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research paper

# Evaluating the impact of oyster (*Crassostrea virginica*) gardening on sediment nitrogen cycling in a subtropical estuary

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ABSTRACT.—To quantify the effect of oysters on sediment N cycling, oyster-free cages and cages with adult or juvenile oysters [*Crassostrea virginica* (Gmelin, 1791)] were suspended above the sediments at two sites in Mobile Bay, Alabama, located in the northern Gulf of Mexico. While hydrogen sulfide ($HS^-$) was below detection limits at Site 2, $HS^-$ exceeded 500 µM prior to the deployment at Site 1 and remained detectable in sediments in the oyster treatments. Oyster mediated N inputs were estimated at 11.4 (SE 0.4) g N $m^{-2}$ and 3.2 (SE 0.2) g N $m^{-2}$ in the juvenile and adult treatments, respectively. The abundances of genes associated with denitrifiers (*nirK)*, sulfate reducers (*dsrB*), and bacterial and archaeal nitrifiers (*bac_amoA*, *arc_amoA*, respectively) varied over the course of the study, but were not affected by the treatments. Similarly, potential denitrification rates measured during the study were similar in all treatments. Net $N_2$ fluxes, determined from $N_2$:Ar ratios using a membrane inlet mass spectrometer were similar among treatments, despite significantly higher sediment chlorophyll-*a* content in the juvenile treatment. We conclude that the commonly assumed enhanced rates of $N_2$ loss from sediments in response to deployment of oysters is not guaranteed and may depend on site-specific biogeochemistry.

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Eutrophication negatively affects the structure and function of many nearshore marine ecosystems (Nixon 1995, Cloern 2001, Howarth and Marino 2006). Excess nutrient input to estuaries leads to higher phytoplankton biomass, resulting in lower light at the benthos (Gallegos and Jordan 2002, Kemp et al. 2005). Benthic light limitation contributes to the decline of submerged aquatic vegetation in impacted ecosystems (Kemp et al. 2005, Waycott et al. 2009). In addition, higher primary production in the water column leads to greater organic matter deposition to the

sediments (Nixon 1981, Heip et al. 1995, Kemp et al. 1997) and lower dissolved oxygen due to mineralization (Kemp et al. 1992, Rabalais et al. 1996, Hagy et al. 2004), which further impacts the benthic community (Rosenberg et al. 1990). Mitigation strategies intended to lower the input of land-derived nutrients to coastal regions have been increasingly explored and implemented (Kemp et al. 2005, Paerl et al. 2006). Reduction of point source inputs of nutrients to ecosystems can ameliorate some symptoms of eutrophication (Conley et al. 2000, Carstensen et al. 2006). In instances when non-point sources account for a large fraction of nutrient input to an estuary, mitigation strategies to lower nutrient concentrations to desired levels remain problematic (Butt and Brown 2000, Boesch 2002).

One option is the use of commercially harvestable bivalves to lower the suspended particle load (Officer et al. 1982, Fulford et al. 2007) and, through harvesting, remove nitrogen (N) from the system via assimilation into soft tissues during growth (Newell 2004, Gifford et al. 2005). This strategy relies on the ability of bivalves to lower phytoplankton biomass via high filtration rates (Newell 1988, Dame and Prins 1997, Nelson et al. 2004, Grizzle et al. 2008, Carlsson et al. 2012). The potential for such efforts to reduce suspended particle loads and diminish nutrient loads near oyster reefs and large scale aquaculture sites prompted calls for restoration of oyster reefs for economic benefit and as a companion strategy to point source reductions to remediate symptoms of nutrient enrichment in estuaries (Breitburg et al. 2000, Cerco and Noel 2007, Carmichael et al. 2012).

In addition to N removal through harvest, bivalves alter organic input to the sediments and contribute to N removal through changes that occur in sediment biogeochemical cycles (Newell et al. 2002, Nizzoli et al. 2006, Carlsson et al. 2012). Bivalves sort and package lower quality particles into psuedofeces that are aggregated with a mucus layer and rejected prior to ingestion (Newell and Jordan 1983). Psuedofeces, along with feces, have fast sinking velocities and reach the benthos much faster than non-aggregated and suspended particles (Widdows et al. 1998). Newell et al. (2002) experimentally showed that high input of pelletized phytoplankton (simulating pseudofeces) altered sediment biogeochemistry increasing the contribution of bivalves to N removal. With input of organic matter, dissimlatory reduction of nitrate to di-nitrogen gas (denitrification) increased, resulting in additional loss of N from the system (Newell et al. 2002). Higher oxygen consumption as a result of aerobic respiration of biodeposits can enhance ammonium flux to the water column (Newell et al. 2002, Christensen et al. 2003, Carlsson et al. 2012). Newell et al. (2004) suggested that this resulting enhancement in denitrification was an additive factor that needed to be considered when calculating net N removal from the system by oysters.

At moderate inputs of organic matter to oxic sediments, the increased production of ammonium is accompanied by higher nitrification rates, a process which supplies the substrate (nitrate) used during denitrification. However, as organic loads to the sediments increase, consumption of oxygen in the sediments can result in a diminished oxic layer and oxygen limitation of nitrification. This reduction in nitrification results in lower rates of coupled nitrification-denitrification, and mineralized N is instead mainly returned to the water column in the form of ammonium (Carlsson et al. 2012). The enhancement of N removal by denitrification in the vicinity of oyster reefs was apparent in a temperate estuary (Piehler and Smyth 2011) where higher rates of denitrification were measured in oyster reef mudflats compared to intertidal sediments, seagrass beds, and marshes. Increased rates of denitrification were also

observed adjacent to natural oyster reefs compared to more distant sediment (15–20 m); however, rates could have been higher due to better oxygen penetration in the shelly sediment rather than due to biodeposits (Hoellein et al. 2015). Lower rates of denitrification also have been associated with aquaculture compared to natural oyster reefs (Higgens et al. 2013, reviewed in Kellogg et al. 2014). The high degree of spatial and temporal variability among measured effects of bivalves on sediment biogeochemistry suggests some mediation by site-specific attributes that are not fully understood (Hatcher et al. 1994, Christensen et al. 2003, Nizzoli et al. 2006, Carlsson et al. 2012).

In the present study, we examined the impact of oysters on sediment N cycling at two sites in Mobile Bay, Alabama, a subtropical estuary. Mobile Bay has extensive oyster beds exceeding 2040 hectares (May 1971) with annual mean (1954–2000) commercial landings estimated at 385,554 kg of oyster meat (Gregalis et al. 2008). The two sites were selected in consultation with the Mobile Bay Oyster Gardening Program, an education and restoration volunteer program that relies on "gardeners" to grow oysters for restoration enhancement efforts in Mobile Bay (http://masgc.org/oyster/). Oyster spat are supplied to the gardeners in June of each year who then maintain the oysters off their dock in suspended cages and conduct weekly removal of predators and algae from the cages. Oysters are collected from the gardeners in November and transported to restoration and enhancement sites within Mobile Bay and field-planted. This program has successfully produced over 500,000 oysters for restoration purposes (http://masgc.org/oyster/). If the oysters also contributed to N removal during the growing season by stimulating denitrification in sediments, there would be an additional benefit to the program.

We deployed juvenile and harvest-sized adult oysters during a 4-mo period and examined abundances of genes associated with key microbial groups in the N cycle along with potential denitrification rates and benthic N fluxes. We predicted that biodeposits from oysters would increase N to sediments and stimulate denitrification compared to sediment without oysters. While the gardening program focuses on growing spat through one summer, gardening adult oysters could result in higher rates of denitrification due to the production of more biodeposits (Carmichael et al. 2012). As the juveniles grew throughout the year, we predicted that their effect on denitrification would increase to levels comparable to that of adults.

## Methods

Sampling Sites.—Mobile Bay is a shallow (mean depth = 3 m), 50 km long and 17–38 km wide subtropical estuary located in Alabama, USA (Schroeder 1977). It has a surface area of 1060 $km^2$ and receives fresh water from the Mobile River with an average discharge rate of 1914 $m^3\ s^{-1}$ (Ward et al. 2005). Two sites were selected off Fort Morgan Peninsula in the bay. These two sites were 2 km apart and are typically subject to similar environmental conditions (salinity, temperature, and dissolved oxygen). Sites 1 (30°16′57″N, 87°45′5″W) and 2 (30°15′14″N, 87°49′22″W) had depths of 1 m at the locations where the cages were deployed.

Experimental Setup.—In June 2011, mesh cages 15 cm tall covering an area of 1.67 $m^2$ were suspended at 0.2 m above the sediment surface under pilings extending approximately 30 m off the shoreline into Mobile Bay, similar to work conducted

by Fertig et al. (2009). At each site, one oyster-free cage served as control, while one cage containing 600 adult oysters [shell length: 98.0 (SE 1.3) mm], and another one containing 1800 juvenile oysters [shell length: 42 (SE 0.3) mm] served as the adult and juvenile treatments, respectively. Treatments were separated by approximately 1 m. The starting density of oysters was 1078 individuals $m^{-2}$ for juveniles and 359 individuals $m^{-2}$ for adults. At each site, in situ water temperature (°C), salinity, and dissolved oxygen (mg $L^{-1}$) were measured biweekly with a calibrated handheld YSI-85 meter. Oyster survival was determined by counting and removing dead oysters after measuring water conditions.

DNA Extraction and Quantitative PCR.—In April 2011, prior to the deployment of the oysters, triplicate sediment cores (0–5 cm) were collected by hand with a cutoff syringe corer (1.5 cm diameter) for analysis. In June, July, August, and September triplicate surface sediment cores were collected from underneath the cages in each treatment. DNA was extracted from 1 g of sediment with phenol:chloroform method (Wilson 1987). Gene copy numbers were measured using Agilent Stratagene MX3500P quantitative PCR instrument and GoTaq qPCR Master Mix (Promega). Several genes were used to characterize N cycling along with sulfide production. Quantified genes included: archaeal and bacterial *amoA* (nitrification), *nirK* (denitrification), and *dsrB* (sulfate reduction). For each gene, a standard curve was used to determine the gene copy number. This curve was generated using a purified plasmid containing the target of interest. Each standard curve and sediment DNA sample was run in triplicate to ensure quality of estimates. Dissociation curves were included to ensure the correct product was quantified. Triplicate estimates for each sample were averaged and the three separate cores from each time point were then averaged to obtain a single estimate for each time point for each site and treatment. The primers used for archeal *amoA*, bacterial *amoA*, *nirK*, and *dsrB* were aramoAF/R (Francis et al. 2005), amoA1F/2R (Rotthauwe et al. 1997), Flacu/R3cu (Hallin and Lindgren 1999), and dsr1F/dsr4R (Wagner et al. 1998) were used for *nirK* and *dsrB*, respectively. Thermal cycling for *amoA* and *nirK* consisted of 95 °C 5 min, 40 cycles of 95 °C 30 s, 53 °C 1 min, 72 °C 1 min, and for *dsrB*, 95 °C 5 min, 40 cycles of 95 °C 30 s, 58 °C 40 s.

Increases and decreases in the abundance of genes detected using qPCR were used as a proxy for the organisms capable of carrying out these biogeochemical processes. While DNA cannot determine if these genes were actively transcribed (reviewed in Smith and Osborn 2009), changes in the abundance of genes detected over time indicate growth and death of specific organisms (Dandie et al. 2007). It is possible that some of the genes detected are associated with dead cells, thus potentially overestimating the abundance of genes in our study.

Chlorophyll *a*, Carbon, and Nitrogen.—Benthic chlorophyll-*a* samples were determined in triplicate from the top 1 cm of sediment collected with a 15-mm ID core tube. Chlorophyll-*a* concentrations were determined with a Turner Designs 700 fluorometer after cold extraction in 90% acetone for 24 hrs (Welschmeyer 1994). Particulate organic carbon and nitrogen content were measured for each core by drying sediments to a constant mass at 60 °C, homogenizing with mortar and pestle, and analyzing by combustion in a PDZ Europa Automatic Nitrogen and Carbon Analyzer-Gas Solid Liquid at the University of California Davis Stable Isotope Facility. The molar C:N ratio was then calculated.

Sediment Porewater and Benthic Fluxes.—Potential denitrification rates were measured with the acetylene inhibition technique (Sorensen 1978) from a subsample of the sediments collected for microbial analysis. Sediments (20 g) were incubated in 70-ml serum vials with 35-ml filtered site seawater amended with $KNO_3^-$ (final concentration of 100 μM) (Dollhopf et al. 2005). The vials were sealed with butyl rubber stoppers and bubbled with $N_2$ gas for 10 min to ensure anaerobic conditions. Each vial was injected with acetylene (10% v/v final concentration), vigorously shaken, and incubated in dark. After 1 hr, vials were shaken vigorously for 2 min and headspace $N_2O$ concentration in each vial was determined with a Shimadzu gas chromatograph (GC-2400) equipped with an electron capture detector. The appropriate Bunsen coefficient was used to calculate the dissolved $N_2O$ concentrations in the liquid phase (Weiss and Price 1980). Potential denitrification rates are reported in μmol N $m^{-2}$ $hr^{-1}$. This technique may underestimate denitrification supported by coupled nitrification-denitrification, as this method has been show to inhibit nitrification (Seitzinger et al. 1993).

On April 29, 2011, prior to deployment of the cages, intact polycarbonate sediment cores (17.5 × 9.5 ID) were collected for determination of sediment porewater dissolved oxygen and hydrogen sulfide ion ($HS^-$) concentrations. A micromanipulator held calibrated solid-state $O_2$ and $HS^-$ microelectrodes (OX500-UW and $H_2S$500-UW) connected to a Unisense® mulitmeter analyzer.

Triplicate intact sediment polycarbonate cores (27cm × 9.5cm ID, 30 cm deep) were collected at each site (August 30, 2011, at Site 1, and September 11, 2011, at Site 2) for measurements of benthic N fluxes, net denitrification rates, and sediment oxygen demand. Core tubes were capped with 5 cm of overlying water (approximately 355 ml), stored in a cooler, and transported to a temperature controlled environmental chamber at the Dauphin Island Sea Lab. In the environmental chamber, cores were uncapped under water in mesocosms filled with site water and set up in a flow through system (Kana et al. 1998, McCarthy et al. 2008). The flow through system consists of a multi-channel proportioning pump sending GF/F (0.7 μm) filtered site water from a common reservoir per site ("inflow") at 0.08 L $hr^{-1}$ to each core. The positive displacement of the overlying water from each core ("outflow") was collected. The volume of water overlying each sediment core was exchanged five times during a 24-hr incubation period to let the cores achieve steady state. The incubations were maintained in darkness and ambient water temperature. Discreet inflow and outflow samples from each core for dissolved gas analysis ($N_2$ and Ar) by membrane inlet mass spectrometry (MIMS) (Kana et al. 1994) were collected by overflowing a 12-ml Exetainer® two tube-volumes prior to preserving it with 250 μl of 50% (w/v) $ZnCl_2$ solution. Additional inflow and outflow samples were collected for nutrient concentrations ($NO_2^-$, $NO_3^-$, $NH_4^+$), filtered, frozen immediately, and analyzed using standard wet chemical techniques modified for the Skalar SAN+ autoanalyzer. A calibrated oxygen microelectrode (OX500-U, Unisense) connected to the Unisense® multimeter analyzer was used to measure dissolved oxygen concentrations in inflow and outflow waters. Sediment oxygen demand (SOD) and N fluxes were calculated by taking into account for inflow and outflow concentrations (μM) of the constituent of interest entering and leaving the core, respectively, the flow rate through the cores (L $hr^{-1}$), and sediment surface area ($m^2$). Positive numbers indicate a flux from the sediments to the water column while negative numbers indicate uptake by the sediments.

Statistical Analyses.—Models were formulated for the C and N data as well as the gene copy numbers for *dsrB*, *nirK*, bacterial *amoA*, archaeal *amoA*, and potential denitrification all of which were measured at multiple time points. These data were collected under a randomized complete block design, with site serving as the block. Therefore, these analyses utilized mixed models via the SAS procedure PROC MIXED, with random effects included to account for blocking and within-block error. Although measurements were taken on the same block over time, sub-samples were not true repeated measures, as they were taken on different experimental units. Therefore, no repeated effect could be estimated; however, a random effect for Site*Treatment*Date was estimated to account for subsampling within each measurement time.

For variables measured when cores were collected for net $N_2$ flux determination, univariate models were first estimated for the variables (chlorophyll *a*, $N_2$, $NO_3^-$, $NH_4^+$, and sediment oxygen fluxes). These analyses utilized mixed models via the SAS procedure PROC MIXED, with random effects included to account for blocking (site) and within-block (subsampling) error. When appropriate, data were transformed to reduce heteroscedasticity. The Kenward-Rogers method was used to adjust the degrees of freedom for the random effect. This adjustment results in a better, small-sample approximation of the covariance of the vector or parameter estimates, which results in more accurate significance tests from mixed-effects models. This approximation adjusts both the *F* statistic and its degrees of freedom, and will result in a more powerful test whenever variance among subsamples is high (i.e., when subsamples are dissimilar). Where significant effects were detected, Tukey's test was utilized to evaluate differences among treatments. Where significant interactions were detected, we utilized a simple effects analysis (Winer 1971) to test each underlying effect.

## Results

Environmental Variabilty.—Water temperature increased from 21.4 °C in April, to a high of 32.7 °C, while salinity increased from a low of 3.5 in April to 13.8 in May, and further increased gradually during the deployment of the oysters to a maximum value of 19.7 (data not shown). Effects on oyster biodeposition were observed in the sediment. Analysis of sediment C and N content detected a Treatment*Date effect on the %C and both date and treatment effects on %N (Table 1). For %N, the interaction term was not significant. Subsequent analyses of simple effects for %C showed that the juvenile treatment was significantly lower than both the control and adult treatment on June 8, 2011 (Fig. 1A, 1B). There were also significant differences between juvenile and control treatments on July 14, 2011, and between juvenile and adult treatments on September 20, 2011. Within treatments, multiple dates showed significant differences from each other, with a general trend of increasing %C in the sediment through time. The %N in the control was significantly higher than in the juvenile treatments, but neither was significantly different from the adult treatment (Fig 1C, 1D). The %N on September 8, 2011, was significantly higher than measurements on June 8, 2011, and July 14, 2011. There were no significant effects for the C:N ratios. On most occasions, the C:N ratios in sediments at Site 1 exceeded the Redfield ratio (Fig. 1E), at Site 2 sediment C:N ratios were, on many occasions, below the Redfield ratio (Figs. 1E, 1F).

Table 1. Type 3 test of fixed effects for C and N content, gene data, and potential denitrification (variables measured at multiple time periods). Significant effects are in bold.

| Variable | Effect | Num DF | Den DF | F Value | Pr > F |
|---|---|---|---|---|---|
| %C | Treatment | 2 | 4.33 | 2.02 | 0.2405 |
| | **Date** | **9** | **26.60** | **3.57** | **0.0050** |
| | **Treatment*Date** | **17** | **26.60** | **1.78** | **0.0897** |
| %N | **Treatment** | **2** | **3.00** | **6.01** | **0.0891** |
| | **Date** | **8** | **24.00** | **2.36** | **0.0500** |
| | Treatment*Date | 16 | 24.00 | 1.16 | 0.3643 |
| C:N | Treatment | 2 | 2.24 | 0.68 | 0.5894 |
| | Date | 9 | 25.90 | 0.74 | 0.6722 |
| | Treatment*Date | 17 | 25.90 | 0.66 | 0.8103 |
| gene_copy_*dsrB* | Treatment | 2 | 2.00 | 3.01 | 0.2496 |
| | **Date** | **4** | **12.00** | **191.19** | **<0.0001** |
| | **Treatment*Date** | **8** | **12.00** | **2.92** | **0.0465** |
| gene_copy_*nirK* | Treatment | 2 | 2.00 | 0.26 | 0.7917 |
| | **Date** | **4** | **12.00** | **19.09** | **<0.0001** |
| | Treatment*Date | 8 | 12.00 | 2.04 | 0.1283 |
| gene_copy_*bac_amoA* | Treatment | 2 | 2.00 | 0.85 | 0.5395 |
| | **Date** | **4** | **12.00** | **50.15** | **<0.0001** |
| | Treatment*Date | 8 | 12.00 | 1.38 | 0.2979 |
| gene_copy_*arc_amoA* | Treatment | 2 | 2.00 | 5.64 | 0.1507 |
| | **Date** | **4** | **12.00** | **23.45** | **<0.0001** |
| | Treatment*Date | 8 | 12.00 | 1.52 | 0.2467 |
| Potential denitrification | Treatment | 2 | 2.00 | 3.46 | 0.2240 |
| | Date | 4 | 12.00 | 2.23 | 0.1267 |
| | Treatment*Date | 8 | 12.00 | 1.37 | 0.3020 |

Microbial Abundances and Potential Denitrification Rates.—Over the course of the deployment, the abundances of quantified genes and potential denitrification rates varied, but differences across oyster treatments were not detected. The abundance of *dsrB* genes exceeded *amoA* and *nirK* abundances by >1000× and >100×, respectively (Fig. 2). The abundance of all targeted genes varied significantly over time (Table 1). Potential denitrification rates over the course of the study ranged from 5.3 to 280.3 μmol N $m^{-2}$ $hr^{-1}$ (Fig. 3), with temporal variability that was much less pronounced than that measured for the microbial gene abundances. No treatment effect on rates of potential denitrification was apparent (Table 1). In the univariate analyses of the gene copy and potential denitrification data, there was a significant Treatment* Date interaction for *dsrB*. Post-hoc least square mean differences, performed on the marginal means within date and within treatment, indicated that for all treatments *dsrB* abundances were significantly different on August 17, 2011 ($P$ = 0.047), with highest abundances in the adult treatment and lowest for the juvenile treatment. No other models indicated a significant effect of treatment (Table 1).

Sediment Oxygen and $HS^-$ Concentrations.—Oxygen and sulfide profiles in the sediments differed between sites both at the beginning and end of the

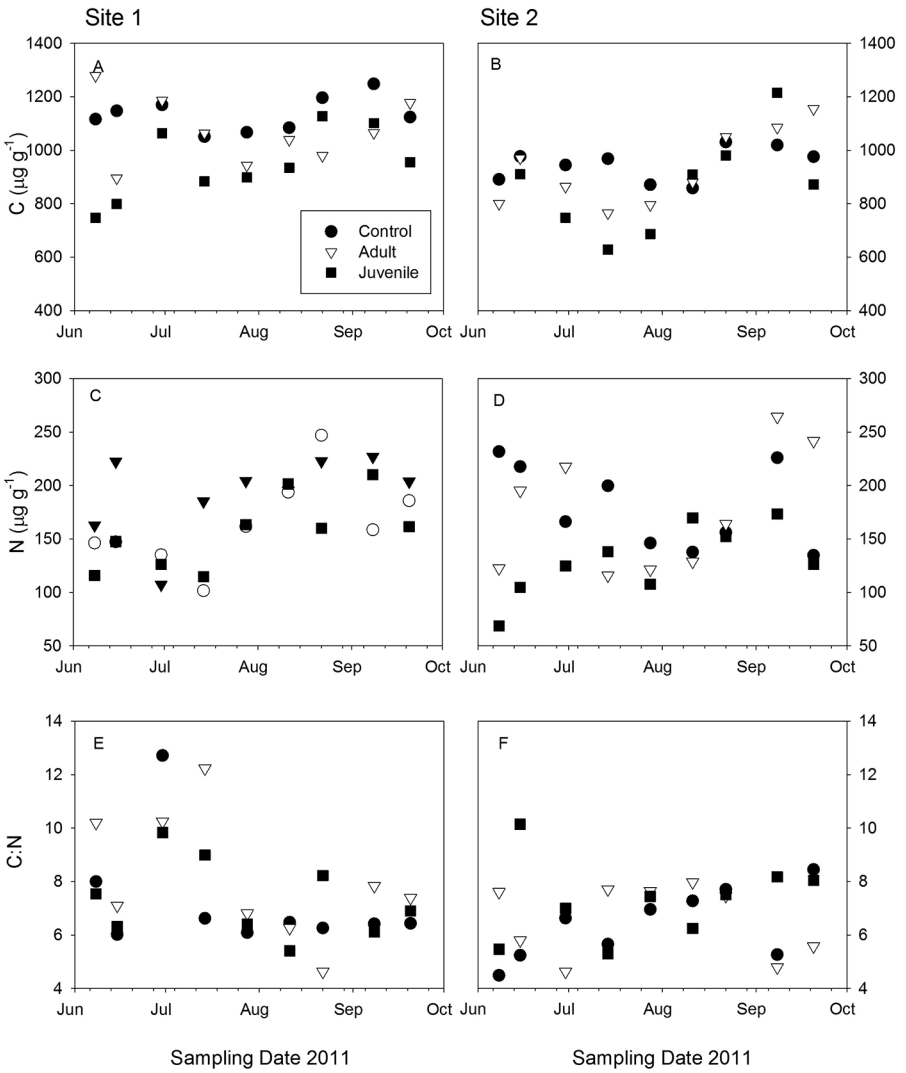


Figure 1. Sediment characteristics at the sites. Particulate carbon content at (A) Site 1 and at (B) Site 2. Particulate nitrogen content at (C) Site 1 and at (D) Site 2. C:N ratio of sediments at (E) Site 1 and at (F) Site 2.

deployment. The depth of the oxic layer in sediments at Sites 1 and 2 approached 4 mm in April (Fig. 4A,C). In September, the depth of oxygen penetration into the sediments declined at Site 1 (Fig. 4B) and in the control treatment at Site 2 (Fig. 4D). In the juvenile and adult oyster treatments at Site 2, slightly higher oxygen concentrations were measured deeper in the sediments compared to the control treatment (Fig. 4D). In April, $HS^-$ concentrations at Site 1 increased from undetectable at the surface to >500 µM in the 4–10 mm zone of the sediments (Fig. 4E). In September, $HS^-$ was absent in the top 10 mm of the sediments at Site 2 (Fig. 4H), but at Site 1, $HS^-$ concentrations in sediments underlying the control and juvenile treatments were higher than in the adult treatment (Fig. 4F).

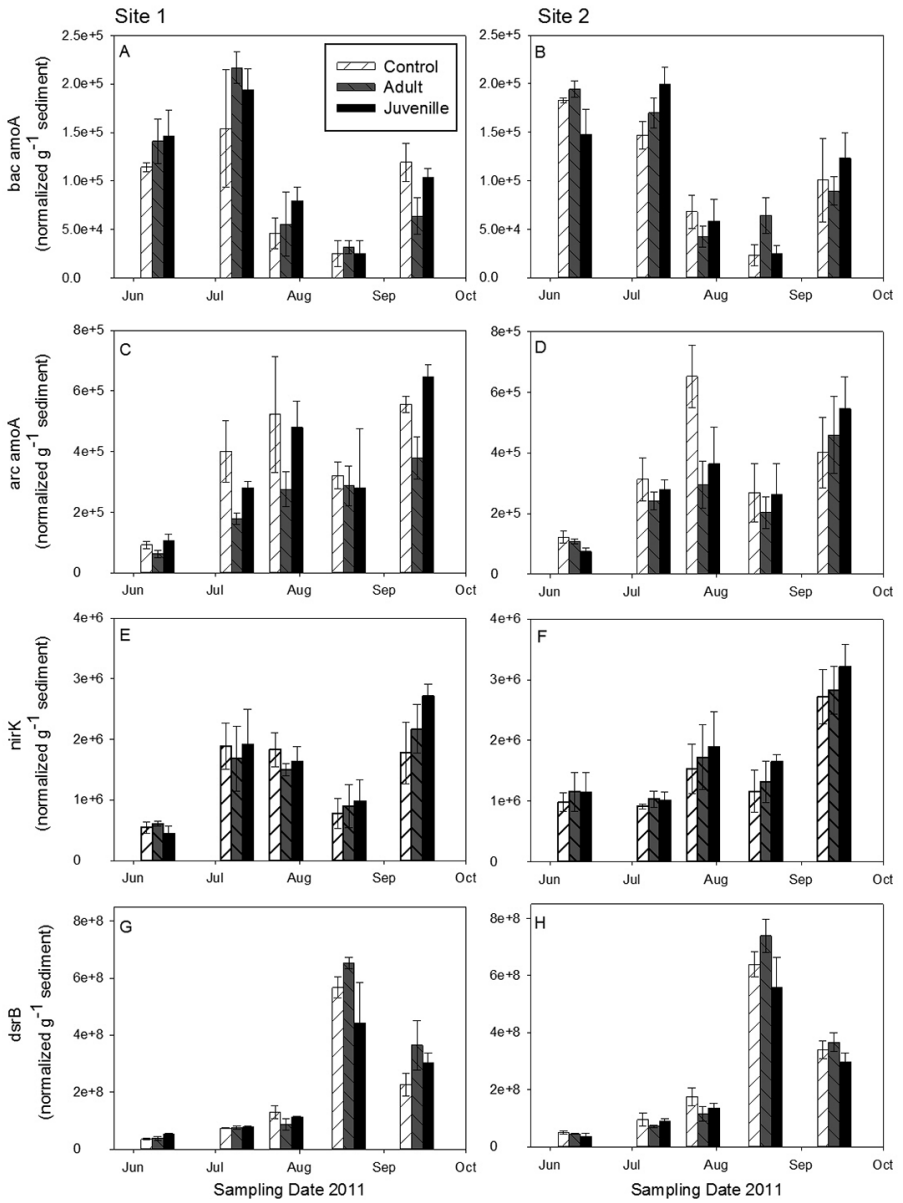


Figure 2. Abundance of targeted genes in the surficial sediments over the course of deployment of the oysters. (A, B) bacterial *amoA*. (C, D) archaeal *amoA*, (E, F) *nirK*, and (G, H) *dsrB*. The error bars are the standard error of the mean.

Sediment chlorophyll *a*, N Fluxes, and SOD.—Univariate analyses of the chlorophyll *a* and sediment flux data (Table 2) detected few treatment effects. A treatment effect was significant for only sediment chlorophyll *a* (Table 3). The result of Tukey's multiple comparison test indicated that both the juvenile ($P = 0.0068$) and adult ($P = 0.022$) treatments had significantly higher sediment chlorophyll *a* than the control.

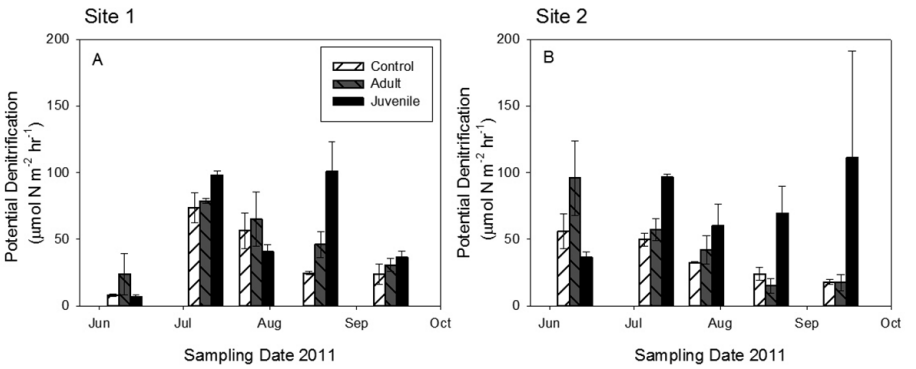


Figure 3. Potential denitrification rates measured prior to and during the deployments of the oysters at (A) Site 1 and (B) Site 2. The error bars are the standard error of the mean.

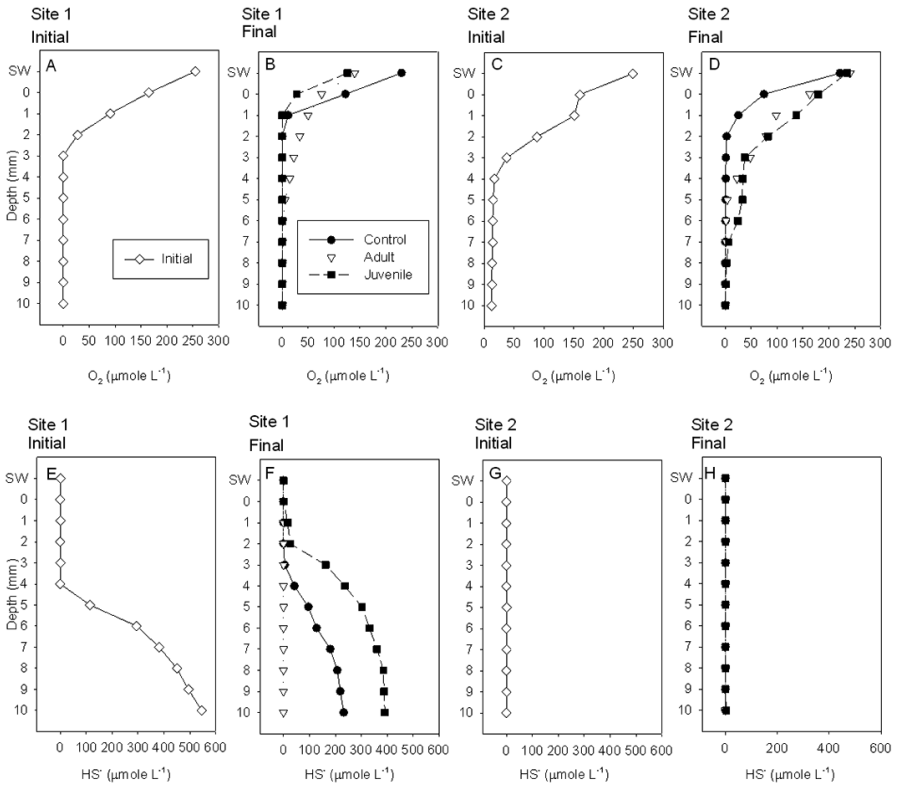


Figure 4. Porewater oxygen concentration profiles at (A, B) Site 1 and at (C, D) Site 2 measured on April 29, 2011, and in September 2011 in the different treatments. Porewater $HS^-$ concentration profiles measured on April 29, 2011, and in September 2011 at (E, F) Site 1 and at (G, H) Site 2.

Table 2. Sediment chlorophyll *a* and fluxes of $O_2$, $NH_4^+$, $NO_3^-$ and $N_2$ in September following the deployment of the oysters at two sites in Mobile Bay, AL. Values are means (1 standard error of the mean) for triplicate cores.

| Treatment | Site 1 | Site 2 |
|---|---|---|
| Chlorophyll *a* (mg $m^{-2}$) | | |
| Control | 165.3 (45.6) | 93.6 (1.7) |
| Adult | 361.0 (22.8) | 99.7 (4.9) |
| Juvenile | 301.7 (21.0) | 104.5 (12.6) |
| $O_2$ (μmol $O_2$ $m^{-2}$ $hr^{-1}$) | | |
| Control | 298.2 (26.8) | 316.8 (11.2) |
| Adult | 274.2 (20.0) | 293.8 (73.8) |
| Juvenile | 258.1 (23.6) | 515.1 (65.0) |
| $NH_4^+$ (μmol N $m^{-2}$ $hr^{-1}$) | | |
| Control | 52.2 (12.1) | 32.7 (4.0) |
| Adult | 99.0 (56.7) | 27.3 (2.8) |
| Juvenile | 71.8 (21.3) | 67.2 (11.1) |
| $NO_3^-$ (μmol N $m^{-2}$ $hr^{-1}$) | | |
| Control | −2.2 (2.9) | −32.2 (0.9) |
| Adult | −20.9 (1.8) | −47.0 (0.4) |
| Juvenile | −38.4 (3.2) | 9.6 (11.1) |
| $N_2$ (μmol N $m^{-2}$ $hr^{-1}$) | | |
| Control | −23.2 (5.3) | −35.5 (7.8) |
| Adult | −8.7 (11.2) | −9.1 (5.0) |
| Juvenile | −49.7 (8.7) | 12.1 (4.2) |

## Discussion

Changes in Sediment Microbial Gene Copy Numbers and Potential Denitrification.—Denitrification has been shown to increase with N input (Seitzinger et al. 2006). Suspension feeding bivalves, by supplying biodeposits to the sediments, can impact N cycling within estuarine sediments (Christensen et al. 2003, Newell et al. 2005) by promoting N removal via denitrification (reviewed in Carmichael et al. 2012). Adult and juvenile oysters subsampled in this experiment released biodeposits at similar rates, approximately 19.6 (SE 1.8) μg N $oyster^{-1}$ $hr^{-1}$ (Dalrymple 2013). Oyster survival was high throughout the experiment, with only 1% and 2% mortality in the juvenile and adult treatments, respectively (Dalrymple and Carmichael 2015). Based on the measured production rate, differences in stocking density, and after accounting for mortality, we estimate that the cumulative biodeposits over the duration of the study amounted to 11.4 (SE 0.4) and 3.2 (SE 0.2) N

Table 3. Type 3 test of fixed effect of treatment for flux data (variables measured at one time period). Significant effects are in bold. Chl = chlorophyll, SOD = sediment oxygen demand.

| Variable | Num DF | Den DF | F Value | Pr > F |
|---|---|---|---|---|
| **Ln (Chl *a* mg $m^{-2}$)** | **2** | **14** | **7.69** | **0.0056** |
| $N_2$ Flux (μmol $m^{-2}$ $hr^{-1}$) | 2 | 14 | 1.29 | 0.3052 |
| Sqrt ($NO_3^-$ Flux +50_μmol $m^{-2}$ $hr^{-1}$) | 2 | 14 | 1.81 | 0.2003 |
| $NH_4^+$ Flux (μmol $m^{-2}$ $hr^{-1}$) | 2 | 14 | 0.61 | 0.5570 |
| SOD (μmol $m^{-2}$ $hr^{-1}$) | 2 | 14 | 1.93 | 0.1812 |

g $m^{-2}$ in the juvenile and adult treatments, respectively. Despite substantial C and N input by the oysters and higher standing stock of microphytobenthos with the presence of oysters, changes in select gene markers and variability in potential denitrification rates could not be attributed to a treatment effect.

In the present study, the temporal variability in the abundance of genes associated with both N and S cycling microbes during the experiment suggested that these members of the microbial community are actively responding to changes in the sediment biochemistry and environmental conditions, but that inputs of N associated with oyster biodeposits were not large enough to override the impact of other environmental factors. Neither the C or N content of the sediments underlying the oyster cages increased substantially either because biodeposits were rapidly mineralized and/or alternatively were dispersed by local physical processes.

Following addition of oysters to a mudflat in Ireland, little response of the microbial community was detected, with the diversity of total Bacteria (16S rRNA), methanogens, and methanotrophs unchanged when compared to sites without oysters (Green et al. 2012). In their study, Green et al. (2012) did detect an increase in the diversity of bacterial *amoA* genes, but only in treatments with the highest number of oysters. It is possible that in our study, oyster densities in the treatments were too low to stimulate a more pronounced response of the microbial community. However, microbial community diversity may have changed without impacting the abundance of targeted genes. In longer-term studies, suspension-feeding bivalves have been shown to significantly contribute C and N to the sediments (Kautsky and Sversker 1987, Hartstein and Stevens 2005, Carlsson et al. 2009). For example, 14 mo after suspended mussel farms were established on the Swedish coast sediment, PCN increased by 3–5 fold (Carlsson et al. 2012). Thus, the 4-mo long deployment of the present study may have been too short to significantly alter the biogeochemistry of the sediments.

Despite substantial changes in the abundance of *nirK* genes and increasing organic content in the water column (data not shown) and sediments during the deployment of the oysters, potential denitrification rates in the control treatment varied little. Similar to the gene copy number data, no significant effect of treatment was apparent (Fig 3, Table 1). In our study, *nirK* was quantified as a proxy for denitrification. *nirS* is often more abundant in marine sediments (e.g., Abell et al. 2010); however, at a similar site west of Mobile Bay, Alabama, *nirK* often outnumbered *nirS* during summer months suggesting that *nirK* is a good representation of the denitrification community at this location (Wang unpubl data). Including both *nirK* and *nirS* in the analysis likely would have shown higher overall abundances of genes associated with denitrification, but still no corresponding increase in potential denitrification rates.

A positive impact of oysters and other suspension feeding bivalves on denitrification does not appear to be ubiquitous. In a much larger study than ours, Higgins et al. (2013) deployed 80,000–120,000 oysters in floating rafts at two sites and found enhanced mineralization, but no impact on N removal through $N_2$ production. Denitrification rates did increase in sediments under mussel farms that had moderate amounts of organic matter inputs to the sediments (Carlsson et al. 2012). In regions where the mussel farms had resulted in the excessive organic matter input, denitrification declined (Carlsson et al. 2012). These observations are consistent with experiments that have shown increases in denitrification following moderate additions of organic matter to the sediments (Caffrey et al. 1993, Sloth et al. 1995), while high organic matter input to the sediments lowered rates of denitrification (Caffrey

et al. 1993, Sloth et al. 1995. Newell et al. 2002). Carlsson et al. (2012) suggested that nitrification and denitrification were inhibited by hydrogen sulfide in regions associated with mussel farms contributing high inputs of organic matter to the sediment.

Despite high potential denitrification rates, $N_2$ fluxes measured with the MIMS suggested that, with the exception of the juvenile treatment at Site 2, nitrogen fixation exceeded denitrification, at a magnitude that was similar to rates measured in a nearby estuary (Mortazavi et al. 2012) and those measured in other temperate estuaries (McCarthy et al. 2008, Fulweiler and Nixon 2012, Smyth et al. 2012). Taken together, high rates of potential denitrification, but low rates of sediment $N_2$ effluxes, suggests that denitrification is $NO_3^-$ limited, as observed for oyster beds in Jamaica Bay, an urbanized and eutrophic estuary in New York (Hoellein and Zarnoch 2014). Hoellein and Zarnoch (2014) noted that increased input of organic matter to the sediments by oysters in Jamaica Bay did not increase denitrification potential because the likely $NO_3^-$ source was the water column and not through mineralization of biodeposits and nitrification.

The data from the deployments in the present study suggest that sediment redox conditions have a large influence on denitrification rates. Hydrogen sulfide was abundant at Site 1 and the oxic layer was shallow, while at Site 2 $HS^-$ was absent within the top 10 mm of the sediments, although the oxic layer was still very shallow, limiting the zone for nitrification. At Site 1, $HS^-$ likely inhibited nitrification (Joye and Hollibaugh 1995), and denitrification could not be enhanced with additional input of organic matter through coupled nitrification-denitrification. While the positive correlation between the abundance of archaeal *amoA* genes and *nirK* genes ($r^2 = 0.65$, $P < 0.0001$) suggested that coupled nitrification/denitrification could be important in N cycling in this system, the abundant microphytobenthos along with the reducing conditions indicate strong competition for $NH_4^+$ by the benthic community. Limitation of nitrification would restrict the influence of oyster mediated N input to sediments that are dependent on coupled nitrification-denitrification.

Some of the highest rates of denitrification in aquatic ecosystems have been measured in oyster beds (Kellogg et al. 2013), with rates in oyster beds exceeding rates in nearby bare sediments by as much as an order of magnitude (Kellogg et al. 2013). But other studies have found no measurable impact of oyster biodeposits on N removal by denitrification (Higgins et al. 2013). In the present study, there appears to be substantial $N_2$ fixation in sediments that are anoxic or have a very limited oxic layer. In reducing sediments, heterotrophic $N_2$ uptake can be mediated by sulfate reducers who have been shown to contribute to $N_2$ fixation in a variety of marine ecosystems (Welsh et al. 1996, Šantrůčková et al. 2010). It remains to be seen if at sites in Mobile Bay with less reducing conditions, the activity of the heterotrophic denitrifiers could potentially be enhanced with moderate inputs of organic matter (Caffrey et al. 1993, Sloth et al. 1995) from oyster biodeposists.

The presence of $HS^-$, uptake of $NO_3^-$ by sediments concurrent with low rates of net denitrification, and high ammonium fluxes indicate that dissimilatory nitrate reduction to ammonium (DNRA) is potentially occurring at Site 1. Hydrogen sulfide can serve as an electron donor to bacteria that reduce $NO_3^-$ to $NH_4^+$ (Sorensen 1978). Experimental results of Christensen et al. (2003) demonstrated that the supply of organic matter and $NO_3^-$ to sediments under mussel farms under reducing conditions enhanced DNRA rather than denitrification. High potential DNRA rates were observed in oyster reef sediments in North Carolina compared to other submerged

habitats (Smyth et al. 2013); however, these oyster reefs were also areas with high rates of denitrification, indicating the two processes are not exclusionary. Future investigations with N isotope labeling experiments could constrain the contribution and response of DNRA at Site 1.

Implications of Oyster Restoration on N cycling.—The amount of N removal through enhancements in denitrification that can be attributed to oysters appears to be site-specific and challenging to generalize to the ecosystem scale. Carmichael et al. (2012) concluded that denitrification and burial could theoretically enhance N removal by 1%–2% after growth of oysters to harvestable size. The use of bivalves in hanging baskets in shallow and protected water bodies is increasingly being considered as a method of reducing eutrophication impacts (Bulmer et al. 2012); however, the impact of oysters beyond direct removal of oysters is not guaranteed. The lack of a ubiquitous increase in denitrification in response to oyster deployments suggests that estimating the impact of oyster aquaculture on sediment biogeochemistry, and specifically denitrification (Beseres Pollack et al. 2013), should proceed with caution. Site selection for oyster aquaculture should take into consideration local hydrodynamics, sediment redox potentials, and other sediments attributes so that the benefit of N removal through denitrification, in addition to environmental services such as habitat creation, improved water clarity, and direct N removal via tissue harvest can be realized.

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