9-2016

Microbial nitrogen processing in hard clam (Mercenaria mercenaria) aquaculture sediments: the relative importance of denitrification and dissimilatory nitrate reduction to ammonium (DNRA)

AE Murphy
Virginia Institute of Marine Science

Iris C. Anderson
Virginia Institute of Marine Science

AR Smyth
Virginia Institute of Marine Science

BK Song
Virginia Institute of Marine Science

Mark Luckenbach
Virginia Institute of Marine Science

LIMNOLOGY
and
OCEANOGRAPHY

ASLO

Limnol. Oceanogr. 61, 2016, 1589–1604

doi: 10.1002/lno.10305

Microbial nitrogen processing in hard clam (*Mercenaria mercenaria*) aquaculture sediments: the relative importance of denitrification and dissimilatory nitrate reduction to ammonium (DNRA)

Anna E. Murphy,* Iris C. Anderson, Ashley R. Smyth, Bongkeun Song, Mark W. Luckenbach
Virginia Institute of Marine Science, College of William & Mary, Gloucester Point

Abstract

As bivalve aquaculture expands worldwide, an understanding of its role in nutrient cycling is necessary to ensure ecological sustainability and determine the potential of using bivalves for nutrient mitigation. Whereas several studies, primarily of epifaunal bivalves, have assessed denitrification, few have considered nutrient regeneration processes such as dissimilatory nitrate reduction to ammonium (DNRA), which competes with denitrification for nitrate and results in nitrogen retention rather than loss. This study compares sediment nitrogen cycling including mineralization, DNRA, and denitrification within U.S. clam aquaculture sediments to nearby uncultivated sediments, seasonally. Clam aquaculture significantly increased sediment ammonium and phosphate effluxes relative to uncultivated sediments. Both DNRA and denitrification were significantly enhanced at clam beds compared to uncultivated sediments in July and November, while in May only DNRA was increased. The ratio of DNRA to denitrification was significantly higher at clam beds compared to uncultivated sediments, demonstrating that DNRA may be favored due to a ready supply of labile organic carbon relative to nitrate and perhaps sulfidic conditions. Functional gene abundances, *nrfA* (DNRA) and *nirS* (denitrification) followed similar patterns to nitrate respiration rates with highest *nrfA* abundances in the clam sediments and similar *nirS* abundances across seasons and sediment type. Ultimately clam sediments were found to be a significant source of nutrients to the water column whereas uncultivated sediments retained ammonium produced by microbial mineralization. Thus, clam cultivation may promote local eutrophication (i.e., increased primary production) by facilitating nutrient regeneration and retention of ammonium in the sediments.

Global aquaculture production more than doubled from 2000 to 2012 (FAO 2014) with bivalve production accounting for about 70% of total mariculture production (Campbell and Pauly 2013). The continued growth of the bivalve aquaculture industry globally, concurrent with increased coastal eutrophication, has prompted recent interest in the potential role bivalves may play in removing bioreactive nitrogen (N) (Stadmark and Conley 2011; Bricker et al. 2014; Petersen et al. 2014). Sediments associated with high densities of suspension feeding bivalves are often characterized as having high rates of denitrification, the microbially mediated removal of bioreactive nitrogen (N), relative to local reference sediments (Newell 2004; Kellogg et al. 2013; Smyth et al. 2013). Due to their impressive capacity to remove particulates from the water column and potentially enhance denitrification, increasing bivalve populations through either restoration or aquaculture has been proposed as an effective in-water strategy to reduce N in aquatic environments and subsequently mitigate eutrophication (Lindahl et al. 2005; Rose et al. 2014). However, by delivering reactive organic carbon to anaerobic sediments through filtration and biodeposition, bivalves may also create favorable conditions for dissimilatory nitrate reduction to ammonium (DNRA), the recycling of nitrate (NO_3^-) back to ammonium (NH_4^+) (Hardison et al. 2015). In fact, a recent study showed high densities of *Macoma balthica*, an infaunal bivalve, stimulated DNRA and decreased denitrification (Bonaglia et al. 2014). Thus, the effect of bivalves on the partitioning of NO_3^- between the two competing pathways is complex and dependent on a number of factors but ultimately dictates the fate of nitrogen in clam beds.

The competition for NO_3^- between denitrification and DNRA, is of significant ecological importance due to the outcomes of the processes: N removal vs. retention, respectively. Denitrification occurs widely in coastal anoxic sediments where both organic matter and NO_3^- are available (Seitzinger et al. 2006); DNRA has been less studied and its distribution

*Correspondence: annie@vims.edu

across aquatic systems is not fully understood (Burgin and Hamilton 2007; Giblin et al. 2013). Further, the factors that control the partitioning of NO_3^- between denitrification and DNRA are complex and not well defined across all systems. However, environmental factors such as NO_3^- supply, sulfide concentrations, and organic carbon quality have been shown to affect NO_3^- respiration rates and determine the dominant pathway (i.e., denitrification or DNRA) (Magni et al. 2000; Hiwatari et al. 2002; Gibbs et al. 2005; Burgin and Hamilton 2007). The ratio of DNRA to denitrification is often correlated with the ratio of available labile C to NO_3^-; DNRA is dominant when this ratio is high as this process utilizes NO_3^- more efficiently than denitrification (i.e., transfers more electrons) (Tiedje 1988). A recent laboratory study, which supported results from a modeling study (Algar and Vallino 2014), showed DNRA exceeded denitrification with high C loading and low NO_3^- availability, with the ratio of C decomposition rates to NO_3^- reduction rates being important in partitioning between the two pathways (Hardison et al. 2015). Sulfide accumulation may favor DNRA bacteria that use sulfide as an electron donor (Brunet and Garcia-Gil 1996). Further, a recent study demonstrated sulfide had a more general influence on the two pathways, causing a decrease in nitrite (NO_2^-) production relative to NO_3^- and subsequently favoring DNRA (Kraft et al. 2014).

The relative importance of denitrification and DNRA in bivalve-dominated systems is likely to differ depending on environmental factors, the physiology and behavior of the bivalve species, and the ecological setting (i.e., natural or aquaculture). Few studies have fully characterized N cycling rates within bivalve aquaculture systems, with many neglecting to consider recycling processes including DNRA, microbial mineralization, and direct bivalve excretion (as reviewed in Burkholder and Shumway 2011). Furthermore, the majority of previous studies have focused on epifaunal bivalves (i.e., oysters and mussels) with few considering the effects of infaunal bivalve species on NO_3^- respiration rates (but see Nizzoli et al. 2006; Welsh et al. 2015). In fact, this is the first study to our knowledge to characterize N cycling rates at a U.S. commercial clam aquaculture site.

Clam activities such as biodeposition, bioturbation, and excretion likely influence the dominant NO_3^- respiration pathway by altering NO_3^- and dissolved oxygen (DO) supply, sulfide concentrations, and organic carbon quantity and quality. For example, nitrification, a two-step aerobic process in which NH_4^+ is oxidized to NO_2^- and NO_3^-, may be enhanced by clam bioturbation and excretion, which supply DO and NH_4^+, respectively to the sediments (Hammen 1980; Henriksen et al. 1983; Nizzoli et al. 2006). Nitrification is often tightly coupled to NO_3^- reduction pathways in estuarine sediments, serving as an important NO_3^- source (Seitzinger et al. 2006); thus, by potentially increasing nitrification, clams may enhance denitrification and/or DNRA. Alternatively, clam biodeposition may suppress nitrification by fueling microbial mineralization and increasing sediment oxygen demand (SOD), resulting in reduced sediments with high sulfide. Low DO and sulfide accumulation may inhibit nitrification (Joye and Hollibaugh 1995; Giles and Pilditch 2006; Carlsson et al. 2010), causing NO_3^- limitation, which concurrent with high organic carbon concentrations, may favor DNRA over denitrification (Tiedje 1988; Algar and Vallino 2014). Additionally, sulfide may directly inhibit the last step in denitrification, the conversion of N_2O to N_2 (Sorensen et al. 1980; Brunet and Garcia-Gil 1996), while enhancing chemoautotrophic DNRA, in which sulfide rather than organic matter serves as the electron donor during NO_3^- respiration. Thus, high densities of clams associated with aquaculture will likely have significant and complex effects on NO_3^- respiration, affecting both the rates and the dominant pathway.

The overall objective of our study was to determine the effects of clam aquaculture on sediment nutrient dynamics including rates of N removal and N recycling. Specifically, we were interested in determining the relative importance of DNRA vs. denitrification in clam beds compared to nearby uncultivated sediments. The functional genes encoding nitrite reductase, cytochrome cd nitrite reductase (*nirS*) and cytochrome C reductase (*nrfA*), were selected to quantify abundance of denitrifying and DNRA communities, respectively. The relationships between *nirS* and *nrfA* gene abundances to rates of denitrification and DNRA were investigated. Our experimental design aimed to capture a range of sediment conditions (i.e., porewater sulfide and sediment organic content) across a clam aquaculture lease to account for variability due to clam size-class and time since clams were planted. We hypothesized that by delivering labile organic matter to the sediments, clam cultivation will enhance overall nitrate respiration rates above control sediments; however, DNRA will be dominant over denitrification, which will be reflected in higher *nrfA* abundances than *nirS* abundances.

Methods

Site description

Located on the bayside of the Eastern Shore of Virginia, Cherrystone Inlet is a shallow tributary of Chesapeake Bay, where shellfish have been cultivated for about three decades (Fig. 1). Infaunal hard clams (*Mercenaria mercenaria*) are cultured in the shallow subtidal regions of the estuary ($\sim$ 0.2 m to 1 m, below mean low water). Approximately 145 million cultivated clams inhabit the private shellfish leases across the 5.6 km^2 embayment at any given time. Juvenile clams (8–15 mm), reared in land-based hatcheries and nurseries, are planted directly in the sediments. A plastic net, set flush to the sediment surface, is used to protect the clams from natural predators. Macroalgae proliferate on the predator-exclusion nets and are swept from the nets periodically by the

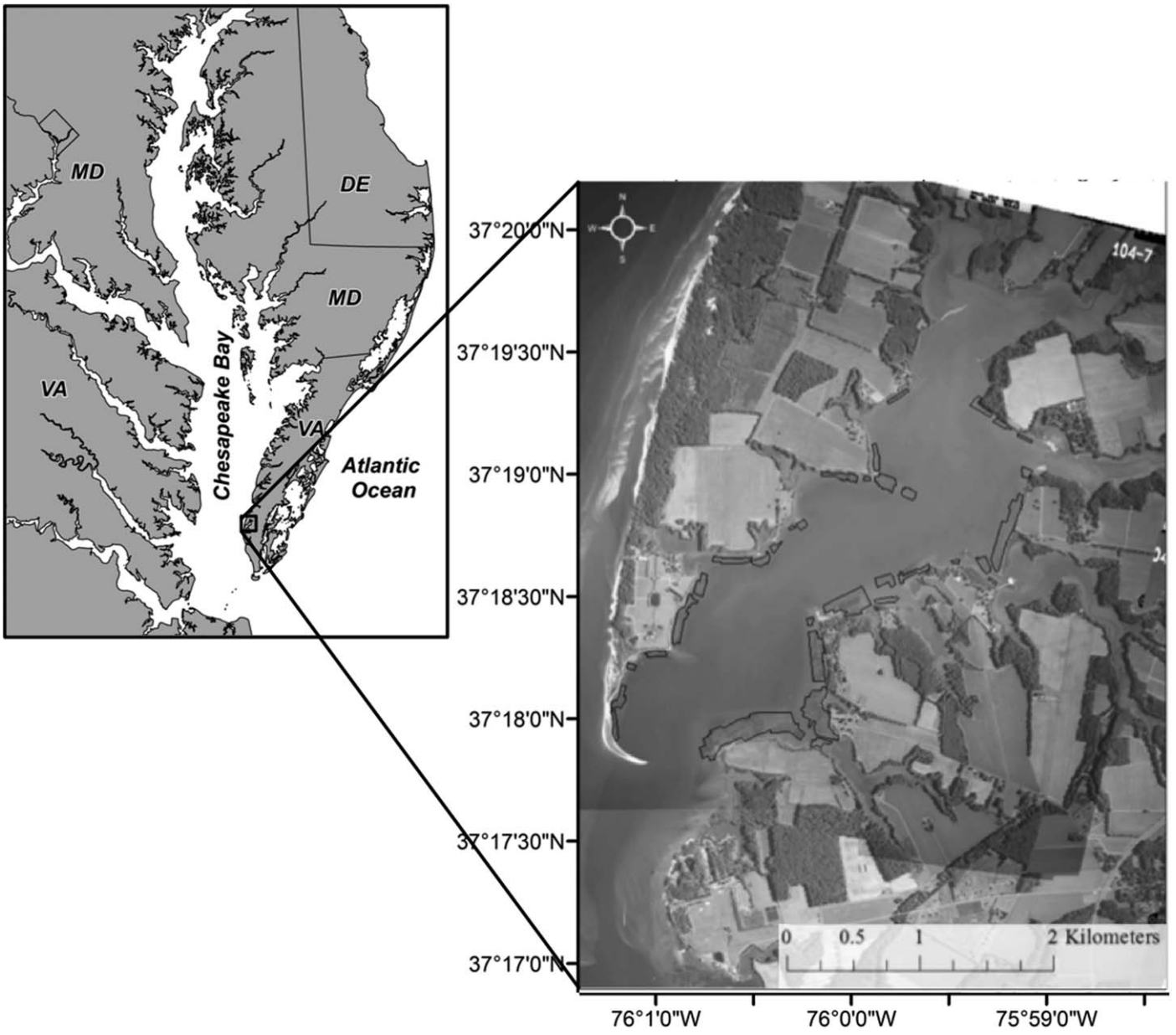

Fig. 1. Cherrystone Inlet, Chesapeake Bay, U.S.A. Aerial photograph of Cherrystone Inlet taken in 2012, black polygons delineate active clam aquaculture operations.

aquaculturists (Murphy et al. 2015). After about 2 yr, market-sized clams (~ 40 mm shell length) are mechanically harvested from the sediments (Castagna and Kraeuter 1981). After harvest the sediments remain fallow for a season prior to planting more clams (T. Rapine, Cherrystone Aquafarms, pers. comm.).

Sampling design

The sampling design aimed to capture a range of sediment conditions varying both seasonally and spatially across a leased area. Each clam bed (approximately 4 m × 18 m) consists of approximately 50,000 clams of a homogenous age class, as the clams within each bed are all planted at the same time. As a result, we anticipated that the clam beds across the lease would have varying levels of organic matter enrichment as well as porewater sulfide and nutrient concentrations, depending on clam metabolism and length of time clams had occupied the space. In May and July 2013, 16 randomly selected clam beds and 4 uncultivated sites and in

November 2013, 7 randomly selected clam beds, and 3 uncultivated sites were sampled. Uncultivated sites, which to the best of our knowledge had not been previously cultivated, were located adjacent to, approximately 20 m, from the clam beds, a distance chosen to reduce any influence of aquaculture on the control sediments and at a water depth similar to that of the clam beds.

Sediment and water column characteristics

At each clam bed and uncultivated site sampled, porewater was collected at 5–7 cm below the sediment surface using a stainless steel push-point sampler (MHE Products, East Tawas, Michigan, U.S.A) for nutrient and hydrogen sulfide analysis. Porewater sulfide samples were immediately fixed in zinc acetate, filtered, and stored until analysis on a spectrophotometer within a week of collection (Cline 1969). In addition to the porewater nutrient samples, triplicate water column grab samples, collected over the clam beds and uncultivated sites, were filtered (0.45 μm Whatman polyethersulfone (PES)) and frozen until analysis for dissolved inorganic nitrogen (DIN) (including NO_3^-, NO_2^-, and NH_4^+) (Liao 2001) and soluble reactive phosphorus (SRP) (Knepel and Bogren 2001) on a Lachat QuikChem 8000 automated ion analyzer (Lachat Instruments, Milwaukee, Wisconsin, U.S.A.).

A sediment core (2.2 cm i.d.) was collected at each clam bed and uncultivated site, sub-sectioned horizontally at 0–2 cm and 2–5 cm and analyzed for porosity, as loss of wet weight after drying at 70°C, and sediment organic matter (SOM), as loss on ignition after combustion at 500°C for 5 h. Prior to combustion, subsamples of dried sediments were acidified and analyzed on a Carlo Erba elemental analyzer (Thermo Electron Corp. Flash EA 1112 Series) for organic C and total N content.

Surface sediment samples (0–3 cm) were collected at a subset of clam beds ($n = 7$, 6, 6 in May, July, and November, respectively) and uncultivated sites ($n = 3$, 2, 3 in May, July, and November, respectively) using a small core (2.2 cm i.d.) for gene abundance analysis. Molecular samples were placed in liquid nitrogen in the field and stored at −80°C upon return to the lab until DNA extraction and molecular analysis were performed (see below for details).

Benthic metabolism and nutrient flux measurements

At each clam bed and uncultivated site three sediment cores (9.5 cm i.d., with approximately 10 cm overlying water and 8 cm sediment depth) were collected for determinations of benthic metabolism, nutrient fluxes and N cycling rates; thus 60 cores were collected in May and July (20 total sites during each month), and 30 cores were collected in November (10 total sites). Cores were not treated as replicates, but were used to conduct concurrent incubations in the light ($\sim$ 50–100 μE m^{-2} s^{-1}) and dark (paired cores) and for measurement of N cycling rates by isotope-pairing (with a T_0 core; see details below). Cores were transported to the Virginia Institute of Marine Science Eastern Shore Laboratory (VIMS ESL) in Wachapreague, Virginia, within 3 h of collection, placed in a water bath with continuously flowing water maintained at ambient conditions of the sampling location, and allowed to equilibrate overnight. Flowing water from the adjacent Wachapreague Inlet provided a continuous supply of oxygen and phytoplankton to the clams and sediments overnight. Gradient formation was prevented by suspending a magnetic stir bar (2.5 cm) in each core, spinning at 60 rpm powered by a central battery-operated motor (6V).

The following day, one core from each site was illuminated while the other two were kept dark (dark core and T_0 core). The T_0 cores were capped but not sampled during the initial flux incubation. Cores were capped with lids that contained an inflow and outflow port, avoiding any air bubbles; the overlying water was sampled using a 60 mL syringe through the outflow port while replacement water was allowed to enter through the inflow port from the water bath. Cores were sampled approximately hourly for 3–4 h for NH_4^+, SRP, NO_x^- (combined NO_2 and NO_3) and dissolved inorganic carbon (DIC). Samples collected for NH_4^+, SRP, and NO_x^- were immediately filtered (0.45 μM Whatman PES) and frozen until analysis (as described above). DIC samples were placed in 8 mL hungate tubes, pre-spiked with 15 μL saturated mercuric chloride, and stored cold underwater until analysis using a Li-Cor 6252 infrared gas analyzer within a month of collection as described by (Neubauer and Anderson 2003). During the incubation a Hach LDO101 Luminescent DO sensor (Hach Co., Loveland, Colorado, U.S.A.), secured in the lids of 12 randomly selected cores, continuously monitored DO in real time to determine the duration of the isotope pairing incubation which followed (necessary to keep DO above 70% of the original concentration; (Dalsgaard et al. 2000)).

Hourly and daily fluxes for each analyte were calculated as:

$$\text{Hourly Flux} = (m \times V)/A \quad (1)$$

$$\text{Daily Flux} = (F_l \times h_l) + (F_d \times h_d) \quad (2)$$

where m is equal to the slope of the linear regression of concentration (μM or mM) vs. time (hours); V is equal to the volume of water in the flux chamber (liters); A is the sediment surface area within the chamber (m^2); F_d and F_l are hourly fluxes in the dark and light, respectively (mmol m^{-2} h^{-1}), h_d and h_l are the number of hours of dark and light in a day, which varied by season. A flux from the sediment to the water column is positive (production) while a flux to the sediment from the water column is a negative value (consumption).

Denitrification and DNRA rate measurements

After the flux incubation, the sediment cores were uncapped and allowed to re-equilibrate in the oxygenated water bath for at least an hour. Water level was dropped to

just below the lip of the cores and each core was spiked with $^{15}NO_3^-$ (98.9 atom%; 1.1 mL added to each core of a 50 mM stock made from $Na^{15}NO_3$ to obtain a final concentration of approximately 100 μM). After spiking, a water sample was collected from each core and analyzed on the Lachat for total NO_3^- ($^{14}NO_3^- + {}^{15}NO_3^-$). The cores were left uncapped and each gently bubbled for about an hour to allow $^{15}NO_3^-$ to diffuse to the zone of active denitrification and DNRA in the sediments. The diffusion time for $^{15}NO_3^-$ to reach the anoxic zone, estimated using calculations based on Fick's law, described in the NICE handbook (Dalsgaard et al. 2000) and the DO penetration depth of approximately 2 mm obtained using an oxygen microsensor and micromanipulator (OX100, Unisense, Aarhus N, Denmark) (A. E. Murphy, unpubl.), was approximately 16 min. At the completion of the pre-incubation period, the T_0 core from each site was sampled to account for any $^{29}N_2$ and $^{30}N_2$ produced prior to sealing the cores (see below for details on postincubation-sampling). The remaining light and dark cores from each site were capped and incubated for 2–4 h depending on the SOD determined in the previous flux incubation allowing DO to drop no more than 70% of the initial concentration.

At the end of the incubation each core was uncapped, gently homogenized, and sampled for $^{29}N_2$, $^{30}N_2$, and extracted $^{15}NH_4^+$ (see below). Afterward all clams were removed from each core counted, measured, and the tissue ash-free dry weight (DW) determined by the difference in DW prior to and after combusting at 500°C for 5 h. Samples were collected for $^{29}N_2$ and $^{30}N_2$ by siphoning the slurry into a 12 mL exetainer vial and preserving the sample with 100 μL of 7M $ZnCl_2$. Samples were analyzed within a month on a membrane inlet mass spectrometer (MIMS) (Kana et al. 1994). For $^{15}NH_4^+$ analysis, approximately 120 mL of the core slurry was collected in a whirlpak bag with potassium chloride (KCl) (final concentration of 2M), shaken for one hour, centrifuged, filtered (0.45 μM Whatman PES), and stored frozen until diffusion. Samples were diffused and trapped for analysis of $^{15}NH_4^+$ enrichment and concentration using a method modified from Brooks et al. (1989). Water samples were placed in specimen cups, which held an acidified (25 μL of 2.5M sulfuric acid) GFF filter (1 cm, i.d.), threaded onto a stainless steel wire, suspended on the lip of the cup. Sample volumes ranged from 5 mL to 60 mL, depending on the NH_4^+ concentration obtained on the Lachat prior to diffusion, to obtain a target mass for analysis of 30 μg. Magnesium oxide was added and the samples were allowed to diffuse for 2 weeks, after which samples were encapsulated in tin capsules and analyzed on an EA-IRMS at the University of California Davis Stable Isotope Facility.

Denitrification rates were calculated as described by Nielsen (1992) as follows:

$$D_{15} = p29 + 2p30 \quad (3)$$

$$D_{14} = D_{15} \times (p29/2p30) \quad (4)$$

where D_{15} represents denitrification of the added $^{15}NO_3^-$; p29 and p30 are equal to the rates of production of $^{29}N_2$ and $^{30}N_2$, respectively, and D_{14} is the denitrification rate of ambient $^{14}NO_3^-$. Direct denitrification of NO_3^- from the water column, (D_w), and coupled denitrification (D_n) were calculated as described in Nielsen (1992):

$$D_w = (^{14}NO_3^-/{}^{15}NO_3^-) \times D_{15} \quad (5)$$

$$D_n = D_{14} - D_w \quad (6)$$

where $^{14}NO_3^-$ is equal to the ambient unlabeled NO_3^- concentration (μM) and $^{15}NO_3^-$ is equal to the isotopically-labeled NO_3^- concentration at the start of the incubation. Preliminary manipulation experiments in which denitrification rates were measured with varying concentrations of $^{15}NO_3^-$ addition, revealed D_{14} was independent of the concentration of $^{15}NO_3^-$ added. Additionally, a time series experiment was conducted in which cores were sacrificed over time to ensure linear production of $^{29}N_2$ and $^{30}N_2$. These results confirmed the IPT assumptions were met and the equations are valid for this system (Nielsen 1992).

Actual DNRA rates were calculated according to Risgaard-Petersen and Rysgaard (1995) as:

$$DNRA_t = p^{15}NH_4^+ \times (D_{14}/D_{15}) \quad (7)$$

where $p^{15}NH_4^+$ is equal to the production of $^{15}NH_4^+$. This assumes that DNRA occurs in the same sediment horizon as denitrification (Rysgaard et al. 1993). DNRA coupled to nitrification ($DNRA_n$) and direct from water column NO_3^- ($DNRA_w$) were calculated as:

$$DNRA_w = (^{14}NO_3^-/{}^{15}NO_3^-) \times p^{15}NH_4^+ \quad (8)$$

$$DNRA_n = DNRA_t - DNRA_w \quad (9)$$

Gross ammonification measurements

Gross ammonification rates, which include NH_4^+ production from organic matter mineralization and some contribution from DNRA and heterotrophic N fixation, were measured using the isotope pool dilution method (Anderson et al. 1997). Two paired cores (5.7 cm i.d, with approximately 5 cm overlying water and 5 cm sediment depth) were collected at each sampling site, transported to the laboratory, and placed in a water bath filled with site water. Prior to collection in the field, clams were carefully removed from the area to obtain sediments void of clams in order to measure microbial ammonification independent of the contribution of clam excretion. Cores were uncapped and held underwater overnight in the dark with gentle mixing and aeration. The following day the sediments were homogenously spiked with $^{15}N\text{-}NH_4^+$ (3.6 mL of $[NH_4]_2SO_4$, 30 at.%, 10 mM) by injecting 100 μL of the stock solution into 36

silicone-filled holes through the vertical sediment column. Prior to sacrificing, the cores were sectioned horizontally 0–2 cm and 2–5 cm, although rates did not differ between the two horizons and therefore only rates associated with the top 2 cm are reported. One of the paired cores from each site, T_0, was immediately sacrificed after spiking by shaking in 2M KCl for an hour; the extractant was filtered and frozen until analysis. The remaining core from each site, T_f, was capped and incubated for 24 h in the dark at in situ temperatures. After the incubation, the T_f cores were extracted. NH_4^+ in the extracts was trapped, diffused, and analyzed as described above for the DNRA samples. Rates of gross ammonification were calculated using a model described by Wessel and Tietema (1992) as

$$\text{Ammonification} = \frac{\ln\left(\text{Tf}_{\text{atm}\%} - k\right) / \left(\text{T0}_{\text{atm}\%} - k\right)}{\ln\left[\text{NH}_4^+\, T_f\right] / \left[\text{NH}_4^+\, T_0\right]} \times \frac{\left[\text{NH}_4^+ T_0\right] - \left[\text{NH}_4^+ T_f\right]}{\text{time}} \quad (10)$$

where $\text{Tf}_{\text{atm}\%}$ and $\text{T0}_{\text{atm}\%}$ refers to the $^{15}NH_4^+$ enrichment of the T_f and T_0 cores; k is equal to natural abundance of $^{15}NH_4^+$ expressed as atom %; $[NH_4^+ T_f]$ and $[NH_4^+ T_0]$ are the concentrations of NH_4^+ in the T_f and T_0 cores, and time is the incubation time.

DNA extraction and quantitative PCR

Sediment DNA was extracted from homogenized surface sediments (0–3 cm) using the PowerSoil DNA Kit (Mo-Bio Laboratories, Inc., Carlsbad, California. U.S.A.), following the manufacturer's protocol with the following modifications: 0.5 g of wet sediment was used and Thermo Savant Fast Prep FP 120 Cell Disrupter (Qbiogene Inc. Carlsbad, California, U.S.A.) was used for cell disruption. Sediment DNA concentration was measured using Qubit double-stranded DNA High Sensitivity assay kit and a Qubit fluorometer according to the manufacturer's protocol (Life Technologies, Grand Island, New York, U.S.A.). Samples were subsequently diluted to a concentration of 1 ng μL^{-1}.

Quantitative PCR (qPCR) assays were carried out to quantify the abundance of genes responsible for denitrification (*nirS)* and DNRA (*nrfA)*. The *nirS* primers used were NIRS1F and NIRS-Q-R (Braker et al. 1998; Mosier and Francis 2010) and the *nrfA* primers were NRFAF2 and NRFA1R (Mohan et al. 2004; Welsh et al. 2014). Each qPCR incubation mixture (total volume 20 μL) contained Go-Taq qPCR Master Mix (Promega Corporation, Madison, Wisconsin, U.S.A.), the primers (0.5 uM), and sediment DNA (3 ng). The *nrfA* mixture also contained 0.5 μL of MgCl for amplification optimization. Preparation of qPCR standards and PCR cycling were previously reported in (Song et al. 2014) and Lisa et al. (unpubl.). All qPCR analyses were conducted in triplicate. PCR specificity and primer dimer were assessed using dissociation curves. The R^2 values for the standard curves were 0.986 and 0.997 for *nirS* and *nrfA*, respectively.

Clam respiration and excretion estimates

Clam respiration rates were estimated using an equation reported by Hofmann et al. (2006) and adjusted by Wiseman (2010) using data collected in Cherrystone Inlet, Virginia (Condon 2005); the estimate takes into account clam biomass per m^2 and temperature. Clam excretion rates, primarily composed of NH_4^+ (Hammen 1980), were estimated stoichiometrically. The ratio of C respired to nitrogen excreted is dependent on the bivalve's rate of catabolism and the composition of the food source (Bayne 1976). The respiration to excretion ratio was estimated at 7.0, which is the theoretical minimum signifying protein catabolism (Mayzaud and Conover 1988; Dame 2012). This may overestimate excretion as a higher respiration to excretion ratio may occur if carbohydrate and lipid catabolism are significant. Nonetheless this ratio provides a reasonable estimate for excretion (Dame 2012).

Annual sediment N budgets

Sediment N budgets were constructed for the clam bed and uncultivated sediments by scaling the seasonal sediment fluxes and N transformation rates (i.e., DNRA, denitrification, and ammonification) to annual rates. These budgets provided conservative annual estimates as negligible winter rates were assumed due to low temperatures, which a preliminary study at this site revealed. Nitrification, which was not directly measured in this study, was estimated as rates of coupled nitrification-DNRA plus coupled nitrification-denitrification plus the net NO_x^- flux. Immobilization of NH_4^+ into microbial biomass and benthic microalgal uptake was estimated as gross ammonification plus the net NH_4^+ flux.

Statistical analysis

To determine whether significant differences existed between the uncultivated sediments and the clam sediments, the increase or decrease of a clam bed measurement relative to the mean for the uncultivated site for each season was calculated as

$$\Delta_r = C_{is} - U_s \quad (11)$$

where the C_{is} is the response measurement at clam bed i during season s, and U_s is the mean response measurement at the uncultivated sediments for each season (s). *t*-tests were conducted to determine if the mean Δ_r was significantly different from zero. Prior to running the *t*-tests normality was checked and data were transformed using Box-Cox when appropriate. This approach was used to assess sediment characteristics (e.g., porewater nutrients, sulfide, sediment organic matter) as well as rate measurements (e.g., net fluxes, DNRA, denitrification rates). If the mean Δ_r is significantly different than zero ($p < 0.05$), this implies that sediments exposed to clam

Table 1. Environmental conditions in Cherrystone Inlet during sampling. Clam metrics including clam lengths (mm), biomass (ash free DW, g m^{-2}), and densities (ind m^{-2}).

Month	Temperature (°C)	Salinity	Shell length	Biomass	Density
May	17	24.5	11.8–58.1	3.5–419.8	46.9–1126.8
Jul	25	23.0	21.0–48.8	23.1–539.8	140.8–2441.3
Nov	12	23.0	15.3–54.0	76.3–497.2	328.6–3333.3

Table 2. Water column nutrient concentrations (μM) at the clam site and uncultivated site ($n = 3$ per season and site). Standard errors are provided in parentheses. * indicates significantly higher concentrations above the clam beds compared to uncultivated sites within each month.

Month	Site	NO_x^-	NH_4^+	SRP
May	Clam	*0.46(0.04)	*0.48(0.05)	*0.09(0.01)
	Uncultivated	0.04(0.003)	0.18(0.01)	0.07(0.001)
Jul	Clam	0.22(0.01)	*4.04(0.35)	*0.26(0.01)
	Uncultivated	0.19(0.04)	0.48(0.17)	0.10(0.01)
Nov	Clam	0.05(0.01)	0.96(0.19)	0.03(0.01)
	Uncultivated	0.06(0.01)	1.41(0.05)	0.02(0.01)

aquaculture behave differently than uncultivated sediments (Kellogg et al. 2014). Linear regression analyses were used to investigate the relationships between clam metrics (including size, biomass, and density) and sediment characteristics (porewater sulfide, nutrients, and organic content). Linear regressions were also used to assess the relationship between functional gene abundances and rates of denitrification and DNRA. To investigate the potential mechanisms driving the proportion of DNRA relative to denitrification linear regressions were conducted for the ratio of DNRA/denitrification against porewater sulfide concentrations and gross ammonification measurements. All error estimates are reported as standard error. A significance value of $p < 0.05$ was used for all statistical tests, which were conducted in R studio software (version 0.98.1091 and R version 3.0.2).

Results

Environmental characteristics

Water temperatures ranged from 12°C in November to 25°C in July, with an intermediate of 17°C in May. Salinity did not vary across seasons, with an average of 23.5 (Table 1). Water column nutrients were generally low (< 1 μM) across all months and sites. Despite the close proximity of the clam and control sites, water column SRP and NH_4^+ were significantly higher above the clam beds compared to the control sites in May and July; water column NO_x^- was significantly higher above the clam beds than the control sites in May (Table 2).

Sampling sites included a range of clam sizes, with shell lengths ranging from 11.8 mm to 58.1 mm (Table 1). Clam density ranged widely (46.9–3333.3 individuals m^{-2}) and was dependent on clam size; higher densities were observed at recently planted clam beds with smaller individuals while lower densities occurred at clam beds planted 1–2 yr prior to sampling, which had larger individuals.

Within the clam beds, no significant relationships were observed between clam metrics (size, density, or biomass) and porewater nutrients, sediment organic content, or sulfide concentrations. However, mean porewater NH_4^+, SRP, and sulfide concentrations were generally higher in clam compared to uncultivated sediments during July and November (Table 3). In May, mean porewater NH_4^+, SRP, and sulfide, were all significantly higher at the uncultivated control sites than the clam beds.

Sediment organic content was low and similar between the clam and uncultivated sediments (Table 3). However, in July clam beds had significantly higher organic content than the uncultivated sediments. Sediment C : N was similar at the clam and uncultivated sediments across all months.

Nutrient fluxes

Daily NH_4^+ fluxes were significantly higher at clam compared to uncultivated sediments during all months, with the highest efflux rates (23.3 ± 2.8 mmol m^{-2} d^{-1}) from clam beds in July (Fig. 2a; Table 4). Clam excretion was estimated to contribute approximately 42, 21, and 38% of the daily NH_4^+ flux in May, July, and November, respectively (Fig. 2a). Whereas a net release of NH_4^+ from the clam sediments to the water column was observed during all months, a net uptake occurred in the uncultivated sediments in July and November with a small release in May. Increased NH_4^+ fluxes from clam beds relative to those from uncultivated sediments on average ranged from 2.74 mmol N m^{-2} d^{-1} in May to 24.4 mmol N m^{-2} d^{-1} in July (Table 4).

SRP fluxes followed similar trends to those of NH_4^+ with net effluxes occurring at the clam sediments and net uptake in the uncultivated sediments. SRP fluxes were significantly higher in the clam sediments than the uncultivated sites except in May (Fig. 2b; Table 4).

NO_x^- fluxes were generally low relative to the NH_4^+ fluxes and highly variable across sites and seasons, with similar rates at clam and uncultivated sediments. In both sediment types there was net uptake of NO_x^- in May and November,

Table 3. Mean sediment characteristics at uncultivated and clam sediments seasonally including porewater DOC (μM), nutrients (μM), and sulfide (μM) (collected at 5–7 cm depth); percent sediment organic matter (SOM) and molar C to N ratio in the surface 0–2 cm sediment horizon (C: N); benthic chlorophyll (B. Chla) and phaeophytin (B. Phaeo) (μg cm^{-2}); and *nir*S and *nrf*A gene abundances (copy number g sediment^{-1}). *n* refers to the number of sites sampled. Standard errors are in parentheses. * denotes significant difference between uncultivated and clam sediments within each month.

	May		Jul		Nov	
Site	**Uncultivated**	**Clam**	**Uncultivated**	**Clam**	**Uncultivated**	**Clam**
n	4	16	4	16	3	7
DOC	371.6(51.8)	404.2(51.6)	607.8(372.2)	393.3(101.4)	226.3(14.2)	287.8(19.3)
NO_x^-	0.1(0.01)	*0.3(0.1)	0.3(0.02)	0.3(0.02)	0.1(0.01)	0.5(0.2)
NH_4^+	107.4(17.0)	*49.2(3.5)	47.9(4.0)	*59.3(4.7)	37.2(9.3)	64.7(16.5)
SRP	7.3(0.8)	*4.6(0.8)	2.9(0.4)	*6.3(1.2)	0.9(0.6)	*5.0(1.5)
Sulfide	241.6(36.1)	*101.2(21.0)	47.8(10.2)	141.1(50.8)	13.5(13.5)	38.0(16.3)
SOM	1.2(0.2)	1.0(0.1)	0.8(0.1)	*1.3(0.1)	0.7(0.1)	1.1(0.3)
C : N	6.8(0.3)	6.9(0.3)	7.4(0.5)	7.2(0.1)	7.6(1.7)	7.2(0.6)
B. Chl*a*	2.7(0.4)	3.3(0.4)	2.0(0.7)	4.2(0.5)	3.7(0.43)	4.3(0.38)
B. Phaeo	1.7(0.5)	3.5(0.7)	4.0(2.3)	8.2(0.6)	3.7(0.31)	7.4(1.23)
nirS	3.69×10^7	3.65×10^7	2.67×10^7	4.21×10^7	2.33×10^7	2.44×10^7
	(7.1×10^6)	(6.3×10^6)	(2.9×10^6)	(9.7×10^6)	(2.7×10^6)	(5.2×10^6)
nrfA	1.07×10^8	$*2.24 \times 10^8$	3.81×10^7	$*3.56 \times 10^8$	4.43×10^7	1.16×10^8
	(1.6×10^7)	(3.5×10^7)	(5.0×10^6)	(1.2×10^8)	(1.6×10^7)	(3.1×10^7)

averaging -0.29 ± 0.13 mmol m^{-2} d^{-1} and -0.14 ± 0.03 mmol m^{-2} d^{-1}, respectively and a net efflux in July with a mean of 0.74 ± 0.31 mmol m^{-2} d^{-1} (Fig. 2c). There was no significant effect of clams on NO_x^- fluxes (Table 4).

DON fluxes were highly variable with no net flux at the uncultivated sediments during any of the months (Fig. 2d). In July, clam sediments were a net sink for DON averaging -3.7 ± 0.74 mmol m^{-2} d^{-1}, while small DON effluxes were observed in May and November from the clam sediments.

Benthic metabolism

Seasonal variability of daily SOD and DIC fluxes was observed at both the clam and uncultivated sediments, with higher metabolic rates measured in the summer. DO uptake and DIC release was observed at all sites with significantly higher SOD at clam beds compared to uncultivated sites (Fig. 2e,f; Table 4). DIC release was generally higher at clam sites than uncultivated sediments but the difference was only significant in July. Estimated clam respiration contributed 15, 26, and 15% of SOD fluxes in May, July, and November, respectively (Fig. 2e). The respiratory quotient (RQ), which is equal to the net DIC flux divided by SOD, was 1.2, 2.0, and 0.9 at the clam beds in May, July, and November, while at the uncultivated sediments RQ was estimated as 1.0, 1.6, and 2.3 in May, July, and November.

Ammonification rates

Seasonal variation was apparent in gross ammonification with highest rates measured in July followed by May and lowest rates in November. Ammonification rates at clam and uncultivated sediments were not significantly different in May or November averaging 1.53 mmol N m^{-2} d^{-1} and 0.49 mmol N m^{-2} d^{-1}, respectively (Fig. 3); however in July, clam beds had significantly higher rates of ammonification than uncultivated sediments (Table 4). It is important to note that as described in the methods, excretion of NH_4^+ by clams did not contribute to the measured ammonification rates but did contribute to the net NH_4^+ fluxes (Table 2).

Nitrate respiration rates

Total nitrate respiration rates (denitrification plus DNRA) varied seasonally and were significantly higher at the clam beds than uncultivated sediments in July and November, with no significant difference in May (Fig. 4). At both the clam and uncultivated sediments an average of 96.5%, 94.6%, and 99.1% of denitrification and DNRA rates were coupled to nitrification in May, July, and November, respectively. Generally denitrification rates were lower than DNRA rates during all seasons and at both the clam and uncultivated sediments (Fig. 4).

DNRA rates were significantly higher at the clam beds compared to uncultivated sediments during all seasons (Fig. 4; Table 4). Overall, across all seasons, clam beds enhanced DNRA rates above the control sediments by a mean of 151.3 μmol m^{-2} d^{-1} (Table 4). Denitrification rates were significantly higher at the clam beds than the uncultivated sediments in July and November, with overall average rates of 42.8 μmol m^{-2} d^{-1} and 19.6 μmol m^{-2} d^{-1}, respectively (Fig. 4; Table 4). However, in May clam and uncultivated sediments had similar denitrification rates, averaging 73.0 μmol m^{-2} d^{-1}.

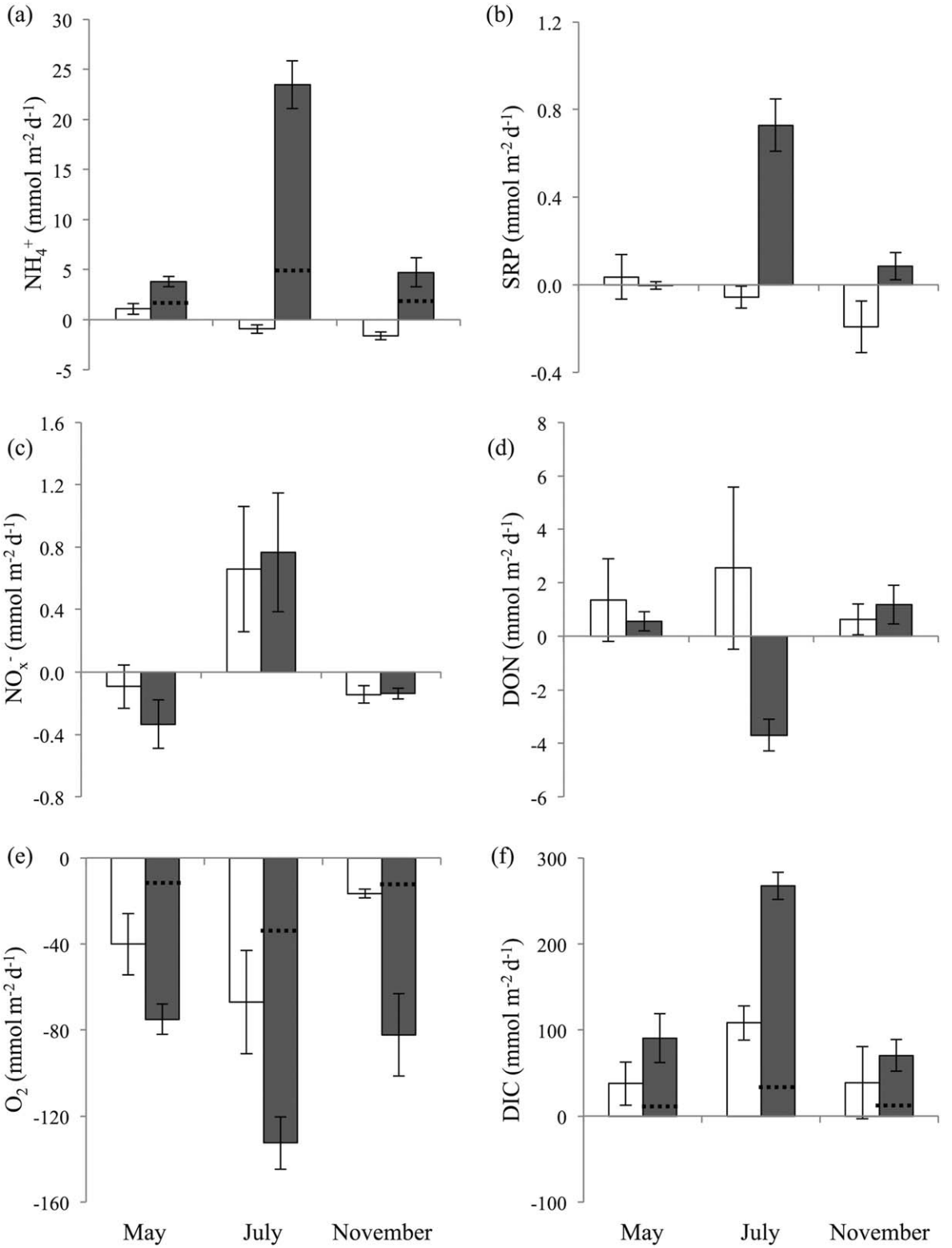

Fig. 2. Seasonal mean daily fluxes of ammonium (NH_4^+) (a), phosphate (SRP) (b), nitrate + nitrite (NO_x^-) (c), dissolved organic nitrogen (DON) (d), dissolved oxygen (e), and dissolved inorganic carbon (DIC) (f) at uncultivated sediments (white) ($n = 4$ in May and July, $n = 3$ in November) and clam beds (gray) ($n = 16$ in May and July, $n = 7$ in November). Dotted lines in (a), (e), and (f) represent estimated clam metabolic contribution to the net fluxes. Error bars represent standard errors.

Table 4. The mean difference between the clam beds and the average uncultivated sediments for each season as well as overall (across all seasons; All) of ammonium (NH_4^+), nitrate+nitrite (NO_x^-), phosphate (SRP), dissolved organic nitrogen (DON), dissolved inorganic carbon (DIC), and sediment oxygen demand (SOD), denitrification, dissimilatory nitrate reduction to ammonium (DNRA), and gross ammonification (mmol m^{-2} d^{-1}). Additionally, the mean difference in the ratio of DNRA relative to denitrification (DNRA: DNF) between the clam beds and uncultivated sites for each month (unit-less). A positive value represents a higher measurement at the clam beds relative to the uncultivated sediments while a negative value reflects a lower rate. Bold text denotes values are significantly different than zero (*t*-test; alpha = 0.05).

Response	May	Jul	Nov	All
NH_4^+	**2.74**	**24.42**	**6.36**	**11.96**
NO_x	−0.24	−0.36	0.01	−0.06
SRP	**−0.04**	**0.79**	**0.28**	**0.34**
DON	**−0.78**	**−6.25**	0.55	**−2.79**
DIC	52.74	**159.53**	31.69	**92.05**
SOD	**34.92**	**65.52**	**65.65**	**52.99**
Denitrification	−0.010	**0.020**	**0.014**	**0.006**
DNRA	**0.108**	**0.260**	**0.043**	**0.151**
Ammonification	−0.18	**0.76**	0.36	0.30
DNRA : DNF	**1.15**	**5.02**	−0.15	**2.11**

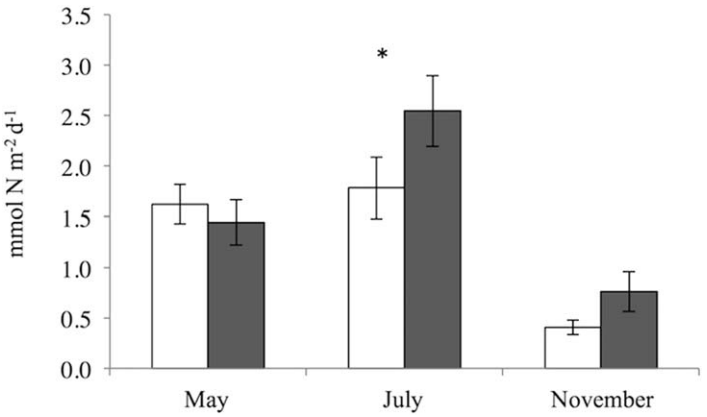

Fig. 3. Gross ammonification rates at uncultivated (white) and clam sediments (gray) seasonally. Error bars represent standard errors. * denotes clam sediments are significantly higher than uncultivated sediment within each month.

DNRA and denitrification rates were positively correlated with each other at both the clam beds and uncultivated sediments (Fig. 5). However, the relative proportion of DNRA to denitrification (i.e., the slope of the regression) was significantly higher at the clam beds than the uncultivated sediments (Table 4). A positive relationship between the relative proportion of DNRA to denitrification and gross ammonification rates, a proxy for organic C quality and availability was observed, although the trend was not statistically significant. Additionally, DNRA relative to denitrification generally increased with increasing porewater sulfide when all seasonal data were pooled, although not significantly.

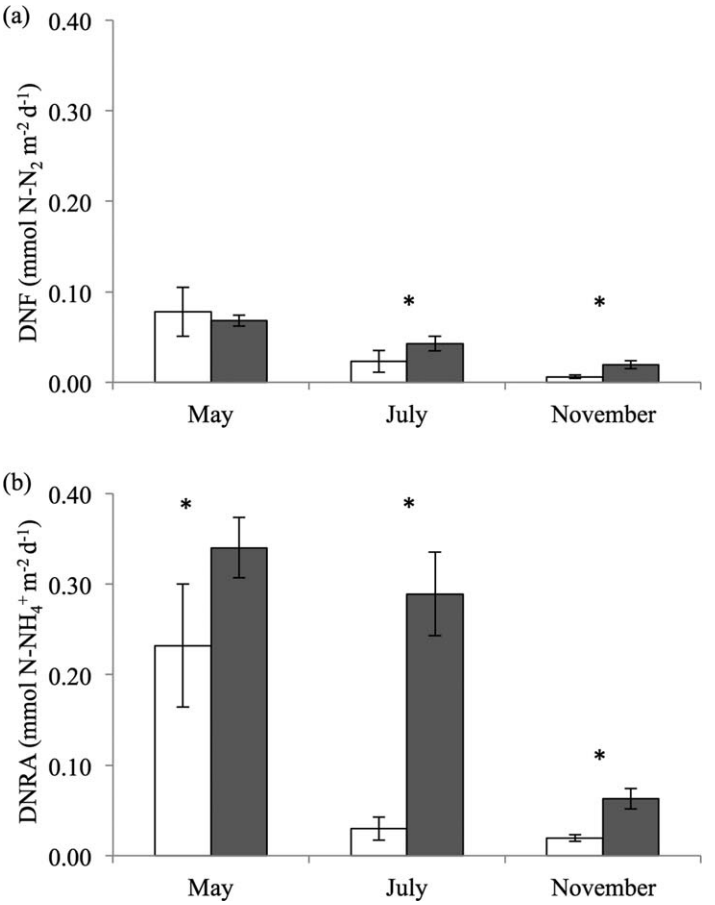

Fig. 4. Denitrification (A) and DNRA (B) in May, July, and November 2013 at uncultivated sediments (white) and clam sediments (gray). Error bars are standard errors. Uncultivated sediments, $n = 4$ in May and July, $n = 3$ in November. Clam sediments, $n = 16$ in May and July, $n = 7$ in November. * denotes rates are significantly higher at the clam sediments relative to the uncultivated sites within each month.

Functional gene abundances

Abundances of *nirS*, encoding cytochrome cd nitrite reductase in denitrification, were similar between the clam and uncultivated sediments throughout all seasons with no seasonal variation (Table 3). However, abundances of *nrfA*, which encodes for cytochrome C nitrite reductase in DNRA, were significantly higher at the clam beds compared to the uncultivated sediments during all months sampled. At the clam beds, *nrfA* abundances were an order of magnitude higher than at the uncultivated sediments, with highest mean *nrfA* abundance observed at the clam site in July (3.56×10^8 gene copies g sed^{-1}). Strong significant relationships between functional gene abundances and process rates were

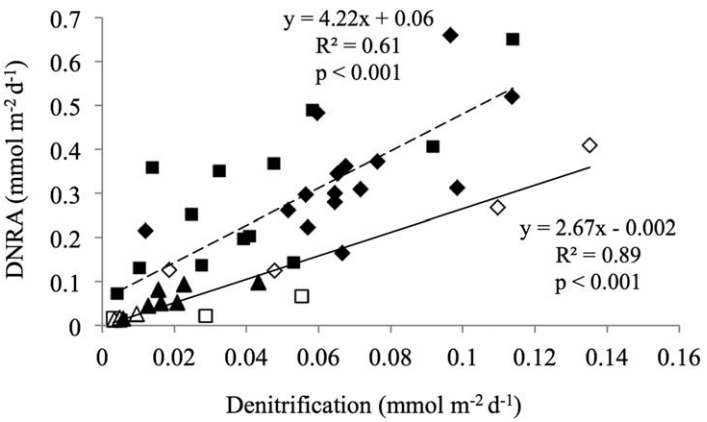

Fig. 5. Relationship of DNRA and denitrification (mmol m^{-2} d^{-1}) at clam sediments (black symbols) and uncultivated sediments (open symbols). Regression statistics include all seasons within each sediment type; dashed line is linear regression of clam beds and solid line is linear regression of uncultivated sediment. May, July, and November samples are shown as diamonds, squares, and triangles, respectively.

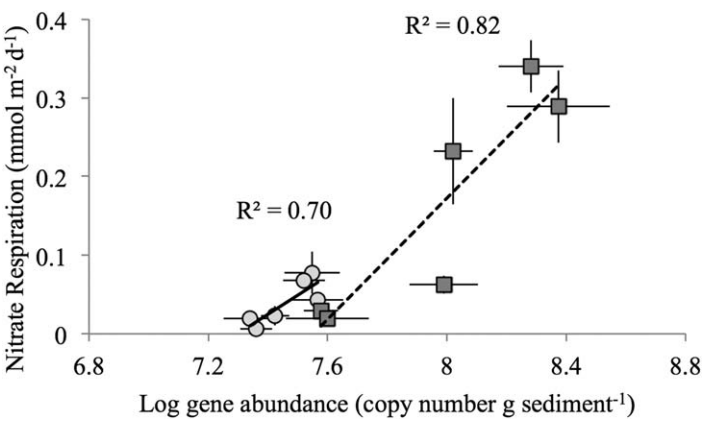

Fig. 6. Nitrate respiration rates (DNRA (squares) and denitrification (circles)) as a function of log-transformed *nrfA* and *nirS* gene abundance, respectively. Solid lines is linear regression of denitrification and *nirS*; dashed line is linear regression of DNRA and *nrfA*.

observed with *nirS* and *nrfA* abundances and denitrification and DNRA rates, respectively (Fig. 6).

Sediment N budget

The estimated sediment N budgets at the clam beds and uncultivated site are provided in Fig. 7. On an annual scale, assuming negligible rates in the winter, clam aquaculture increased denitrification by 1.2-fold compared to the uncultivated sites. However, clam aquaculture facilitated increased nutrient regeneration in the benthos through enhanced DNRA, microbial mineralization, and clam excretion, with the net NH_4^+ flux enhanced from -133 μmol m^{-2} yr^{-1} at the uncultivated sites to 2884 μmol m^{-2} yr^{-1} at the clam sites.

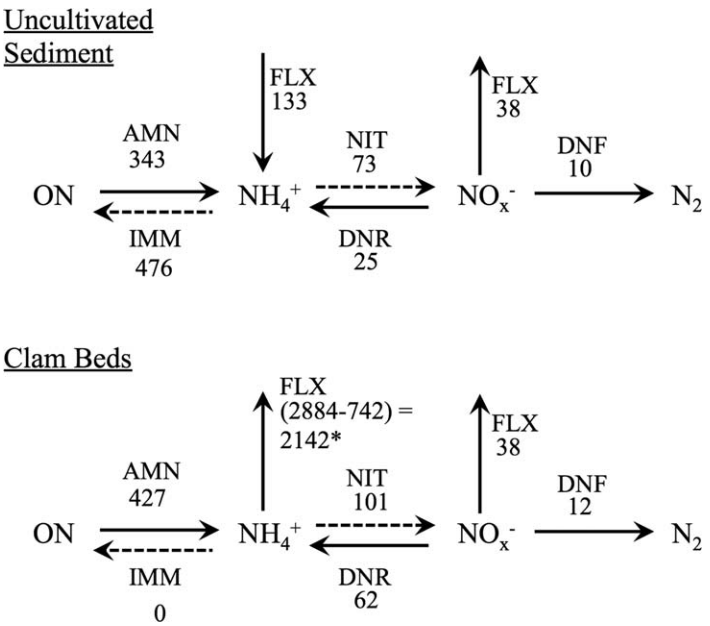

Fig. 7. Annual microbial N cycling rates (mmol N m^{-2} yr^{-1}) within the sediments/porewater at the uncultivated and clam sediments, including ammonification (AMN), nitrification (NIT), denitrification (DNF), DNRA (DNR), net fluxes of NH_4^+ and NO_x^- (FLX), and immobilization of NH_4^+ into microbial and benthic microalgal biomass (IMM). Solid arrows represent processes directly measured in this study while dashed lines represent calculated rates. Vertical arrows show exchanges between the sediment and water column. *clam excretion rates were estimated as described in the text and subtracted from the net NH_4^+ flux to determine the amount contributed by microbial processes at the clam sediments (2142); the discrepancy between this number and gross ammonification may be due to excretion by other infaunal organisms.

Discussion

Enhanced nutrient regeneration at clam beds

This study demonstrates that clam aquaculture significantly affects sediment N cycling rates, favoring retention rather than removal of N in shallow coastal ecosystems. Similar findings were reported in a previous study at this site, which measured in situ fluxes of nutrients and metabolism in clam beds of close to market size individuals ($\sim$ 40 mm shell length) and included a clam plus macroalgae treatment (Murphy et al. 2015). The present study builds on these findings by directly quantifying benthic microbial processes contributing to benthic N cycling (e.g., DNRA and denitrification). This study found generally lower net NH_4^+, SRP, and metabolic fluxes than Murphy et al. (2015), likely because sampling included sediments from clam beds with varying clam sizes (11.8–58.1 mm shell length), not just large individuals, which impacted the contribution of clam metabolism to overall benthic rates. Additionally, Murphy et al. (2015) reported net autotrophy at the uncultivated sediments while this study showed slightly heterotrophic control sediments, potentially due to the greater availability

of light in the field than in the lab. Increased benthic nutrient recycling processes resulted in elevated nutrient release from the clam sediments to the water column in both studies, which may serve as an important subsidy for local primary production in the ecosystem (Murphy et al. 2015).

By sampling intensively across a leased area, the present study captured the natural variability in metabolism and N transformation rates due to season and spatial differences in sediment properties as related to time-since planted and clam size. As a result we were able to scale our results across the farm to construct an annual sediment N budget for clam and uncultivated sediments, assuming negligible rates in the winter (not sampled). The sediment N budgets (Fig. 7) highlight the major difference between the clam and uncultivated sediments: clam sediments are a net source of regenerated nutrients to the water column while uncultivated sediments are a net sink for DIN. Although microbial ammonification rates were only slightly higher at the clam sediments, the fate of the NH_4^+ produced in the benthos differed between the two sediment types. During all seasons, NH_4^+ was released from the clam beds while either little release or uptake occurred in uncultivated sediments. High DIN efflux has previously been observed in infaunal bivalve-dominated sediments, particularly when bivalves are included in the incubations at both natural (e.g., Doering et al. 1987; Sandwell et al. 2009; Jones et al. 2011) and aquaculture settings (e.g., Bartoli et al. 2001; Nizzoli et al. 2006). In our study, this NH_4^+ was sourced from microbial mineralization of organic matter, DNRA, and clam excretion. Even after clam excretion was subtracted from the net NH_4^+ flux, our data showed little to no retention of microbial-derived DIN in the clam sediments perhaps due to reduced benthic microalgal activity.

Previous studies in shallow coastal bays located on the Eastern Shore of Virginia show that benthic microalgae (BMA) can take up much of the nitrogen produced by sediment microbial mineralization, provided that sufficient light is available (Anderson et al. 2003). Although benthic chlorophyll *a* concentrations were similar between the clam beds and uncultivated sediments, the sources were likely different. In fact, Secrist (2013) found that the bulk sediment Chl *a* biomass at Cherrystone clam beds was composed mainly of detrital macroalgal material as opposed to BMA (i.e., pennate diatoms). At the clam sites the predator-exclusion nets, which sit flush on the sediment surface and are colonized by thick macroalgal mats (Murphy et al. 2015), cause shading, decreasing BMA biomass (Secrist 2013). At the uncultivated sites the majority of the NH_4^+ produced was retained in the benthos (Fig. 7) indicating active BMA. In our study system calculated BMA N demand in the uncultivated sediments, which was estimated using methods described by Anderson et al. 2003 based on gross primary production corrected for autotrophic respiration and a C : N ratio of 9.0 (Sundback et al. 2000), was greater than the measured N mineralization rates in the sediments. Thus, BMA at the uncultivated sites could take up all mineralized N produced in the sediments.

Concurrent with net DIN release, clam aquaculture sediments generally had higher porewater nutrients, sulfide, and organic content than control sites, indicative of highly reduced conditions with limited oxygen penetration, similar to results of other studies that have characterized sediments associated with bivalve aquaculture (e.g., Mazouni et al. 1996; Christensen et al. 2003). Although infaunal clams are often reported to be important sediment bioturbators, allowing oxygen (and other solutes) to penetrate into the sediments (e.g., Welsh 2003), bioturbation is likely limited in these cultivated clam beds due to high clam densities and predator exclusion nets, which may limit movement and water exchange, further promoting sulfide accumulation and oxygen depletion. A recent study reported similar findings; clams (*Macoma baltica*) were associated with the accumulation of reduced metabolites such as sulfide in the sediments, resulting in low nitrification (Bonaglia et al. 2014). Surprisingly during May in Cherrystone, the uncultivated sediments had higher porewater nutrients, sulfide, and organic content than the clam sediments, concurrent with elevated mineralization and NO_3^- respiration rates. A likely explanation is that these sediments, adjacent to clam operations, experienced periodic pulses of organic matter deposition caused by aquaculture practices (e.g., sweeping the predator-exclusion nets of macroalgae and hydraulic clam harvesting).

DNRA exceeded denitrification at clam beds

Clam cultivation in Cherrystone Inlet tended to have enhanced DNRA and denitrification compared to uncultivated sediments, although rates were low overall compared to other rates, such as clam excretion and gross ammonification (Fig. 7). The ratio of DNRA to denitrification was significantly higher at clam beds than the uncultivated sediments. The contribution of DNRA to total NO_3^- respiration, which averaged 82% in the clam sediments, is on the very high-end compared to other estuarine systems, which typically range from 0% to 60% (Tobias et al. 2001; Burgin and Hamilton 2007; Koop-Jakobsen and Giblin 2010) but can be up to 98% (as reviewed in Song et al. 2013). Although a recent synthesis, which compared denitrification and DNRA rates across 55 coastal sites, reported DNRA was the dominant pathway at more than one-third of the sites (Giblin et al. 2013). In the current study, these trends were corroborated with observed functional gene abundances in Cherrystone sediments; *nrf*A was significantly higher in the clam sediments, whereas *nirS* abundances were similar across sediment types and months. Despite the fact that *nrf*A is known to be present in diverse genera of bacteria, capable of a variety of metabolic pathways (Mohan et al. 2004), in Cherrystone sediments *nrfA* abundance was significantly correlated with DRNA rates. This strong relationship indicates that the abundance of DNRA bacteria may be an important microbial

control on the process and, thus, serve as a genetic proxy for DNRA potential (Song et al. 2014).

A number of environmental factors may explain why clam aquaculture favors DNRA and *nrf*A abundance over denitrification and *nir*S abundance. Both DNRA and denitrification depend on concentrations of available electron donors (typically organic carbon) and the electron acceptor, NO_3^- (as reviewed in Seitzinger 2006; Burgin and Hamilton 2007; Kraft et al. 2014), with DNRA dominating when the ratio of labile carbon to NO_3^- is high (Tiedje et al. 1982; Burgin and Hamilton 2007; Ferrón et al. 2009; Algar and Vallino 2014; Hardison et al. 2015). The availability of labile organic carbon, delivered as clam biodeposits to sediments, in Cherrystone Inlet is likely high. Despite the fact that clam beds had similar porewater DOC, sediment organic matter, and sediment C:N as the uncultivated sediments, these measurements are of bulk sediment C and may not provide insight into C quality. Previous laboratory studies have shown that bivalve biodeposits degrade quickly (Giles and Pilditch 2006; Carlsson et al. 2010; Jansen et al. 2012). Additionally, ammonification rates, which may serve as an indicator of C quality and availability, were positively, although not significantly, correlated with the proportion of DNRA to denitrification. At the same time, low water column NO_3^- concentration indicates that both sediment denitrification and DNRA were strongly reliant on nitrification for NO_3^- supply, as our data show that the majority of both DNRA and denitrification were coupled to nitrification ($DNRA_n$ and D_n, respectively). NO_x produced by nitrification was low, compared to NH_4^+ production, in both clam and uncultivated sediments, calculated as 100.6 mmol N m^{-2} yr^{-1} and 73.0 mmol N m^{-2} yr^{-1}, respectively (Fig. 7). Nitrification is generally inhibited by anaerobic and sulfidic conditions (Joye and Hollibaugh 1995). Thus, both DNRA and denitrification in these systems may be regulated by oxygen penetration depth and sulfide concentrations, particularly in warm summer months when clam biodeposition and microbial respiration rates are high. Although one might expect complete inhibition of nitrification at the porewater sulfide concentrations observed in clam sediments in July, we did see a small efflux of NO_3^- suggesting incomplete inhibition. Sulfide samples were collected across a bulk sediment horizon, with the sampler window centered approximately 5-7cm from the sediment surface, and, therefore, do not reflect the sulfide concentrations at the zone of nitrification, which is likely within the top few millimeters of the sediments.

In addition to suppressing nitrification, sulfide may directly enhance DNRA relative to denitrification. Chemolithotrophic DNRA bacteria are capable of oxidizing reduced forms of sulfur, including free sulfide and elemental sulfur, while reducing NO_3^- to NH_4^+ (Brunet and Garcia-Gil 1996; Otte et al. 1999). Additionally the final two steps in denitrification (i.e., NO to N_2O and N_2O to N_2) may be directly inhibited under sulfidic conditions (Sorensen et al. 1980; Brunet and Garcia-Gil 1996). Thus the highly sulfidic sediments associated with clam aquaculture promote DNRA over N_2 production.

Although clam cultivation in Cherrystone Inlet tends to favor DNRA, the overall effect of bivalves on NO_3^- respiration rates is quite variable and highly dependent on the environment and type of bivalve. Some studies report denitrification enhancement in bivalve-dominated sediments compared to reference locations (Kellogg et al. 2013; Smyth et al. 2013; Turek and Hoellein 2015) while other studies show no difference in denitrification across sediment types (Christensen et al. 2003; Jones et al. 2011; Higgins et al. 2013). Still other studies report spatial and/or temporal variability on the effects of bivalves on sediment denitrification (Nizzoli et al. 2006; Carlsson et al. 2012) and recently a study reported a decrease in denitrification in the presence of high clam abundance (Bonaglia et al. 2014). Cherrystone Inlet clam aquaculture had lower rates of denitrification than those observed in other bivalve studies, including those in nearby tributaries of the Chesapeake Bay (e.g., Higgins et al. 2013; Kellogg et al. 2013), although these focused on oyster-dominated systems. DNRA rates in clam beds in Cherrystone Inlet were comparable to previously reported rates associated with cultivated infaunal clams in the Sacca di Goro, Italy (Nizzoli et al. 2006). Most similar studies did not measure DNRA and therefore the ability to determine the mechanisms by which bivalves influence NO_3^- respiration in these studies is limited.

To place the enhanced DIN regeneration at the clam beds into context, we compared it to an estimate of the amount of N removed from the system via clam harvest, assuming an average harvested clam to be 45.7 mm in length (littleneck size; 0.87 g DW, of which 13.1% is N (A.E. Murphy, unpubl. data) and 2 yr to reach market size. Thus, at a density of 700 individuals m^{-2}, approximately 2.85 mol N m^{-2} yr^{-1} is removed through harvest, comparable to the estimated annual NH_4^+ regenerated from the sediments (2.88 mol N m^{-2} yr^{-1}) (Fig. 7). Although requiring higher spatial and temporal resolution, this exercise demonstrates the importance of considering N regeneration processes when estimating the total N removed from a bivalve cultivation system, particularly in systems where this enhanced N recycling may promote local eutrophication. For example, in Cherrystone Inlet the fate of these regenerated nutrients has been shown to promote macroalgal production (Murphy et al. 2015).

Clam aquaculture is a growing industry on the Eastern Shore of Viriginia (Emery 2015) and globally (FAO 2014). As this coastal anthropogenic activity expands, an understanding of how it alters ecosystem functioning such as benthic nutrient cycling is necessary to prevent overexploitation and ecosystem degradation. Numerous studies have suggested suspension-feeding bivalves may promote denitrification and thus serve an important function in reducing bioavailable N and subsequently eutrophication (e.g., Rose et al. 2014).

However, this study, which is, to our knowledge, the first to measure sediment N cycling processes associated with clam aquaculture in the United States, highlights that DNRA can outcompete denitrification in areas of intensive clam farming, promoting DIN turnover and release from the sediments to the water column.

References

Algar, C. K., and J. J. Vallino. 2014. Predicting microbial nitrate reduction pathways in coastal sediments. Aquat. Microb. Ecol. **71**: 223–238. doi:10.3354/ame01678

Anderson, I. C., C. R. Tobias, B. B. Neikirk, and R. L. Wetzel. 1997. Development of a process-based nitrogen mass balance model for a Virginia (USA). Mar. Ecol. Prog. Ser. **159**: 13–27. doi:10.3354/meps159013

Anderson, I. C., K. J. McGlathery, and A. C. Tyler. 2003. Microbial mediation of "reactive" nitrogen transformations in a temperate lagoon. Mar. Ecol. Prog. Ser. **246**: 73–84. doi:10.3354/meps246073

Bartoli, M., D. Nizzoli, P. Viaroli, E. Turolla, G. Castaldelli, E. A. Fano, and R. Rossi. 2001. Impact of Tapes philippinarum farming on nutrient dynamics and benthic respiration in the Sacca di Goro. Hydrobiologia **455**: 203–212. doi:10.1023/A:1011910422400

Braker, G., A. Fesefeldt, and K. P. Witzel. 1998. Development of PCR primer systems for amplification of nitrite reductase genes (nirK and nirS) to detect denitrifying bacteria in environmental samples. Environ. Microbiol. **64**: 3769–3775.

Bricker, S. B., K. C. Rice, and O. P. Bricker. 2014. From headwaters to coast: Influence of human activities on water quality of the Potomac river estuary. Aquat. Geochem. **20**: 291–323. doi:10.1007/s10498-014-9233-z

Brooks, P. D., J. M. Stark, B. B. McInteer, and T. Preston. 1989. Diffusion method to prepare soil extracts for automated nitrogen-15 analysis. Soil Sci. Soc. Am. Proc. **53**: 1707–1711. doi:10.2136/sssaj1989.03615995005300060016x

Brunet, R. C., and L. J. Garcia-Gil. 1996. Sulfide-induced dissimilatory nitrate reduction to ammonia in anaerobic freshwater sediments. FEMS Microbiol. Ecol. **21**: 131–138. doi:10.1111/j.1574-6941.1996.tb00340.x

Burgin, A. J., and S. K. Hamilton. 2007. Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways. Front. Ecol. Environ. **5**: 89–96. doi:10.1890/1540-9295(2007)5[89:HWOTRO]2.0.CO;2

Burkholder, J. M., and S. E. Shumway. 2011. Bivalve shellfish aquaculture and eutrophication, p. 155–215. *In* S. E. Shumway [ed.], Shellfish and the environment. Wiley.

Campbell, B., and D. Pauly. 2013. Mariculture: A global analysis of production trends since 1950. Mar. Pol. **39**: 94–100. doi:10.1016/j.marpol.2012.10.009

Carlsson, M. S., R. N. Glud, and J. K. Petersen. 2010. Degradation of mussel (Mytilus edulis) fecal pellets released from hanging long-lines upon sinking and after settling at the sediment. Can. J. Fish. Aquat. Sci. **67**: 1376–1387.

Carlsson, M. S., P. Engström, O. Lindahl, L. Ljungqvist, J. K. Petersen, L. Svanberg, and M. Holmer. 2012. Effects of mussel farms on the benthic nitrogen cycle on the Swedish west coast. Aquac. Environ. Interact. **2**: 177–191.

Castagna, M., and J. N. Kraeuter. 1981. Manual for growing the hard clam Mercenaria. Special report in applied marine science and ocean engineering (249). Virginia Institute of Marine Science, Gloucester Point.

Christensen, P. B., R. N. Glud, T. Dalsgaard, and P. Gillespie. 2003. Impacts of longline mussel farming on oxygen and nitrogen dynamics and biological communities of coastal sediments. Aquaculture **218**: 567–588. doi:10.1016/S0044-8486(02)00587-2

Condon, E. D. 2005. Physiological ecology of the cultured hard clam, *Mercenaria mercenaria*: A case study in Cherrystone Inlet, Virginia. M.Sc. thesis. Virginia Institute of Marine Science, College of William & Mary.

Dalsgaard, T., L. P. Nielsen, V. Brotas, and P. Viaroli. 2000. Protocol handbook for NICE-Nitrogen Cycling in Estuaries: A project under the EU research programme: Marine Science and Technology (MAST III).

Dame, R. 2012. Ecology of marine bivalves: An ecosystem approach, 2nd ed. CRC Press.

Doering, P. H., J. R. Kelly, C. A. Oviatt, and T. Sowers. 1987. Effect of the hard clam Mercenaria mercenaria on benthic fluxes of inorganic nutrients and gases. Mar. Biol. **94**: 377–383. doi:10.1007/BF00428243

Emery, K. 2015. Coastal bivalve aquaculture carbon cycling, spatial distribution and resource use in Virginia, USA and Baja California, Mexico. M.Sc. thesis. Univ. of Virginia.

FAO. 2014. The state of the world fisheries and aquaculture 2014. Food and Agricultural Organization of the United Nations, Rome.

Ferrón, S., T. Ortega, and J. M. Forja. 2009. Mar. Chem. **113**: 50–62.

Gibbs, M., G. Funnell, and S. Pickmere. 2005. Benthic nutrient fluxes along an estuarinegradient: Influence of the pinnid bivalve Atrina zelandica in summer. Mar. Ecol. Prog. Ser. **288**: 151–164. doi:10.3354/meps288151

Giblin, A., C. Tobias, B. Song, N. Weston, G. Banta, and V. Rivera-Monroy. 2013. The importance of dissimilatory nitrate reduction to ammonium (DNRA) in the nitrogen cycle of coastal ecosystems. Oceanography **26**: 124–131. doi:10.5670/oceanog.2013.54

Giles, H., and C. A. Pilditch. 2006. Effects of mussel (Perna canaliculus) biodeposit decomposition on benthic respiration and nutrient fluxes. Mar. Biol. **150**: 261–271. doi:10.1007/s00227-006-0348-7

Hammen, C. S. 1980. Marine invertebrates. University Press of New England.

Hardison, A. K., C. K. Algar, A. E. Giblin, and J. J. Rich. 2015. Infuence of organic carbon and nitrate loading on

partitioning between dissimilaroty nitrate reduction to ammonium (DNRA) and N2 production. Geochim. Cosmochim. Acta **164**: 146–160. doi:10.1016/j.gca.2015.04.049

Henriksen, K., M. B. Rasmussen, and A. Jensen. 1983. Effect of bioturbation on microbial nitrogen transformations in the sediment and fluxes of ammonium and nitrate to the overlaying water. Ecol. Bull. **35**: 193–205.

Higgins, C. B., C. Tobias, M. F. Piehler, A. R. Smyth, R. F. Dame, K. Stephenson, and B. L. Brown. 2013. Effect of aquacultured oyster biodeposition on sediment N_2 production in Chesapeake Bay. Mar. Ecol. Prog. Ser. **473**: 7–27. doi:10.3354/meps10062

Hiwatari, T., K. Kohata, and A. Iijima. 2002. Nitrogen Budget of the Bivalve Mactra veneriformis, and its Significance in Benthic—pelagic Systems in the Sanbanse Area of Tokyo Bay. Estuar. Coast. Shelf Sci. **55**: 299–308. doi:10.1006/ecss.2001.0905

Jansen, H. M., M. C. J. Verdegem, Ø. Strand, and A. C. Smaal. 2012. Seasonal variation in mineralization rates (C-N-P-Si) of mussel Mytilus edulis biodeposits. Mar. Biol. **159**: 1567–1580. doi:10.1007/s00227-012-1944-3

Jones, H. F. E., C. A. Pilditch, D. A. Bruesewitz, and A. M. Lohrer. 2011. Sedimentary environment influences the effect of an infaunal suspension feeding bivalve on estuarine ecosystem function. PLoS One **6**: e27065. doi:10.1371/journal.pone.0027065

Joye, S. B., and J. T. Hollibaugh. 1995. Influence of sulfide inhibition of nitrification on nitrogen regeneration in sediments. Science **270**: 623–625. doi:10.1126/science.270.5236.623

Kana, T. M., C. Darkangelo, M. D. Hunt, J. B. Oldham, G. E. Bennett, and J. C. Cornwell. 1994. Membrane inlet mass spectrometer for rapid high-precision determination of N_2, O_2, and Ar in environmental water samples. Anal. Chem. **66**: 4166–4170. doi:10.1021/ac00095a009

Kellogg, M. L., J. C. Cornwell, and M. S. Owens. 2013. Denitrification and nutrient assimilation on a restored oyster reef. Mar. Ecol. Prog. Ser. **480**: 1–19. doi:10.3354/meps10331

Kellogg, M. L. 2014. Use of oysters to mitigate eutrophication in coastal waters. Estuar. Coast. Shelf Sci. **151**: 156–168.

Knepel, K., and K. Bogren. 2001. Determination of orthophosphorous by flow injection analysis in seawaters: QuickChem Method 31-115-01-1-H, *In* Saline Methods of Analysis.

Koop-Jakobsen, K., and A. E. Giblin. 2010. The effect of increased nitrate loading on nitrate reduction via denitrification and DNRA in salt marsh sediments. Limnol. Oceangr. **55**: 789–802. doi:10.4319/lo.2009.55.2.0789

Kraft, B., and others. 2014. The environmental controls that govern the end product of bacterial nitrate respiration. Science **345**: 676–679. doi:10.1126/science.1254070

Lindahl, O., and others. 2005. Improving marine water quality by mussel farming: A profitable solution for Swedish society. Ambio **34**: 131–138. doi:10.1579/0044-7447-34.2.131

Magni, P., S. Montani, C. Takada, and H. Tsutsumi. 2000. Temporal scaling and relevance of bivalve nutrient excretion on a tidal flat of the Seto Inland Sea, Japan. Mar. Ecol. Prog. Ser. **198**: 139–155. doi:10.3354/meps198139

Mazouni, N., J.-C. Gaertner, J.-M. Deslous-Paoli, S. Landrein, and M. Geringer d'Oedenberg. 1996. Nutrient and oxygen exchanges at the water-sediment interface in a shellfish farming lagoon (Thau, France). J. Exp. Mar. Bio. Ecol. **205**: 91–113. doi:10.1016/S0022-0981(96)02594-4

Mohan, S. B., M. Schmid, M. Jetten, and J. Cole. 2004. Detection and widespread distribution of the nrfA gene encoding nitrite reduction to ammonia, a short circuit in the biological nitrogen cycle that competes with denitrification. FEMS Microbiol. Ecol. **49**: 433–443. doi:10.1016/j.femsec.2004.04.012

Mosier, A. C., and C. A. Francis. 2010. Denitrifier abundance and activity across the San Francisco Bay estuary. Environ. Microbiol. Rep. **2**: 667–676. doi:10.1111/j.1758-2229.2010.00156.x

Murphy, A. E., I. C. Anderson, and M. W. Luckenbach. 2015. Enhanced nutrient regeneration at commercial hard clam (Mercenaria mercenaria) beds and the role of macroalgae. Mar. Ecol. Prog. Ser. **530**: 135–151. doi:10.3354/meps11301

Neubauer, S. C., and I. C. Anderson. 2003. Transport of dissolved inorganic carbon from a tidal freshwater marsh to the York River estuary. Limnol. Oceangr. **48**: 299–307. doi:10.4319/lo.2003.48.1.0299

Newell, R. I. 2004. Ecosystem influences of natural and cultivated populations of suspension-feeding bivalve molluscs: A review. J. Shellfish Res. **23**: 51–62.

Nielsen, L. P. 1992. Denitrification in sediment determined from nitrogen isotope pairing. FEMS Microbiol. Lett. **86**: 357–362. doi:10.1016/0378-1097(92)90800-4

Nizzoli, D., D. T. Welsh, E. A. Fano, and P. Viaroli. 2006. Impact of clam and mussel farming on benthic metabolism and nitrogen cycling, with emphasis on nitrate reduction pathways. Mar. Ecol. Prog. Ser. **315**: 151–165. doi:10.3354/meps315151

Otte, S., J. G. Kuenen, L. P. Nielsen, and H. W. Paerl. 1999. Nitrogen, carbon, and sulfur metabolism in naturalthioploca samples. Appl. Environ. Microbiol. **65**: 3148–3157. doi:10388716

Petersen, J. K., B. Hasler, K. Timmermann, and P. Nielsen. 2014. Mussels as a tool for mitigation of nutrients in the marine environment. Mar. Pollut. Bull. **82**: 137–143. doi:10.1016/j.marpolbul.2014.03.006

Risgaard-Petersen, N., and S. Rysgaard. 1995. Nitrate reduction in sediments and waterlogged soil measured by 15N techniques, p. 1–13. *In* Methods in applied soil microbiology. Academic Press.

Rose, J. M., S. B. Bricker, M. A. Tedesco, and G. H. Wikfors. 2014. A role for shellfish aquaculture in coastal nitrogen

management. Environ. Sci. Technol. **48**: 2519–2525. doi: 10.1021/es4041336

Rysgaard, S., N. Risgaard-Petersen, L. P. Nielsen, and N. P. Revsbech. 1993. Nitrification and Denitrification in Lake and Estuarine Sediments Measured by the 15N Dilution Technique and Isotope Pairing. Appl. Environ. Microbiol. **59**: 2093–2098.

Sandwell, D. R., C. A. Pilditch, and A. M. Lohrer. 2009. Density dependent effects of an infaunal suspension-feeding bivalve (Austrovenus stutchburyi) on sandflat nutrient fluxes and microphytobenthic productivity. J. Exp. Mar. Biol. Ecol. **373**: 16–25. doi:10.1016/j.jembe.2009.02.015

Secrist, R. G. 2013. Food availability and utilization for cultured hard clams. M.Sc. thesis. Virginia Institute of Marine Science, College of William & Mary.

Seitzinger, S. 2006. Denitrification across landscapes and waterscapes: A synthesis. Ecol. Appl. **16**: 2064–2090. doi: 17205890

Smyth, A. R., N. R. Geraldi, and M. F. Piehler. 2013. Oyster-mediated benthic-pelagic coupling modifies nitrogen pools and processes. Mar. Ecol. Prog. Ser. **493**: 23–30. doi: 10.3354/meps10516

Song, G. D., S. M. Liu, H. Marchant, M. M. M. Kuypers, and G. Lavik. 2013. Anammox, denitrification and dissimilatory nitrate reduction to ammonium in the East China Sea sediment. Biogeosciences **10**: 6851–6864. doi:10.5194/bg-10-6851-2013

Song, B., J. A. Lisa, and C. R. Tobias. 2014. Linking DNRA community structure and activity in a shallow lagoonal estuarine system. Front. Microbiol. **5**: 460.

Sorensen, J., J. M. Tiedje, and R. B. Firestone. 1980. Inhibition by sulfide of nitric and nitrous oxide reduction by denitrifying Pseudomonas fluorescens. Appl. Environ. Microbiol. **39**: 105–108. doi:6766699

Stadmark, J., and D. J. Conley. 2011. Mussel farming as a nutrient reduction measure in the Baltic Sea: Consideration of nutrient biogeochemical cycles. Mar. Pollut. Bull. **62**: 1385–1388. doi:10.1016/j.marpolbul.2011.05.001

Tiedje, J. M. 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium, p. 179–244. *In* A. Zehnder [ed.]. Biology of anaerobic microorganisms.

Tiedje, J. M., A. J. Sexstone, D. D. Myrold, and J. A. Robinson. 1982. Denitrification: Ecological niches, competition and survival. Antonie Leewenhoek J. Microbiol. **48**: 569–583. doi:6762848

Tobias, C. R., I. C. Anderson, and E. A. Canuel. 2001. Nitrogen cycling through a fringing marsh-aquifer ecotone. Mar. Ecol. Prog. Ser. **210**: 25–39. doi:10.3354/meps210025

Turek, K. A., and T. J. Hoellein. 2015. The invasive Asian clam (Corbicula fluminea) increases sediment denitrification and ammonium flux in 2 streams in the midwestern USA. Freshw. Sci. **34**: 472–484. doi:10.1086/680400

Welsh, D. T. 2003. It's a dirty job but someone has to do it: The role of marine benthic macrofauna in organic matter turnover and nutrient recycling to the water column. Chem. Ecol. **19**: 321–342. doi:10.1080/0275754031000155474

Welsh, A., J. C. Chee-Sanford, L. M. Connor, F. E. Loffler, and R. A. Sanford. 2014. Refined NrfA Phylogeny Improves PCR-Based nrfA Gene Detection. Appl. Environ. Microbiol. **80**: 2110–2119. doi:10.1128/AEM.03443-13

Welsh, D. T., D. Nizzoli, E. A. Fano, and P. Viaroli. 2015. Direct Contribution of clams (*Ruditapes philippinarum*) to benthic fluxes, nitrification, denitrification, and nitrous oxide emission in a farmed sediment. Estuar. Coast. Shelf Sci. **154**: 84–93. doi:10.1016/j.ecss.2014.12.021

Wessel, W. W., and A. Tietema. 1992. Calculating gross N transformation rates of 15N pool dilution experiments with acid forest litter: Analytical and numberical approaches. Soil Biol. Biochem. **24**: 931–942. doi:10.1016/0038-0717(92)90020-X

Acknowledgments

Many thanks to the aquaculturists who allowed us access to their lease; Jennifer Stanhope and Hunter Walker for logistics and laboratory support; PG Ross, Edward Smith, Alan Birch, Sean Fate, Paige Smith, and Linda Ward for their endless support and assistance in the field and lab. Discussions with Mark Brush, Lisa Kellogg, and Anne Giblin greatly improved this manuscript. This work was supported by Virginia Sea Grant (NA10OAR4170085, #R/71515W), The National Science Foundation GK12 Fellowship (NSF DGE-0840804), NSF Grant (OCE1321373), NSF Virginia Coast Reserve Long Term Ecological Research (DEB-0621014, DEB-1237733), and the Strategic Environmental Research and Development Program – Defense Coastal/Estuarine Research Program Project SI-1413. This paper is Contribution No. of the Virginia Institute of Marine Science, The College of William and Mary.

Submitted 21 August 2015
Revised 25 January 2016; 8 March 2016
Accepted 15 March 2016

Associate editor: Bo Thamdrup