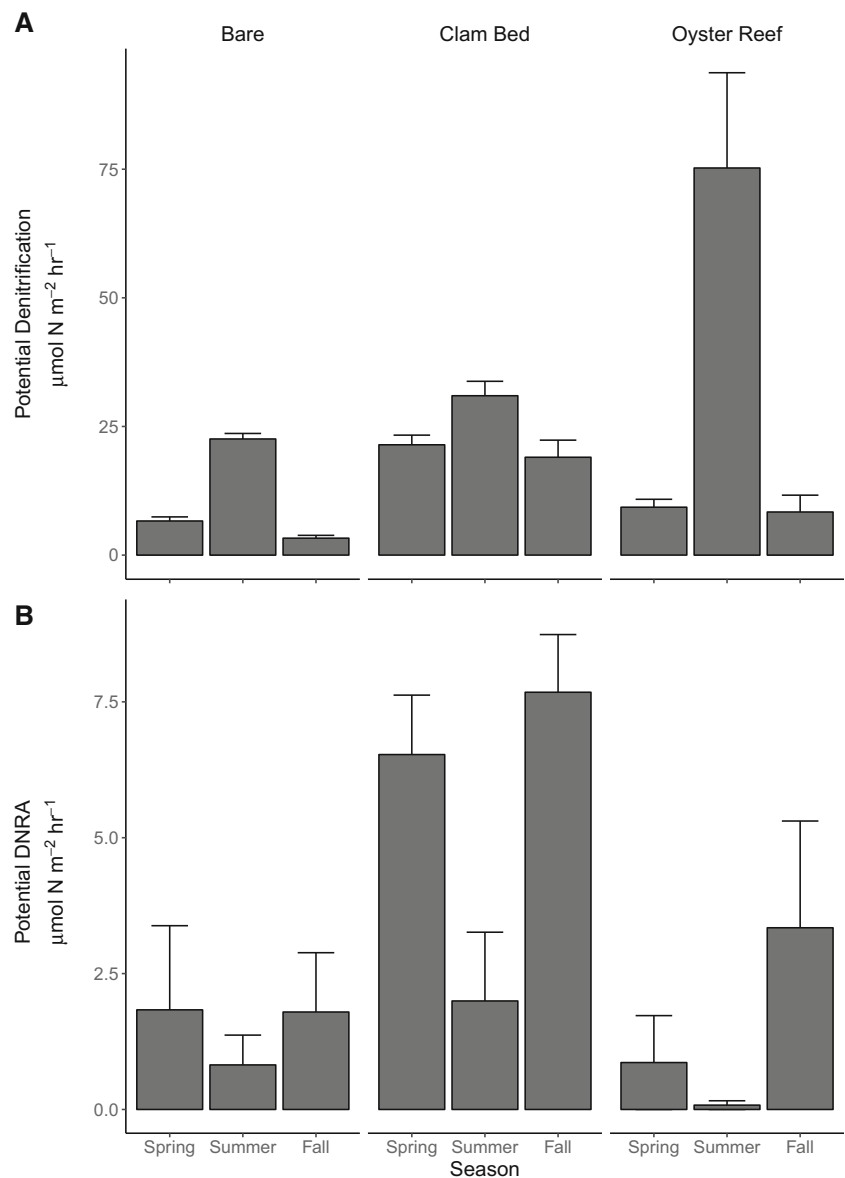
There were both seasonal ( $p < 0.001$ ) and site ( $p = 0.020$ ) differences in SOD, but the interaction was not significant ( $p = 0.105$ ). The lowest SOD ( $324.75 \pm 23.19 \mu\text{mol O}_2 \text{m}^{-2} \text{h}^{-1}$ ) was measured from the bare sediment during the fall. The clam aquaculture site had the highest SOD ( $1883.69 \pm 368.06 \mu\text{mol O}_2 \text{m}^{-2} \text{h}^{-1}$ ), measured during the spring (Table 4). SOD was higher in the oyster reef sediment and clam aquaculture site compared to the bare sediments (Table 4); however, the only significant increase was between the bare sediment and the clam aquaculture site (Tukey HSD;  $p = 0.021$ ). SOD across all of the sites was significantly lower

in the fall compared to those in spring (Tukey HSD;  $p < 0.001$ ) and summer (Tukey HSD;  $p < 0.001$ ).

### Correlations

SOD was significantly and positively related to total ambient nitrate reduction ( $p = 0.017$ ), calculated nitrification ( $p = 0.019$ ), and  $\text{NH}_4^+$  flux ( $p = 0.015$ ). SOD explained 21% of the variance in total ambient nitrate reduction, calculated as the sum of  $DNRA_{14}$  plus  $D_{14}$ , 17% of the variance in calculated nitrification, and 31% of the variance in  $\text{NH}_4^+$  flux (Fig. 4). SOM also explained 10% of the variance in  $DNRA_{14}$  and 20% of the variance in  $D_{14}$  (Fig. 5).  $D_{14}$  was significantly and positively correlated with SOM ( $p = 0.018$ ).  $DNRA_{14}$  was positively correlated with SOM, but this relationship was not significant ( $p = 0.107$ ).

**Fig. 3** Potential denitrification ( $D_{15}$ ) (a) and DNRA ( $DNRA_{15}$ ) (b) rates for each site and season. Error bars are the standard error of the mean ( $n = 3$ )



## Discussion

Clam aquaculture and restored oyster reefs enhanced N transformations via their effect on sediment carbon and nitrification. N cycling pathways were related to SOD (Fig. 4) and SOM controlled denitrification (Fig. 5). These relationships indicate the importance of carbon availability as a predictor of nitrate reduction in the oligotrophic Smith Island Bay ecosystem (Eyre et al. 2013). While carbon availability contributes to the differences between the sites, the high percent of nitrate reduction, coupled to nitrification (both  $DNRA_n$  and  $D_n$  were greater than 95% of  $DNRA_{14}$  and  $D_{14}$ ), indicates the importance of nitrification as a source of nitrate for all sites.

Previous studies have attributed increased rates of N cycling processes in sediments associated with bivalves to high organic matter loading due to biodeposition, high surface area of the

oyster reef substrate compared to bare sediment, bioturbation activity, and gut and shell biofilm communities and, when present, excretion by the animals themselves (Newell et al. 2002; Smyth et al. 2013a; Kellogg et al. 2014; Welsh et al. 2015). Given the higher SOM at the clam aquaculture site and oyster reef sediment compared to the subtidal bare sediment, biodeposition of labile organic carbon likely contributed to the observed enhanced denitrification. In addition, because denitrification rates increased with added  $NO_3^-$  at the bivalve sites but not at the bare sediment, we conclude that  $NO_3^-$  also limited denitrification at bivalve sites. In contrast, denitrification rates were not primarily limited by  $NO_3^-$  at the bare sediment locations, which may be more strongly driven by organic C, anoxic conditions, or microbial community structure.

While oyster reef sediments and clam aquaculture sites increase denitrification compared to the bare sediment, the

**Table 4** Seasonal mean ( $n = 3$ ) fluxes of ammonium ( $\text{NH}_4^+$ ), nitrate + nitrite ( $\text{NO}_x^-$ ), calculated nitrification, and sediment oxygen demand (SOD). Data are mean  $\pm$  SE for all parameters

		$\text{NH}_4^+$ flux ( $\mu\text{mol N m}^{-2} \text{h}^{-1}$ )	$\text{NO}_x^-$ flux ( $\mu\text{mol N m}^{-2} \text{h}^{-1}$ )	Nitrification ( $\mu\text{mol N m}^{-2} \text{h}^{-1}$ )	SOD ( $\mu\text{mol O}_2 \text{m}^{-2} \text{h}^{-1}$ )
Spring	Bare	8.24 $\pm$ 2.52	-0.58 $\pm$ 0.03	4.78 $\pm$ 1.22	537.19 $\pm$ 77.49
	Clam bed	70.75 $\pm$ 13.38	-0.19 $\pm$ 0.29	8.69 $\pm$ 0.20	1883.69 $\pm$ 368.06
	Oyster reef	-17.26 $\pm$ 4.95	0.08 $\pm$ 0.41	4.70 $\pm$ 1.21	1443.94 $\pm$ 356.35
Summer	Bare	46.92 $\pm$ 16.04	2.35 $\pm$ 0.84	4.93 $\pm$ 1.20	1124.74 $\pm$ 15.30
	Clam bed	32.84 $\pm$ 7.15	5.67 $\pm$ 3.26	10.95 $\pm$ 2.93	1258.32 $\pm$ 270.33
	Oyster reef	35.20 $\pm$ 2.15	2.25 $\pm$ 1.15	18.25 $\pm$ 3.68	1624.84 $\pm$ 228.21
Fall	Bare	-16.93 $\pm$ 5.35	-3.42 $\pm$ 0.43	1.54 $\pm$ 0.42	324.75 $\pm$ 23.19
	Clam bed	26.97 $\pm$ 17.33	3.97 $\pm$ 2.90	12.38 $\pm$ 5.41	582.43 $\pm$ 204.68
	Oyster reef	-5.58 $\pm$ 5.65	1.15 $\pm$ 1.14	3.13 $\pm$ 0.77	336.56 $\pm$ 60.77
Mean	Bare	12.74 $\pm$ 10.51	-0.55 $\pm$ 0.88	3.74 $\pm$ 0.75	662.23 $\pm$ 121.96
	Clam bed	43.52 $\pm$ 9.55	3.15 $\pm$ 1.53	10.65 $\pm$ 1.85	1241.48 $\pm$ 236.99
	Oyster reef	4.12 $\pm$ 8.26	1.16 $\pm$ 0.57	8.69 $\pm$ 2.66	1135.11 $\pm$ 236.15

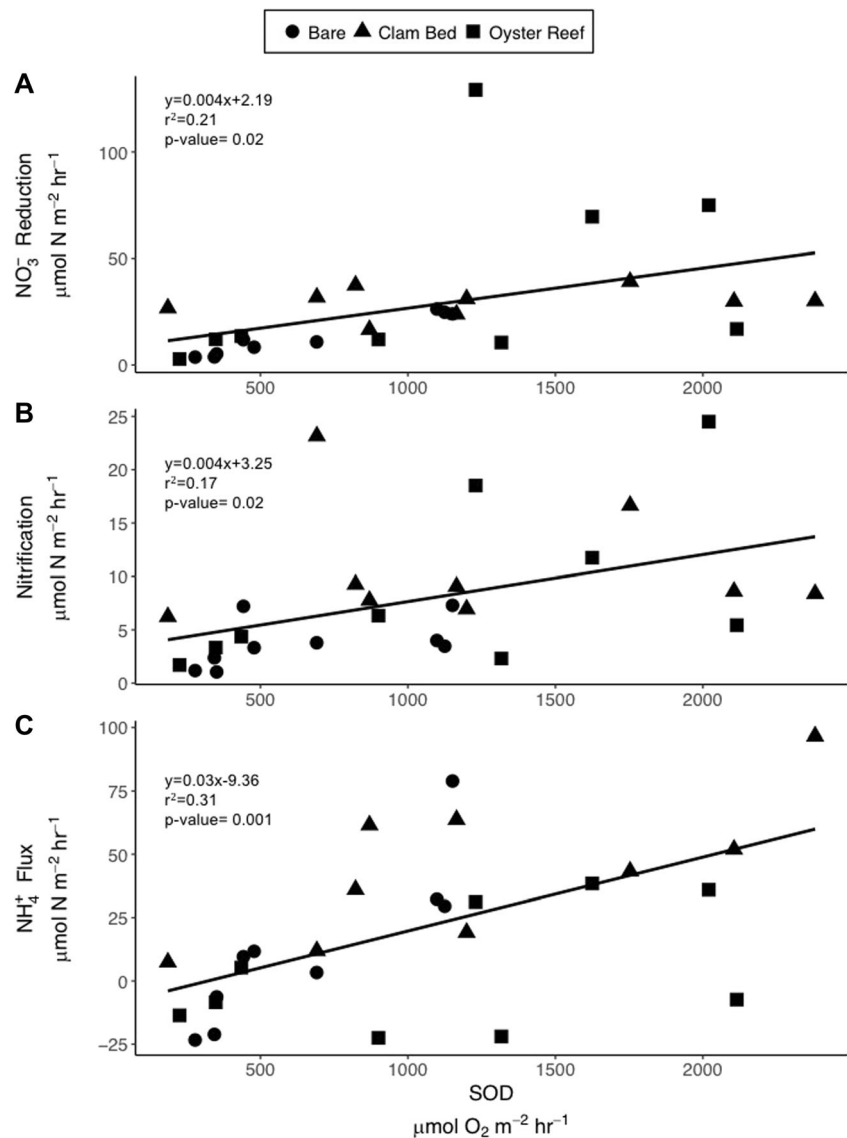
effect varied seasonally and was dependent on bivalve species. The largest bivalve effect on denitrification was observed at the oyster reef sediment in the summer. During the spring and fall, denitrification was enhanced more at the clam aquaculture site than the oyster reef sediment. In contrast, there were no seasonal differences within the clam aquaculture site, suggesting farm management practices may override seasonal effects. The seasonal differences possibly associated with variation in the bivalve response to food availability (i.e., filtration rates). In warmer months when seston is high, oysters tend to increase biodeposit production while clams decrease clearance rates (Langdon and Newell 1989; Hawkins et al. 1998; Newell et al. 2005). However, there were no clams inside the sediment cores in the summer. Because denitrification associated with the clam shells, gut, and gills, which can be very important (Welsh and Castadelli 2004), was unaccounted for during this time, and these measurements may be an underestimate.

When assessing the role of bivalves for enhanced N removal through denitrification, it is important to consider the magnitude of N removal versus N recycling. Overall, denitrification efficiency ( $D_{14}/(D_{14} + \text{NO}_x + \text{NH}_4^+)$ ) was 15, 47, and 66%, in the clam aquaculture site, bare sediment, and oyster reef sediment, respectively. The lower efficiency at the clam aquaculture site is associated with increased ammonium flux. DNRA accounted for less than 10% of the total  $\text{NH}_4^+$  efflux and is unlikely responsible for the enhanced ammonium flux. Rather, the high ammonium efflux at the clam aquaculture site may be due to microbial mineralization as well as direct excretion of  $\text{NH}_4^+$  by the clams. The difference between the oyster reef sediment and clam aquaculture site is due in part to the fact that live oysters were not included in oyster reef samples. Denitrification efficiency at the oyster reef would likely be lower, and nitrogen regeneration would be higher if

live oysters were included with the sediment, as excretion from live oysters is also source of  $\text{NH}_4^+$  (Smyth et al. 2013a; Caffrey et al. 2016).

Bivalves can have a disproportionately large effect on nitrogen cycling relative to the area. For example, oyster reefs only occupy 2.7% of the area in Bogue Sound, North Carolina, yet remove 4% of the estimated annual nitrogen load through enhanced denitrification (Smyth et al. 2013b). Restoration of oyster reefs in Maryland could remove about half of the external nitrogen inputs (Kellogg et al. 2013), and 26% of N inputs into Ninigret Pond, Rhode Island, could be removed if 5% of the estuary was used for oyster aquaculture (Humphries et al. 2016). However, nitrogen regeneration associated with bivalves can also be disproportionately high relative to the aerial coverage of the bivalves. Clam aquaculture occupies 3% of the subtidal bottom in Cherrystone Inlet, Virginia, but nitrogen regeneration is equal to about half of the watershed nitrogen load (Murphy et al. 2016b). Similarly, at a mussel farm in Sacca di Goro, Italy, mussels covered only 5% of the area but excretion accounted for 25% of total DIN regeneration in the system (Nizzoli et al. 2011). Density is one of the main factors that determine whether bivalves are nitrogen sources or sinks at the ecosystem scale. High densities of bivalves tend to decrease denitrification and increase nitrogen regeneration due to excretion and organic matter loading to the sediments (Newell 2004). The exact density of bivalves that increases nitrogen regeneration and decreases denitrification depends on hydrodynamics and sediment quality. To extrapolate rates from experiments to the ecosystem scale assumes that processes scale linearly with density. We did not capture denitrification directly related to the oyster microbiome itself or account for oyster excretion. Therefore, extrapolating rates from our experiment may underestimate the overall impact of bivalves on N cycling. More work is

**Fig. 4** Regression of total ambient  $\text{NO}_3^-$  reduction (a), nitrification (b), and  $\text{NH}_4^+$  flux (c) against SOD. Pearson correlation coefficient, regression equation,  $r^2$ , and  $p$  value for the relationship, which includes all the data for each site and season, and are included on each graph. Samples from the clam bed are identified as triangles, oyster reef sediments are squares, and bare sediment are circles



needed to determine ways to evaluate the effect of bivalves on processes at the ecosystem scale.

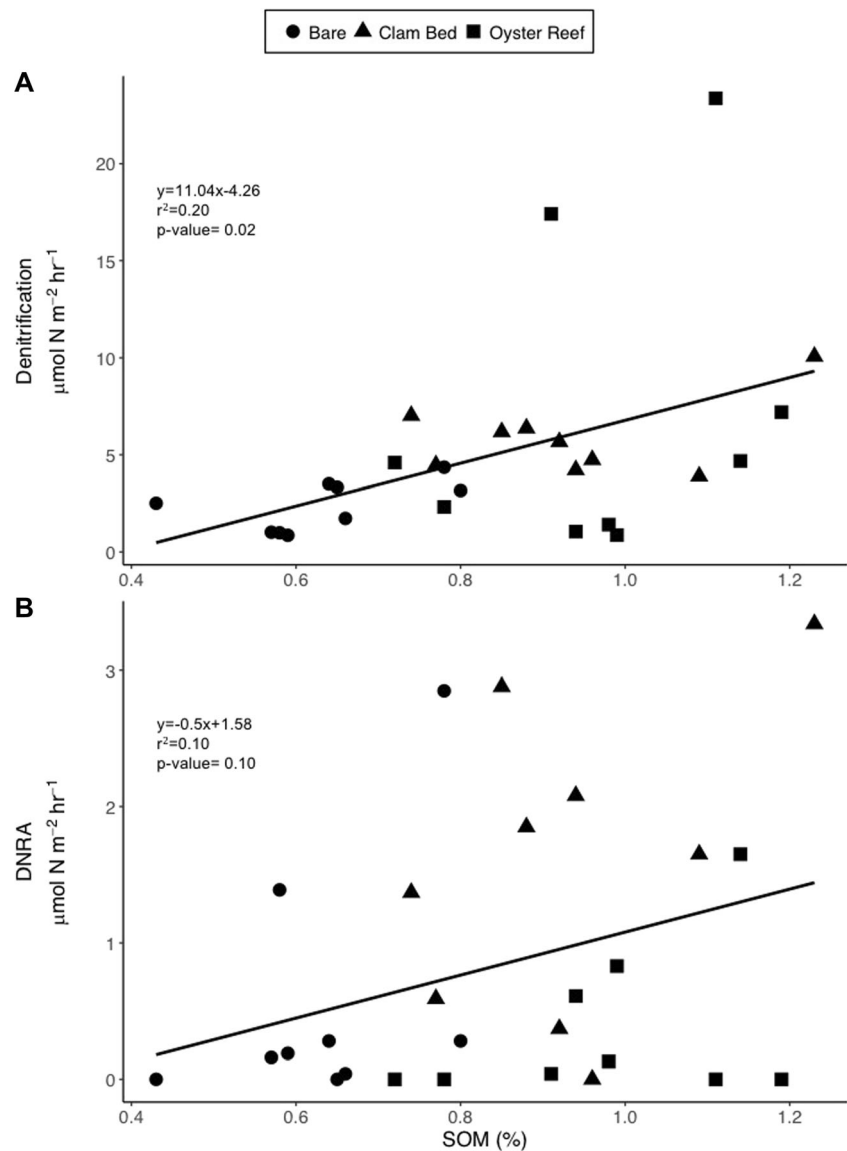
In addition to direct modification of N pools and fluxes, bivalves can affect  $\text{O}_2$  availability. The clam aquaculture site had the highest SOD, which coincided with a larger ammonium efflux. The correlation between SOD and ammonium production for bare sediment and oyster reef sediment reflects demand of oxygen to support nitrification and aerobic decomposition. When live clams are present, SOD also incorporates clam respiration (Murphy et al. 2016a). Thus, clams are major sinks for oxygen and sources of N, in addition to being sites for nitrification and denitrification (Welsh et al. 2015; Benelli et al. 2017).

As observed in other oligotrophic systems with low nutrients, biodeposition enhances denitrification. Biodeposits are a carbon source, and accumulation on the sediment decreases the oxygen penetration depth and diffusive pathway for water column nitrate, leading to increased fluxes (Caffrey et al. 1993;

Smyth et al. 2015). However, there is likely a threshold above which the addition of organic matter from bivalves increases SOD and no longer stimulates denitrification (Newell et al. 2005; Hoellein and Zarnoch 2014; Humphries et al. 2016). This threshold depends on competition for oxygen, which limits nitrification rates and impacts nitrate availability as well as denitrifier abundance and sediment redox conditions. Higher SOD is indicative of increased organic matter oxidation or higher nitrification (Caffrey et al. 1993; Fulweiler et al. 2008); however, a high SOD is also related to sediment anoxia, as the abiotic oxidation of reduced compounds such as sulfide consumes oxygen. Because of the relationship between SOD and nitrate reduction, we conclude that carbon deposition is driving nitrate reduction, particularly at the bivalve sites. The relationship between total nitrate reduction and SOD reflects the complicated relationship between nitrate availability,  $\text{O}_2$ , and carbon for controlling nitrate reduction.



**Fig. 5** Regression of  $D_{14}$  (a) and  $DNRA_{14}$  (b) against sediment organic matter (SOM). Pearson correlation coefficient, regression equation,  $r^2$ , and  $p$  value for the relationship, which includes all the data for each site and season, and are included on each graph. Samples from the clam bed are identified as triangles, oyster reef sediments are squares, and bare sediment are circles



As expected, based on the environmental conditions of Smith Island (i.e., low water column nitrate concentrations, high flow, and an oxygenated water column), the majority (> 95%) of nitrate reduction measured in this study was supported by  $\text{NO}_3^-$  from nitrification ( $D_n$  and  $DNRA_n$ , Fig. 2). The bivalve sites had higher rates of nitrification than the bare sediment, possibly due to organic matter loading and/or ample  $\text{NH}_4^+$  supply. Although nitrification is a chemolithoautotrophic process, which does not rely on organic matter, small amounts of organic matter loading increase nitrification (Caffrey et al. 1993). The mineralization of this organic matter serves as a source of  $\text{NH}_4^+$  to the nitrifying community. However, high SOM, and subsequent increase in SOD, can lead to oxygen limitation for nitrification and the highest rates of nitrification occur at intermediate concentrations of both ammonium and oxygen (Caffrey et al. 1993; Sloth et al. 1995; Blackburn 1996). Alternatively, carbon can affect nitrification if ammonium-oxidizing archaea (AOA), which are

mixotrophs that require a carbon source, rather than ammonium-oxidizing bacteria (AOB), are responsible for nitrification (Qin et al. 2014).

The experimental addition of  $\text{NO}_3^-$  significantly enhanced denitrification rates in both the clam aquaculture site and oyster reef sediments (i.e.,  $D_{15} > D_{14}$ ). The increase in denitrification with increased  $\text{NO}_3^-$  in the water column suggests that denitrification was  $\text{NO}_3^-$ -limited rather than carbon-limited at both bivalve sediments. The increase in denitrification at the bivalve sites supports the hypothesis that bivalve biodeposition supplies organic carbon for  $\text{NO}_3^-$  reduction. At the bare sediment, both DNRA and denitrification were ultimately limited by carbon availability or, potentially, microbial metabolic capacity since the experimental  $\text{NO}_3^-$  addition had no effect on denitrification or DNRA.

Higher water column  $\text{NO}_3^-$  can alleviate the competition between DNRA and denitrification and enhance both processes

(Roberts et al. 2012; Koop-Jakobsen and Giblin 2010). However, in our study, the stimulatory effect of  $\text{NO}_3^-$  addition on DNRA was minimal and only observed at the clam aquaculture site (Fig. 3). The fact that  $\text{NO}_3^-$  stimulated DNRA at the clam aquaculture site may be due to differences in the microbial community structure compared to bare sediment. In a nearby tributary, clam aquaculture sediments had significantly higher abundances of DNRA communities compared with bare sediments based on the quantification of cytochrome C nitrite reductase genes (*nrfA*) (Murphy et al. 2016a). But, despite the increase in DNRA at the clam aquaculture site, the relative importance of DNRA to total  $\text{NO}_3^-$  reduction was unaffected by the added  $\text{NO}_3^-$ . A similar trend has been observed in salt marsh ecosystems (Koop-Jakobsen and Giblin 2010) where the addition of nitrate alleviated competition between DNRA and denitrification for nitrate from nitrification and allowed both processes to increase. Yet, when water column nitrate increased, the ratio of carbon to  $\text{NO}_3^-$  shifted because of increased  $\text{NO}_3^-$ , conditions which favor denitrification resulting in higher denitrification than DNRA (Burgin and Hamilton 2007). The  $\text{NO}_3^-$  addition lowered the ratio of organic carbon to  $\text{NO}_3^-$ , resulting in more energetically favorable conditions for denitrification compared to DNRA (Burgin and Hamilton 2007).

Based on recent studies examining N cycling in clam aquaculture sites and oyster habitats in a tributary of Chesapeake Bay that show that DNRA exceeds denitrification (Murphy et al. 2016a; Lunstrum et al. 2017), it was expected that biodeposition would favor DNRA over denitrification. However, this was not observed in our study; rather, we found that denitrification exceeded DNRA and rates of DNRA were lower than those reported previously. Moreover, unlike other studies, DNRA was not affected by clam aquaculture or oysters although slightly higher DNRA was observed in the clam aquaculture site compared to the oyster reef sediment. The availability of nitrate from nitrification maintains conditions that favor denitrification despite the higher SOM at the clam aquaculture site and restored oyster reefs (Tiedje 1988; Burgin and Hamilton 2007). Differences between our data and previous published studies highlight the fact that the effects of bivalves on sediment biogeochemistry are site specific. More studies are necessary to fully understand how environmental conditions drive the effect of bivalves on N dynamics.

The inclusion of live bivalves in sediment cores can drastically alter benthic nitrogen cycling (Kellogg et al. 2013; Smyth et al. 2013a; Turek and Hoellein 2015; Humphries et al. 2016; Murphy et al. 2016a). While there are advantages to including live bivalves in experiment chambers, especially oysters, which build complex reef ecosystems, it is logistically challenging (Humphries et al. 2016). Our study focused on how oyster reefs and clam aquaculture affect N dynamics at the sediment-water interface. By sampling sediments from the oyster reef and omitting the oyster reef itself, we may underestimate the effects of oyster reef ecosystems. The random

sampling of clam aquaculture sites resulted in an unequal number of clams in each core. The clams were not removed to prevent altering the natural biogeochemical gradients in the sediments, which are critical to maintaining realistic conditions during the incubations. By chance, no clams were contained in the summer sediment cores. To account for this, we analyzed our data using a mixed effects model, which allows for variation due to the number of bivalves present. While the current study used sediments from clam aquaculture sites and restored oyster reefs to investigate the effects of these bivalves on sediment N cycling, studies wishing to compare bivalves would benefit from including a known number of bivalves in experimental chambers.

## Conclusion

Understanding how clam aquaculture and oyster reefs affect sediment N cycling is important when assessing the use of bivalves as management tools for controlling eutrophication. While oyster reefs and clam aquaculture can enhance N removal compared to reference sediments, certain conditions may result in net N regeneration. Clam aquaculture, which utilizes a predator exclusion net in ecosystems with low water column nitrate and short residence time, may be a source of new N from mineralization and excretion (Murphy et al. 2016b). The high efflux of  $\text{NH}_4^+$  at the clam aquaculture site is due in part to the presence of live bivalves in the sediment cores, indicating the direct control that infaunal bivalves and their associated microbiota have on overall benthic flux and N cycle process rates. Conversion of bare sediment to oyster reefs would likely have a similar effect, where oyster reefs enhance sediment denitrification relative to bare sediment and where the shell can be colonized by denitrifying bacteria, but the addition of live oysters to the ecosystem may increase ammonium regeneration through excretion and N regeneration (Kellogg et al. 2013; Smyth et al. 2013a). Factors such as location, hydrodynamics, species, growing conditions, and sediment redox condition likely affect whether the addition of bivalves will remove or enhance N. As N loading and bivalve aquaculture and oyster reef restoration continue to expand in shallow coastal ecosystems, understanding what conditions yield removal of N compared to recycling is important for determining the efficacy of bivalves in controlling N pollution.

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