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The Potential for Eutrophication Mitigation from Aquaculture of the
Native Oyster, *Crassostrea virginica*, in Chesapeake Bay:
Quantitative Assessment of an Ecosystem Service

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of
Philosophy at Virginia Commonwealth University.

by

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Virginia Commonwealth University
Richmond, Virginia
August, 2011

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Abstract

THE POTENTIAL FOR EUTROPHICATION MITIGATION FROM NATIVE OYSTER AQUACULTURE IN CHESAPEAKE BAY, *CRASSOSTREA VIRGINICA*: QUANTITATIVE ASSESSMENT OF AN ECOSYSTEM SERVICE

By Colleen B. Higgins, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Integrative Life Sciences, at Virginia Commonwealth University.

Virginia Commonwealth University, 2011.

Major Director: Bonnie L. Brown, Professor, Department of Biology

Native oysters have been promoted as a means to improve water quality in Chesapeake Bay. This project added important insights into the potential of oyster aquaculture to process and remove nutrients from Bay waters. Results clarified that nutrient removal of nitrogen (N), phosphorous (P), and carbon (C) through harvest of cultivated oyster biomass can be quantified and modeled with high levels of statistical confidence. A simple, yet accurate, method is now available for estimating the amount of nutrients removed *via* harvesting aquacultured oysters. Based on model estimates, 10^6 harvest sized oysters (76 mm TL) remove 132 kg TN, 19 kg TP, and 3,823 kg TC. Previous work suggested that potentially substantial quantities of N may be removed through enhancement of the coupled nitrification-denitrification pathway in sediments as a result of oyster biodeposition. Using ^{15}N and N_2/Ar methods to measure N_2 production in sediments, encompassing direct denitrification (DNF), coupled nitrification-

denitrification, and anaerobic ammonium oxidation (anammox) pathways, at two oyster aquaculture sites and two reference sites (no aquaculture), we found that oyster biodeposition did not accelerate sediment N removal. We estimate sediment N removal rates *via* N_2 production at an oyster cultivation site producing 5×10^5 oysters (1750 m^2) to range from $0.49\text{-}12.60 \text{ kg N yr}^{-1}$, compared to $2.27\text{-}16.72 \text{ kg N yr}^{-1}$ at a reference site of the same area; making the contribution of oyster cultivation to N removal *via* sediment N_2 production inconsequential as a policy initiative for Chesapeake Bay eutrophication mitigation. Molecular approaches and direct abundance measures have improved our understanding of the sediment microbial community response to oyster biodeposition. Overall, sediments impacted by oyster biodeposition had a significantly different denitrifying community composition than sediments a few meters away or at the non-aquaculture reference sites. Bacterial abundance in sediments was determined by site rather than by oyster biodeposition. No apparent effects of oyster biodeposition were evident in nitrifying bacterial abundance patterns at either site, indicating that oyster biodeposition does not enhance coupled nitrification-denitrification by increasing the abundance of nitrifiers in sediments.

Chapter 1

Introduction

The cultural eutrophication of estuaries and coastal ecosystems has focused attention on understanding the role that suspension-feeding bivalves play in nutrient cycling. Population growth in the Chesapeake Bay watershed had accelerated nutrient pollution from drainage of fertilizer-rich agricultural lands, urban/suburban runoff, wastewater effluents, and atmospheric deposition. Bay states face challenges in finding low cost, environmentally effective ways to achieve Bay nutrient reduction goals. The native eastern oyster, *Crassostrea virginica*, has been promoted as a means of removing excess nutrients from bay waters.

Biological and biogeochemically mediated processes for the permanent removal of nutrients from Chesapeake Bay by oysters have been suggested: sequestration of nutrients in oyster biomass and oyster biodeposit stimulated coupled nitrification-denitrification in sediments. The research presented here measures the potential of these two nutrient removal pathways using native oyster aquaculture at two sites in Chesapeake Bay that represent two very different types of environments in which oysters are typically cultivated in the Bay. Chapter 2 quantifies oyster nutrient assimilation and develops the quantification tools and verification protocols that would be useful for the production and sale of nutrient assimilation credits produced through native oyster cultivation and harvest. In Chapter 3, the N removal capacity of oyster

aquaculture through sediment N₂ production is quantified using direct measures, presenting temporal, spatial, and experimental results. In Chapter 4, the impact of oyster biodeposition on the sediment microbial community is examined using molecular approaches, focusing on microbes involved in the nitrification and denitrification pathways. The results of this project provide a scientific basis for assessment of the nutrient removal potential of oyster aquaculture and provide the data needed to determine the policy and economic feasibility of using assimilation credits as a water quality management option.

Chapter 2

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**Nutrient bioassimilation capacity of aquacultured oysters:
quantification of an ecosystem service**

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Abstract

Like many coastal zones and estuaries, the Chesapeake Bay has been severely degraded by cultural eutrophication. Rising implementation costs and difficulty achieving nutrient reduction goals associated with point and non-point sources suggests that approaches supplemental to source reductions may prove useful in the future. Enhanced oyster aquaculture has been suggested as one potential policy initiative to help rid the Bay waters of excess nutrients via harvest of bioassimilated nutrients. To assess this potential, total nitrogen (TN), phosphorous (TP), and carbon (TC) content were measured in oyster tissue and shell at two floating-raft cultivation sites in the Chesapeake Bay. Models were developed based on the common market measurement of total length (TL) for aquacultured oysters, which was strongly correlated to the TN ($R^2 = 0.76$), TP ($R^2 = 0.78$), and TC ($R^2 = 0.76$) content per oyster tissue and shell. These models provide resource managers with a tool to quantify the net nutrient removal. Based on model estimates, 10^6 harvest sized oysters (76 mm TL) remove 132 kg TN, 19 kg TP, and 3,823 kg TC. In terms of nutrients removed per unit area, oyster harvest is an effective means of nutrient removal compared to other non-point source reduction strategies. At a density of 286 oysters m^{-2} , assuming no mortality, harvest size nutrient removal rates can be as high as 378 kg TN ha^{-1} , 54 kg TP ha^{-1} , and 10,934 kg TC ha^{-1} for 76 mm oysters. Removing 1 t N from the Bay would require harvesting 7.7 million 76 mm TL cultivated oysters.

Introduction

The health of the Chesapeake Bay has declined as a result of anthropogenic eutrophication (Boesch et al., 2001), concomitant with the reduction in oyster beds across the Bay to less than 1% of pre-19th century populations (Newell, 1988; Rothschild et al., 1994). A major focus for improving water quality is reduction of nutrients at the source. However at present, nutrient source reductions currently meet only 47% of N and 63% of P targeted watershed goals (USEPA, 2009b). Alongside specific concerns about N and P over-enrichment, the environmental impacts of anthropogenic ocean acidification and climate change have become additional water quality issues in the Bay (Pyke et al., 2008). Thus, new strategies to expand nutrient sinks, including *in situ* nutrient removal by oyster aquaculture, are being considered. Permanent removal of nutrients from the Bay can be achieved by harvesting oysters grown through intensive aquaculture. Oysters assimilate nutrients from phytoplankton biomass into tissue and shell and when harvested, nutrients are permanently removed from the ecosystem. Sustainable oyster aquaculture may re-introduce some of the ecosystem services that were lost with the decline of the wild population and has been suggested as a nutrient mitigation tool by researchers (Ulanowicz and Tuttle, 1992; Gerritsen and Holland, 1994; Jonas, 1997; Newell, 2004; Newell et al., 2005; Gifford et al., 2007).

Nutrient removal through assimilation by aquacultured bivalves with the goal of offsetting terrestrial nutrient sources has been proposed, modeled, or piloted in numerous settings and locations (Haamer, 1996; Landry, 2002; Newell, 2004; Lindahl et al., 2005; Gifford et al., 2005; Cerco and Noel, 2007; Shabman and Stephenson, 2007).

A study of blue mussel (*Mytilus edulis*) farms in Sweden estimated that a farm of 10^6 mussels removes up to 250 kg of N through bioassimilation (Lindhal et al., 2005). Similarly, a study of pearl oysters (*Pinctada imbricata*) in Australia found that up to 19 kg N was removed per 1 t of pearl oyster harvest (Gifford et al., 2005). Estimates of nutrient removal associated with cultivated pearl oyster (9.8% and 0.39% N and 0.74% and 0.03% P per g dry weight tissue and shell, respectively) have been identified as a substantial *in situ* nutrient pollution remediation tool to balance sewage treatment plant inputs in Australia (Gifford et al., 2005).

Quantification of nutrients sequestered in aquacultured native oyster biomass and the relative magnitudes of nutrients removed through aquaculture are unknown for the Chesapeake Bay. Laboratory studies report that at seston concentrations of 5-20 mg L⁻¹ an Eastern oyster assimilates approximately 50% of filtered particulate organic nitrogen and excretes the remainder (Newell and Jordan, 1983). Previous studies report that a wild Eastern oyster contains ~ 7% and ~0.3% N per g dry weight (DW) of its tissue and shell, respectively, and ~ 0.8% DW and ~0.1% P per g DW, respectively (Newell et al., 2005). Based on these data, it has been estimated that a harvest size (76 mm) wild oyster with 1 g tissue DW and 150 g shell DW contains 0.52 g TN and 0.16 g TP (Newell et al., 2005). However, similarly sized aquacultured oysters have thinner shells than wild oysters and given the variable growth rates of oysters under the wide variety of cultivation conditions in Chesapeake Bay (Brown et al., 2005a, 2005b), the quantity of nutrients sequestered in cultivated tissue and shell may differ from previous estimates.

Implementation of oyster aquaculture as a nutrient capture and removal system may be a viable auxiliary approach to bridge the gap when best management practice (BMP) implementation fails to meet targeted source reduction milestones. Therefore, we examined the nutrient content of oysters reared and harvested from two commercial-scale floating raft aquaculture oyster production sites to quantify the total nutrient content (TN, TP, and TC) of aquacultured oysters. Direct measures of TN, TP, and TC in aquacultured oyster biomass were used for regression models that related nutrient content to oyster shell total length (TL) and to provide Bay managers with a reliable nutrient removal quantification tool based on the oyster aquaculture market characteristic, TL at harvest. Using these models we extrapolated the potential nutrient removal benefits to Chesapeake Bay.

Materials and Methods

Study areas

Two sites were selected that represented typical areas where oysters are commonly reared in the Chesapeake Bay; one with high flow and low sedimentation and another with low flow and high sedimentation. Oysters were cultivated in two shallow tributaries (1-2 m) of separate sub-watersheds in the Chesapeake Bay, Spencer's Creek (Virginia) within the Little Wicomico River (37°54'22"N, 76°17'27"W; HUC 02070011) and in St. Jerome Creek (Maryland) between the mouths of Patuxent and Potomac Rivers (38°07'13"N, 76°20'53"W; HUC 02060004). The study sites represented two distinct estuarine environments subject to different physicochemical regimes and were selected on the basis of salinity, flushing rate, and ease of access (Fig. 2.1). Spencer's Creek is

a low energy site with poor hydrodynamic exchange, approximately 60 m wide with a soft silt bottom sediment and salinity ranging from 5-15 ‰. St. Jerome Creek is a high energy site that is approximately 800 m wide with a low organic matter sandy bottom sediment and salinity ranging from 12-15 ‰.

Oyster cultivation

Oysters were spawned in the Chesapeake Bay (with the exception of 2000 North Carolina spat described below) and cultchless oyster spat were reared to a size averaging ≥ 12 mm TL in land-based upwelling tanks using ambient water from within the Bay watershed. Oysters were then deployed in floating rafts to limit exposure to predators, siltation, and other consequences of benthic habitation as described by Brown et al. (1998) with the following exceptions. Floating rafts consisted of a rectangular PVC frame approximately 1.8 m x 0.9 m with three polyethylene mesh bags (2 cm diamond mesh) suspended across the long edges, each bag containing 200 oysters, such that each raft contained 600 oysters. Oysters were spread 1-2 oysters deep to maximize access to phytoplankton and reduce access to pre-filtered water from neighboring oysters. When tied together in an array, the floating rafts were ~ 0.6 m apart, with each raft covering ~ 2.1 m² of bottom sediment, equating to a maximum oyster density of 286 oysters m⁻². During Nov. 2006 and Aug. 2007, oyster strains were deployed as available for a total of 80,000 oysters deployed at Spencer's Creek, and 100,000 oysters deployed at St. Jerome Creek during May-July 2007. Monthly tending of the oysters every May-Oct. ensured that fouling was minimized, predators inside the bags were eliminated, and oysters were evenly distributed. Oysters were monitored

seasonally for growth throughout the cultivation period and harvested on or before Oct. 2009 at or above three commonly marketed sizes: cocktail (50.8 mm), regular (76.2 mm), and jumbo (101.6 mm).

Native oysters of various genetic lineages (strains) cultivated at the study sites were deployed in replicate and in separate rafts. Two commercially bred native oyster strains (Circle C Oyster Ranchers Association, Inc., Ridge, MD; Shooting Point Oyster Company, Franktown, VA) and two wild strains (Louisiana F₂s and North Carolina F₁s) were raised from spat to harvest size at Spencer's Creek. Two replicate oyster strains were grown at St. Jerome Creek (Circle C and Louisiana).

Morphometric and nutrient analyses

To quantify the net amount of nutrient removal through bioassimilation in tissue and shell, various sizes of oysters were collected between April-May 2008 (prior to gonadal maturation and natural spawning). The following measurements were taken for each oyster ($n=91$): total drained wet weight (g), total shell length (TL, mm), total shell height (mm), total shell width (mm), tissue DW (g) and shell DW (g). After the oysters were opened, the tissue and shell were separated, weighed to the nearest 0.001g, and dried at 60°C for a period of time necessary to dehydrate completely (≥ 7 days). A representative sample of 84 aquacultured oysters were analyzed for total N, P, and C content in tissue and shell, $n=47$ from Spencer's Creek and $n=37$ from St. Jerome Creek. Individuals in each set of oysters were analyzed for N, P, and C using standard methods. The dehydrated tissue and shell were ground to fine powder with a clean

mortar and pestle and analyzed for N and C by combustion using a Perkin-Elmer 2400 CHN elemental analyzer (Perkin Elmer Corp., Norwalk, CT). Phosphorus was assayed using USEPA method SW 846-3051/6010B which involves acid digestion followed by inductively coupled plasma-atomic emission spectrometry. The nutrient contents for oyster tissue and shell were recorded as percent of DW. Because 99% of the oysters deployed were spawned in Chesapeake Bay, it was assumed that the total nutrient assimilation (from the time oysters were spawned through to harvest) was from Chesapeake Bay waters.

Statistical analyses

To determine whether there were significant differences for N, P, and C in oyster tissue and shell between aquaculture sites and between aquacultured oyster strains, one way ANOVA was performed (SigmaStat 4 and SPSS v. 17.0) where data (or \log_e transformed data) were normally distributed and had equal variance. Alternatively, when data did not conform to parametric assumptions, the Kruskal-Wallis test was used. Predictive variables for nutrient removal were determined via a multiple linear regression stepwise procedure (significance level $p < 0.05$). Models for nutrient removal on a per-oyster basis were developed via linear regression of \log_e transformed data. The models were then used to extrapolate findings to the Bay in terms of oyster aquaculture harvest per year based on TL. This in turn promoted consideration of the mass load removed through oyster aquaculture and the potential policy application of oysters as a nutrient removal mechanism. Statements of significance in the text indicated observed p-values of ≤ 0.02 .

Results and Discussion

Morphometrics of aquacultured oysters

Samples ($n=91$) of aquacultured oysters harvested during this study exhibited mean TL of 76.30 mm, mean tissue DW of 1.13 g, mean shell DW of 29.77 g, shell width of 45.18 mm, and shell height of 24.62 mm (Table 2.1). The observed TL and tissue DW were comparatively similar to that reported by Newell et al. (2005) where a 76 mm oyster was cited as having 1g tissue DW. However, shell DW was five times less for aquacultured oysters than previously reported for wild oysters (150 g shell DW for a 76 mm oyster, Newell et al., 2005). Because cultivated oysters were grown in mesh bags, much of the growth was concentrated in tissue biomass rather than developing thick shells for protection as is the case for wild oysters (Paynter and Dimichele 1990).

For relevant TL categories of marketable oysters, significant differences were observed for shell dry weight, tissue dry weight, and percent water loss between sites. The level of variation in growth across sites was consistent with prior observations of oysters aquacultured in Chesapeake Bay (Brown et al. 1998, Brown et al. 2005b). Although final sizes differed across sites, the proportion of total DW attributed to the shell (96%) was not significantly different across all strains at both sites (Table 2.1). Strong positive relationships were observed (Fig. 2.2) between oyster TL and shell DW ($R^2 = 0.77$) and between oyster TL and tissue DW ($R^2 = 0.71$). This novel information was used to develop models that estimate the total nutrient content of aquacultured oysters, informing Bay policymakers of the nutrient loads that can be captured and removed from the system at harvest.

Nutrient content of aquacultured oysters

The total variation in nutrient content of aquacultured oyster tissue and shell was low, regardless of the cultivation site or oyster strain. The observed N content was slightly higher for oyster tissue and lower for shell than previous estimates for wild oysters. Specifically, the mean N content of aquacultured oyster tissue was 7.9% DW (Table 2.2) which is 13% greater than the previously reported value for wild oysters of 7% DW (Newell et al., 2005). The N content of aquacultured shell was 0.2% DW, 37% lower than the prior reported estimate of 0.3% DW for wild oysters (Newell et al., 2005). The mean P content in tissue of aquacultured oysters was 0.8% DW, not different from previous estimates for wild oysters. However, the P content of shell, 0.04% DW, was 2.5 fold less than the previous reported estimate for wild oysters which was 0.1% DW (Newell et al., 2005). The mean C content of aquacultured oyster tissue was 44.5% DW and the C content of shell was 12.3% DW; estimates for which there are no published data to compare.

For aquacultured oyster strains grown in replicate at the two sites, there was no significant difference among the strains in the N or P content of either tissue or shell. The aquaculture site had no significant impact on the N or P content of either tissue or shell. However, significant differences in C were found for both tissue and shell with respect to site. The higher salinity site, St. Jerome Creek, had significantly higher C content in oyster tissue and shell, 46.2% DW and 12.3% DW, as compared to Spencer's Creek, 43.7% DW and 12.2% DW, respectively (Table 2.2).

The nutrients assimilated in biomass by marketable regular oysters (mean TL 85.5 mm in this study) were observed to be 0.18 g TN, 0.03 g TP, and 5.4 g TC (Table 2.3). Notably, for all harvest size classes, more than half (55-64%) of the total mass of N was in the tissue, even though the tissue made up only about 4% of the total dry weight. Likewise, nearly half (40-48%) of the total mass of P was in the tissue. However, a relatively small amount (only 11-14%) of the total mass of C per oyster was contained in the oyster tissue.

For all three nutrients analyzed, the mean nutrient content of an oyster increased >1.5 fold between each marketable size class resulting in a 3-fold difference in total mass load of nutrient between cocktail and jumbo oysters. The mean TN content of cocktail sized oysters in this study (mean TL 64.8 mm) was 0.1 g TN, versus jumbo oysters (mean TL 117.8 mm) which had 0.4 g TN; a ~4-fold increase in TN. The mean TP content of cocktail oysters was 0.02 g TP versus jumbo oysters with 0.05 g TP; a ~3-fold increase in TP. Similarly, the mean TC content of cocktail size versus jumbo oysters increased ~3-fold, from 3.4 g TC to 10.0 g TC per oyster.

Relationship of shell length to nutrient removal

Total mass loads of N, P, and C in aquacultured oysters were found to be strongly associated with shell TL measurements. Linear regression showed that the shell TL was a highly significant predictor for the total nutrient content per individual oyster and explained 76.6%, 77.6%, and 75.8% of the variation in TN, TP and TC, respectively. Neither site nor strain showed significant differences for TN or TP. Both site and strain

exhibited significant differences for TC. However, significant differences observed between sites for C content of tissue and shell explained very little of the total variation among oysters, 4.5% and 1.8% respectively, determined by multiple linear regression analysis. Therefore separate predictive equations were developed for the total nutrient content (TN, TP, and TC per oyster including both tissue and shell) based on a TL (mm) measurement using data collected for all aquacultured oysters sampled at both sites.

Simple linear specifications between shell TL and oyster TN, TP, and TC in tissue and shell failed normality and constant variance tests. Natural log transformed models generated more statistically valid estimates (Fig. 2.3). The predictive equations were developed based on a TL measurement and allow for reliable estimates of the TN, TP, and TC content per individual aquacultured oyster that includes both tissue and shell:

$$\text{Eq. [1]} \quad TN = e^{(-14.1569 + 2.7994 * \ln(TL))} \quad R^2 = 0.76 \quad SE = 0.47$$

$$\text{Eq. [2]} \quad TP = e^{(-15.6926 + 2.7061 * \ln(TL))} \quad R^2 = 0.78 \quad SE = 0.44$$

$$\text{Eq. [3]} \quad TC = e^{(-10.5076 + 2.7343 * \ln(TL))} \quad R^2 = 0.76 \quad SE = 0.47$$

Using the above equations, the predicted total nutrient mass load of one harvest sized (76.2 mm TL) aquacultured oyster is 0.13 g TN, 0.02 g TP, and 3.8 g TC. A jumbo (101.6 mm TL) aquacultured oyster is estimated to contain 0.30 g TN, 0.04 g TP, and 8.4 g TC. Thus, 25.4 mm of additional growth in an oyster increases the amount of TN, TP, and TC in the oyster by a factor of ~2.2 for each element.

Using Eq. [1-3], the predicted nutrient content of a 76.2 mm TL oyster cultivated in Chesapeake Bay is 4 times lower for N and 8 times lower for P than the estimates previously reported by Newell et al. (2005) for a typical wild Chesapeake Bay oyster (0.52 g N and 0.16 g P per oyster). This disparity is reasonably attributable to the great difference in shell DW (150 g previously reported for a wild oyster versus 30 g observed in this study of aquacultured oysters) and to the lower nutrient content in aquacultured oyster shells, previously reported as 0.3% N and 0.1% P (Newell et al., 2005) versus 0.19% N and 0.04% P observed in this study.

Implications for managing Chesapeake Bay water quality

Currently, ~10-12 million aquacultured oysters yr⁻¹ are brought to market in the Chesapeake Bay (Murray and Oesterling, 2009). Our models estimate that an oyster aquaculture operation with densities similar to the sites used in this study (deploying 286 oysters m⁻² in floating rafts), harvesting 76 mm oysters, and experiencing 12.5% mortality (data not shown) removes 331 kg ha⁻¹ TN, 47 kg ha⁻¹ TP, and 9,567 kg ha⁻¹ TC per unit of time to commercial oyster harvest, typically 12-24 months. It would take 2.9×10^6 regular oysters to fill 1 ha area, representing ~three large-scale aquaculture farms each harvesting 10^6 regular oysters yr⁻¹, and each removing 132 kg TN, 19 kg TP, and 3,823 kg TC (Table 2.4). The nutrient removal value of harvesting 2.9×10^6 regular oysters is equivalent to harvesting $\sim 1.3 \times 10^6$ jumbo oysters. On a hectare-per-hectare basis, aquacultured oysters remove greater amounts of nutrients as compared to agricultural BMPs such as planting early cover crops or implementing continuous conservation tillage which reduce TN loads between 0.04 - 2.25 kg ha⁻¹ and 2.7 - 0.8 kg

ha⁻¹, respectively, depending on the location of the land in the watershed (VADEQ, 2008). Converting agricultural land to forest cover can reduce TN by 3.6 - 23.2 kg ha⁻¹ (VADEQ, 2008). Compared to these agricultural non-point source BMPs, oyster cultivation removes 29 – 1,257 times the amount of TN per unit area.

There is not currently a consensus on the trophic implications of increasing or restoring the Bay oyster population (Pomeroy et al. 2006, Newell et al. 2007, Pomeroy et al. 2007). However, given the loss of over 99% of the Bay oyster population, concomitant with nutrient excesses that stimulate phytoplankton growth, there is little evidence that enhanced oyster cultivation will alter the trophic dynamics. Our models estimate that it would take 8.5×10^9 (76 mm) or 4.0×10^9 (102 mm) oysters to remove 1% of annual TN and 4.3×10^9 (76 mm) or 2.0×10^9 (102 mm) oysters to remove 1% of annual TP inputs into Bay waters. At the ecosystem level, even a dramatic increase of oyster aquaculture in the Bay would still only be a fraction of harvest levels just prior to the wide spread impacts of overharvesting and disease. Were the oyster aquaculture industry to increase Bay-wide to harvest 200×10^6 oysters yr⁻¹ (just under half the oyster harvest from private-leased grounds in the 1950s and 1960s, an antecedent to oyster aquaculture, Alford 1973), our models predict that the watershed-scale nutrient removal would increase from 0.001 to 0.023 % of the 115.3×10^6 kg TN yr⁻¹ input and from 0.002 to 0.047 % of the 8.1×10^6 kg TP yr⁻¹ input to the Bay. Although there is a market for expanded oyster production, an immense scale-up of oyster aquaculture is currently constrained by relatively high input costs and oyster prices, which would likely fall in the face of expanded production (Lipton 2008; Boesch et al. 2010). Additional financial

incentives from nutrient reduction service payments or favorable changes in the underlying demand and cost structure of the oyster aquaculture industry may help to facilitate scale-up of oyster aquaculture production.

Conclusions

Based on model estimates, harvesting 7.7×10^6 cultivated 76 mm oysters removes 1 t of N from the Chesapeake Bay, a small percentage of the total nutrient reductions needed to achieve Bay water quality goals. Oyster aquaculture offsets can be meaningful, however, in terms of policy initiatives at the basin level where nutrient allocations are issued and reduction milestones are set. In some basins, production of 200×10^6 cultivated oysters yr^{-1} (76 mm TL) could offset ~10-15% of the excess TN load. On a per-area basis, oyster aquaculture removes a relatively large quantity of nutrients from ambient waters compared with nonpoint source controls. In contrast to many non-point source reductions, e.g., agricultural BMPs, the nutrient content of cultivated oysters can be accurately quantified with high precision using the common aquaculture market measurement of shell TL. Use of oyster aquaculture as a water quality management tool would require verification procedures to distinguish oyster aquaculture production from wild oyster harvests, and modification of existing public cost-share programs or inclusion in economic nutrient trading programs.

Implementation of a feasible system of ecosystem service payments may have the additional benefit of enhancing public awareness of water quality issues, shifting attitudes toward stewardship, and stimulating local economies.

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Figure Captions

Figure 2.1. Aquaculture study sites in Chesapeake Bay where Eastern oysters were cultivated then analyzed for biomass loads of N, P, and C in tissue and shell.

Figure 2.2. Plots of total length (TL, mm) versus shell dry weight (DW, g, black triangles) and tissue dry weight (gray circles) for Chesapeake Bay aquacultured Eastern oysters. Linear regression line and 95% prediction bands are shown only for shell TL, which is a significant predictor ($p < 0.001$) of total nutrient content per oyster, including tissue and shell.

Figure 2.3. Linear regressions of \log_e transformed TL (mm) versus \log_e transformed total nutrient content (g) for all size classes of aquacultured Eastern oysters cultivated in Chesapeake Bay. Shown are total nitrogen (TN, squares), total phosphorous (TP, triangles), and total carbon (TC, circles).

Table 2.1. Morphometrics of aquacultured Eastern oysters harvested from two sites in Chesapeake Bay. Mean values \pm standard error are given for shell length (TL, mm), shell width (W, mm), shell height (H, mm), tissue and shell wet weight (g), tissue dry weight (DW, g), shell dry weight (DW, g), water loss (%), and shell dry weight as percent of total oyster dry weight.

Study Site	<i>n</i>	TL	W	H	Tissue & Shell	Tissue DW	Shell DW	Water Loss	Shell percent of total DW
Spencer's Cr.	43	64.37 \pm 2.15	36.17 \pm 1.21	18.82 \pm 1.02	24.16 \pm 2.99	0.75 \pm 0.10	19.43 \pm 2.82	36.87 \pm 0.72	96.03 \pm 0.20
St. Jerome Cr.	48	82.25 \pm 0.36	55.66 \pm 1.17	31.22 \pm 1.01	65.59 \pm 5.64	1.63 \pm 0.18	43.27 \pm 3.53	30.28 \pm 1.3	96.52 \pm 0.22
Total	91	76.30 \pm 2.48	45.18 \pm 1.15	24.62 \pm 0.87	46.74 \pm 4.08	1.13 \pm 0.11	29.77 \pm 2.56	34.01 \pm 0.78	96.25 \pm 0.15

Table 2.2. Percent nitrogen, phosphorus, and carbon content per gram dry weight of representative aquacultured Eastern oyster tissue and shell cultivated at two sites in Chesapeake Bay. Spencer's Creek averages include four strains; St. Jerome Creek averages include two strains; two strains were cultivated in replicate at both sites. Significance values for the two strains are shown in the text.

	<i>n</i>	Nitrogen		Phosphorus		Carbon	
		%	Range	%	Range	%	Range
Tissue							
Spencer's Cr.	47	8.10±0.13†	5.80–9.97	0.83±0.01	0.60–1.05	43.74±0.30	37.96–47.15
St. Jerome Cr.	37	7.37±0.19	5.43–10.36	0.82±0.02	0.53–1.07	46.20±0.40	35.66–48.20
Mean		7.86±0.11	5.43–10.36	0.82±0.01	0.53–1.07	44.50±0.26	35.66–48.20
Shell							
Spencer's Cr.	47	0.20±0.01	0.11–0.39	0.04±0.00	0.03–0.05	12.24±0.19	7.55–17.40
St. Jerome Cr.	37	0.20±0.02	0.11–0.48	0.04±0.00	0.03–0.05	12.30±0.15	10.26–14.97
Mean		0.19±0.01	0.11–0.48	0.04±0.00	0.03–0.05	12.27±0.13	7.55–17.40

[†] Values are means ± SE.

Table 2.3. Observed mean values per Eastern oyster for total length (TL) given in mm, tissue and shell DW reported as g, and nutrient reported as percent of dry weight (%) and as total mass load (g) of TN, TP, and TC in tissue and shell for various size classes of aquacultured oysters:

	<i>n</i>	TL†	DW	Nitrogen		Phosphorus		Carbon	
				%	g	%	g	%	g
Tissue									
Submarket ‡	12	43.64±1.67§	0.20±0.03	8.15±0.21	0.016±0.003	0.83±0.03	0.002±0.000	43.29±0.58	0.083±0.014
Cocktail	45	64.80±1.12	0.80±0.07	8.06±0.14	0.064±0.005	0.83±0.01	0.007±0.001	44.28±0.32	0.361±0.032
Regular	16	85.53±1.25	1.58±0.18	7.28±0.26	0.113±0.012	0.82±0.03	0.012±0.001	45.12±0.77	0.729±0.089
Jumbo	11	117.77±5.67	3.00±0.31	7.37±0.41	0.216±0.020	0.82±0.04	0.024±0.002	46.20±0.33	1.387±0.145
Shell									
Submarket	12	43.64 ±1.67	4.81±0.50	0.18±0.02	0.009±0.002	0.04±0.00	0.002±0.000	11.84±0.39	0.564±0.060
Cocktail	45	64.80 ±1.12	24.28±2.07	0.19±0.01	0.048±0.005	0.04±0.00	0.010±0.001	12.36±0.18	3.030±0.275
Regular	16	85.53±1.25	37.58±2.93	0.17±0.01	0.062±0.005	0.04±0.00	0.014±0.001	12.43±0.24	4.646±0.344
Jumbo	11	117.77±5.67	71.88±8.40	0.26±0.04	0.177±0.025	0.04±0.00	0.026±0.003	12.04±0.11	8.624±0.976
Total nutrient per oyster									
Submarket	12				0.025±0.004		0.003±0.000		0.647±0.171
Cocktail	45				0.112±0.010		0.016±0.001		3.391±0.302
Regular	16				0.176±0.014		0.026±0.002		5.375±0.409
Jumbo	11				0.394±0.033		0.050±0.004		10.011±1.069

† DW, dry weight; TC, total carbon; TL, total length; TN, total nitrogen; TP, total phosphorus.

‡ Submarket, <50.8 mm; cocktail, 50.8–76.2 mm; regular, 76.3–101.6 mm; jumbo, >101.6 mm.

§ Values are mean ± SE.

Table 2.4. Nutrient mass load predictions for total nitrogen, total phosphorus, and total carbon bioassimilated by 10^6 aquacultured Eastern oysters of various harvest sizes, generated by models for nutrient content of an average aquacultured oyster based on shell total length.

	For 10^6 aquacultured oysters	Nutrient
	mm	kg
TN†	50.8	42
	76.2	132
	101.6	298
TP	50.8	6
	76.2	19
	101.6	41
TC	50.8	1262
	76.2	3823
	101.6	8396

† TN, total nitrogen; TP, total phosphorus; TC, total carbon.

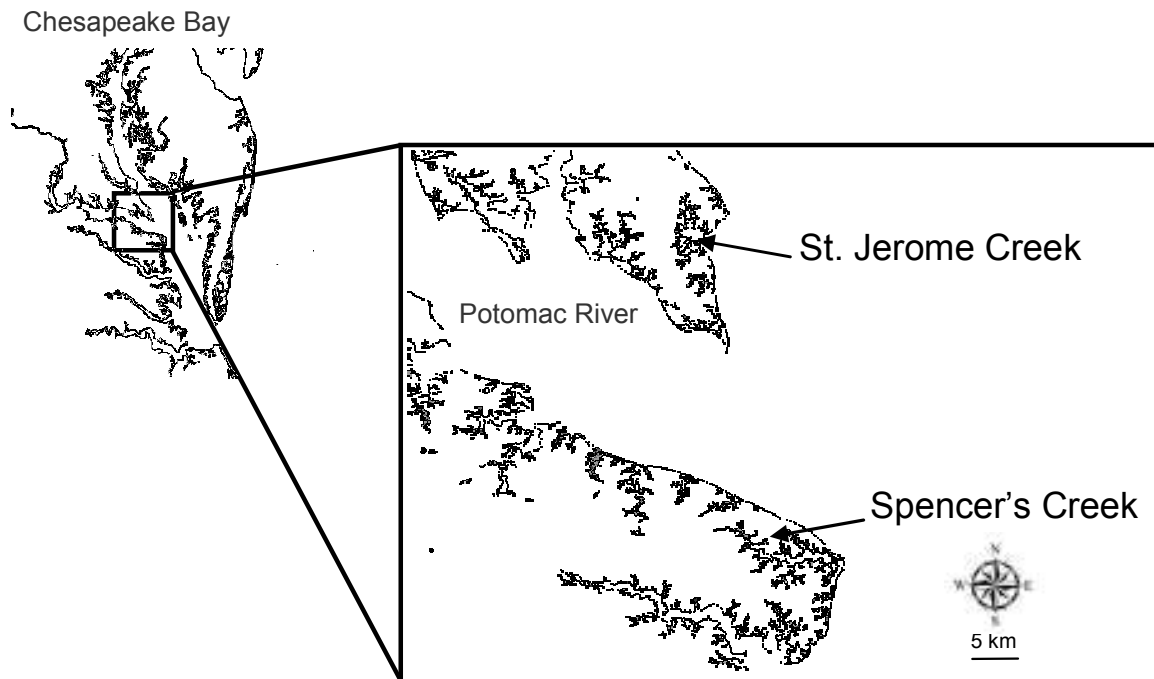


Figure 2.1.

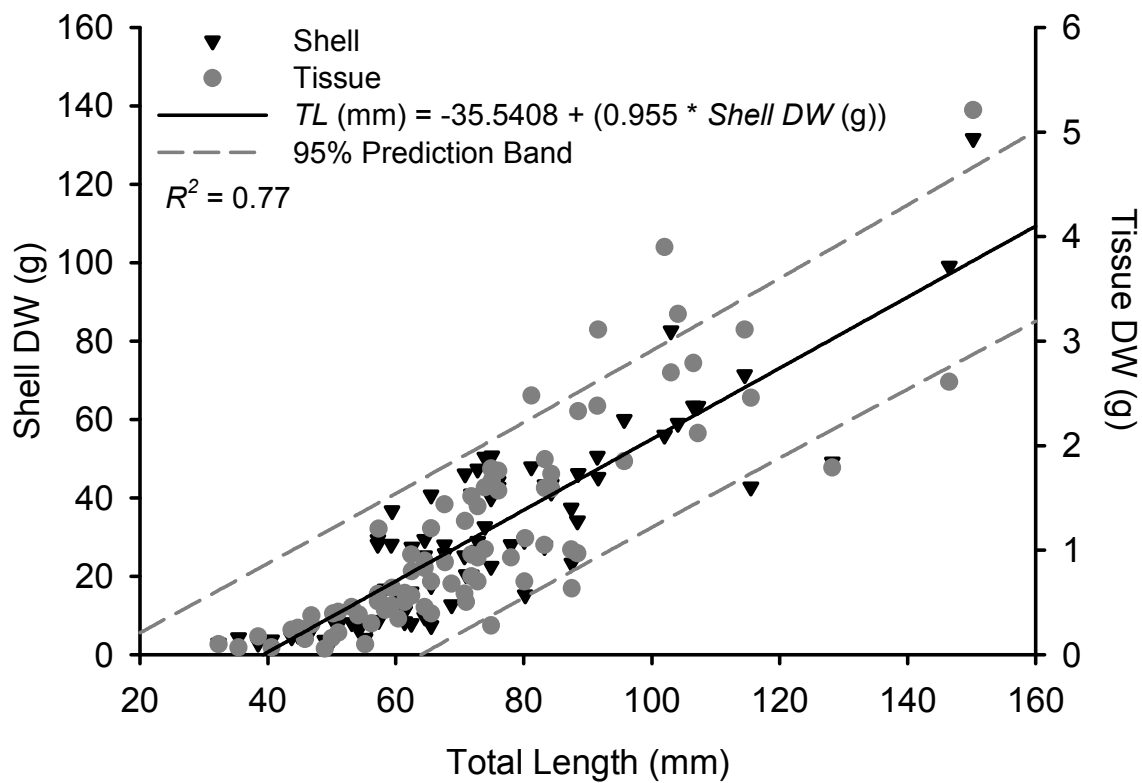


Figure 2.2.

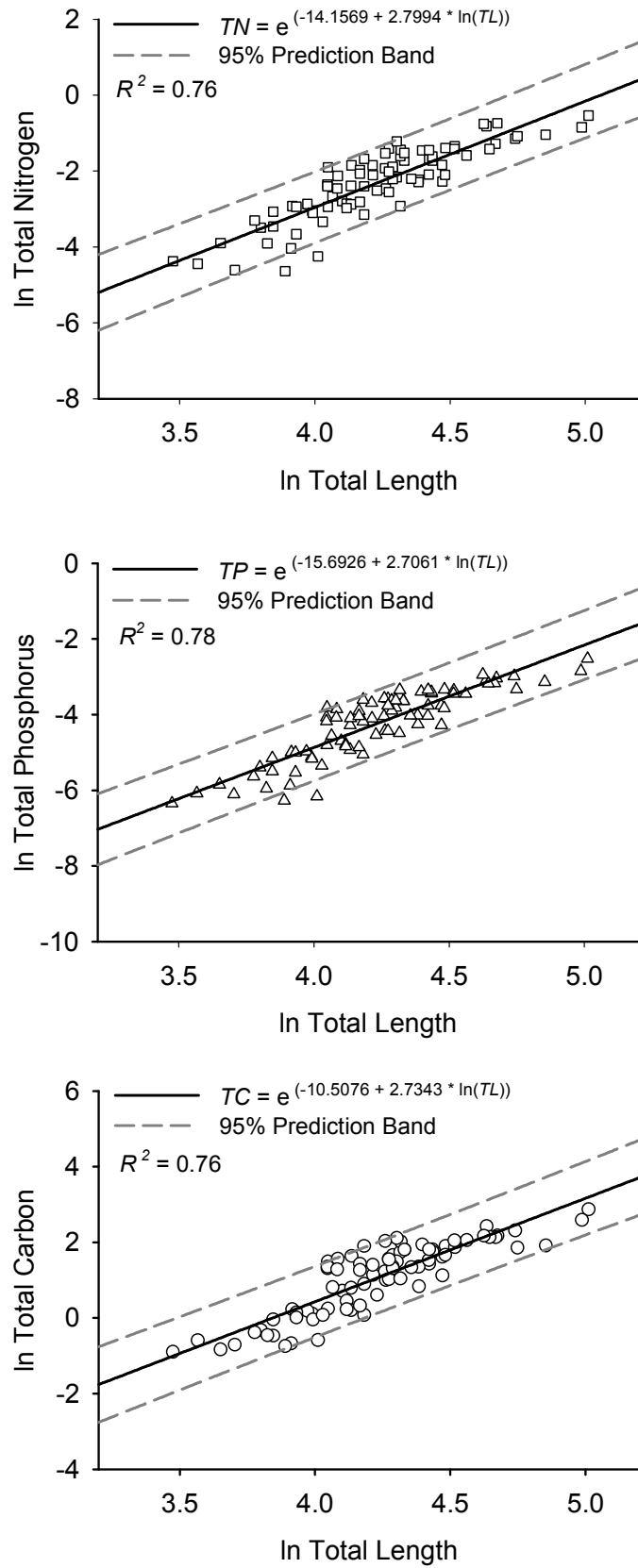


Figure 2.3.

Chapter 3

Impact of Oyster Aquaculture Biodeposition on N₂ production in Chesapeake Bay Sediments

Impact of Oyster Aquaculture Biodeposition on N₂ production in Chesapeake Bay Sediments

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RUNNING TITLE: Oyster biodeposition impact on estuarine sediment nitrogen removal

KEY WORDS: Eastern oyster, *Crassostrea virginica* (Gmelin), denitrification, Anammox, sediment nitrogen removal, oyster biodeposition, Chesapeake Bay

ABSTRACT

Estuarine and coastal eutrophication has heightened interest in understanding the role suspension-feeding bivalves play in nutrient cycling. We conducted an integrated field and laboratory examination of the effect of oyster biodeposition on sediment denitrification (DNF) and anammox (AMX) rates to quantify the nitrogen (N) removal potential of floating raft oyster, *Crassostrea virginica*, aquaculture at two commercial-scale sites in Chesapeake Bay. We quantified the rate of oyster biodeposition and N load to sediments to determine seasonal estimates and found the oyster cultivation sites supplied $\sim 18.2 \text{ kg m}^{-2} \text{ yr}^{-1}$ (dry weight) of biodeposits and $\sim 21.6 \text{ g N m}^{-2} \text{ yr}^{-1}$ to the sediments. We used two methods, ^{15}N isotope tracer and N_2/Ar , to measure seasonal sediment N_2 production rates (DNF and AMX production combined) during periods of active suspension-feeding. Oyster biodeposition did not induce increased sediment N_2 production rates above baseline (no aquaculture) reference rates at either field site. Sediment N_2 production rates underneath oyster rafts ranged from $0.00\text{--}1.56 \text{ mmol N m}^{-2} \text{ d}^{-1}$, and were generally lower than reference sites with the exception of mid-summer when rates were $0.85\text{--}1.14 \text{ mmol N m}^{-2} \text{ d}^{-1}$ higher. Oyster biodeposit N delivery explained only 21% of variation in sediment N_2 production. Based on laboratory biodeposit addition and forced biodeposit accumulation field experiments, we estimate $\sim 2.50 \text{ mmol N m}^{-2} \text{ d}^{-1}$ to be the maximum capacity of the sediments to remove N through DNF and AMX, regardless of increasing organic N or labile organic carbon delivery rates. We estimate sediment N removal rates *via* N_2 production at an oyster cultivation site of 5×10^5 oysters (1750 m^2) to range from $0.49\text{--}12.60 \text{ kg N yr}^{-1}$, compared to $2.27\text{--}16.72 \text{ kg N yr}^{-1}$ at a reference site of the same area; indicating that

the contribution of oyster cultivation to N removal *via* sediment N₂ production is inconsequential as a policy initiative for Chesapeake Bay eutrophication mitigation.

INTRODUCTION

A major consequence of anthropogenic changes to the global nitrogen (N) cycle is the accumulation of reactive N in estuaries and coastal marine ecosystems (Vitousek et al. 1997; NRC 2000; Howarth et al. 2000). Estuarine watersheds that drain large agricultural areas and/or are in close proximity to dense population centers receive substantial N loads through a variety of point and non-point sources, leading to a complex array of ecosystem-scale conservation and management issues. Chesapeake Bay is a highly impacted estuary in which N pollution has contributed to chronic hypoxia-anoxia, reduced water clarity, and loss of biodiversity (Kemp et al. 2005; Boesch et al 2001; Diaz 2001). In addition to limiting N inputs, finding ways to enhance natural N sinks within the Bay is a promising approach being investigated to manage this ecosystem. Denitrification (DNF) and anaerobic ammonium oxidation (AMX) are microbially-mediated biogeochemical processes that permanently remove N in the form of inert N₂ gas. DNF is a natural N sink that removes an estimated 20-50% of N inputs to estuaries (Seitzinger 1988). Sediment DNF rates have been measured across a number of coastal marine systems and generally represent N removal on the order of 1-2 mmol N m⁻² d⁻¹ (Fennel et al. 2008; Seitzinger et al 2006). DNF may occur as direct DNF of water column NO₃⁻ fueled by labile organic carbon, or through the coupled mineralization, nitrification, and DNF of organic N. There is evidence that moderate organic matter (OM) loading may drive higher DNF rates compared to high OM loading which can inhibit coupled nitrification-denitrification (Sloth et al. 1995; Laursen & Seitzinger 2002; Eyre and Ferguson 2009). AMX is less well characterized, typically constituting a smaller contribution to N removal *via* N₂ production, but can be non-trivial

contributor to total N_2 production (Devol 2003; Francis et al. 2007). If these biogeochemical N_2 removal pathways have the potential to be expanded in the Bay, they could serve as tools for mitigating nutrient pollution in impacted marine and estuarine ecosystems.

Eutrophication in these systems has heightened interest in understanding the role that suspension-feeding bivalves play in nutrient cycling. Within Chesapeake Bay, radical increases in nutrient loads and loss of over 99% of the native Eastern oyster population (*Crassostrea virginica*) have occurred simultaneously over the last century, making it difficult to disentangle the impact of these two factors and understand how they are inter-related. Suspension-feeding bivalves affect the distribution of nutrients and energy by filtering the water column and depositing a mixture of feces and pseudofeces (biodeposits), thereby coupling benthic and pelagic processes (Dame et al. 1984). Loss of keystone suspension-feeders has arguably shifted the trophic structure in these ecosystems from one dominated by benthic-pelagic coupling to one that is almost entirely dominated in the pelagic zone by nutrient accelerated primary production (Dame 1996, Jonas 1997, Kemp et al. 2005, Newell et al. 2005). Feces and pseudofeces combine into mucus-coated aggregates that have a higher sinking velocity than the surrounding seston (Giles & Pilditch 2004), effectively increasing sedimentation rates of nutrient enriched OM and promoting benthic-pelagic coupling.

A plausible hypothesis is that suspension feeding bivalves (e.g., oysters) stimulate increased sediment N_2 production rates by accelerating OM sedimentation *via* biodeposition, thereby expanding a N sink resource by facilitating accelerated permanent N removal (Haven and Morales 1966, Kaspar et al. 1985, Kautsky and

Evans 1987, Deslous-Paoli et al. 1992, Newell et al. 2002). Experimental laboratory evidence indicates that coupled nitrification-denitrification rates increase with the addition of pelletized algal cells (used as a biodeposit analog), yielding estimates that 0.5 g N oyster⁻¹ yr⁻¹ may be removed in Chesapeake Bay *via* oyster biodeposit stimulation of increased coupled nitrification-denitrification rates (Newell et al. 2002). Field measurements of DNF rates have been made for sediments associated with bivalve cultivation (clams and mussels) in other marine systems (Christensen et al. 2003, Minjeaud et al. 2009, Nizzoli et al. 2006), yielding results that are equivocal or indicate inhibition of DNF. Alternatively, rather than promoting net N removal, bivalve systems may facilitate N retention (Dame & Libbes 1993). Studies of both natural reefs and aquaculture systems (clams and mussels) have indicated significant increases in water column NH₄⁺ as the deposited organic N is remineralized and returned to the water column as reactive N (Dame 1996, Porter et al. 2004, Gibbs et al. 2005).

In this study, we tested the hypothesis that oyster biodeposition stimulates increased DNF and/or AMX rates in estuarine sediments from two commercial-scale floating-raft oyster aquaculture sites (80,000 – 120,000 oysters) and two reference (no aquaculture) sites 250-300 m away in Chesapeake Bay. We used two methods, ¹⁵N tracer and N₂/Ar measured via membrane inlet mass spectrometry (MIMS), to estimate the effect of varying levels of oyster biodeposition treatment on sediment N₂ production, considering spatial and temporal variability. We collected oyster biodeposits at each site to estimate the biodeposition and N delivery rates to sediments. We also conducted two different biodeposit addition experiments at both sites seasonally. In a series of field experiments, we forced the accumulation of oyster biodeposits in

sediments directly underneath aquaculture rafts, controlling for the effect of flow, and measured sediment N₂ production rates. In laboratory tests, we collected reference sediment cores and inoculated them with freshly collected biodeposits in increasing quantities, to test for a biodeposit effect on N₂ production rates.

METHODS

Study sites and oyster cultivation. Sediments were examined from two shallow (1-2 m) mesohaline tributaries in separate sub-watersheds of Chesapeake Bay (Fig. 3.1) where oysters were cultivated in floating rafts as described by Higgins et al. 2010. The study sites represent two distinct estuarine environments subject to different physicochemical regimes that are representative of the most commonly utilized oyster aquaculture sites in Chesapeake Bay. Spencer's Creek (Virginia), within the Little Wicomico River (37°54'22"N, 76°17'27"W; HUC 02070011), is a low wave energy site approximately 60 m wide, with poor hydrodynamic exchange, soft bottom sediment high in OM, and salinity ranging from 5-15 ‰. Oysters have been cultivated at this site in varying quantities since 2000. St. Jerome Creek (Maryland) near the mouth of Patuxent River (38°07'13"N, 76°20'53"W; HUC 02060004) is a high wave energy site that is approximately 800 m wide with a low organic matter sandy bottom and salinity ranging from 12-15 ‰. This commercial site has been in operation since 1991. At both locations, a comparable reference station (Reference) 350-500 m away from the aquaculture station (Oyster) was identified and used as a control station. A station 5-10 m outside of each oyster array (Near Oyster) was sampled in one summer season at both sites to estimate the area impacted by biodeposition surrounding an oyster array.

At both sites, oysters were cultivated in floating rafts as described by Brown et al. (1998) with the following exceptions. Floating rafts consisted of a rectangular PVC frame approximately 1.8 m x 0.9 m with three polyethylene mesh bags (2 cm diamond mesh) suspended across the long edges, each bag containing 200 oysters, such that each raft contained 600 oysters. Cultchless oyster spat were reared to a spat size averaging at least 12 mm total shell length (TL) in land-based upwelling flow through tanks using ambient water prior to deploying in floating rafts. Oysters (average final total length 86 mm at the end of the culture period; Higgins et al. 2011) were maintained 1-2 deep to maximize access to seston and reduce access to pre-filtered water from neighboring oysters. When tied together in an array, the floating rafts were ~0.3 m apart, equating to a maximum oyster density of 286 oysters m⁻², covering a total bottom area of approximately 250 m² at Spencer's Creek and 350 m² at St. Jerome Creek.

At each sampling event at Spencer's Creek, 80-100,000 oysters ranging from ~50-85 mm TL were present in the array of oyster rafts. At the time of the 2008 ¹⁵N tracer enrichment experiments at St. Jerome Creek, 100,000-120,000 oysters ranging from ~40-85 mm TL were present in the array of oyster rafts. At the time of the spring 2009 ¹⁵N tracer enrichment experiments and all MIMS experiments at St. Jerome Creek, samples were collected from underneath rafts containing ~200,000 oysters ranging from 15-130 mm TL.

Collection of biodeposits and determination of biodeposition rates. Biodeposits were collected as they settled beneath floating oyster rafts containing ~600 oysters raft⁻¹ to quantify the total mass of nutrients (N, P, and C) that oysters delivered to the

sediments and to determine the percentage of delivered N that was removed compared to the reference sites. Biodeposits and accompanying settled seston collection experiments were conducted in conjunction with the direct measures of DNF and/or AMX collected by ^{15}N stable isotope pairing technique and MIMS. For each sampling of biodeposits, a representative sample of the oysters was analyzed to quantify nutrient mass flux rates to the sediments.

Nutrient content of the collected biodeposits and settled seston per unit of time was used to estimate the nutrient content of materials processed by oysters and made available for sediment biogeochemical N_2 removal. Quantification of the properties and amounts of oyster biodeposition was accomplished by making replicate collections of biodeposits seasonally for 3-5 days at both aquaculture stations using a 100 μm nylon mesh device secured under oyster rafts (3.2 A) that captured biodeposits as they settled and before they reached the sediment. Background particulate OM settling out of the water column due to the effect of a raft without oysters deployed at the reference stations was collected using the same technique over the same time period as the oyster biodeposit samples and was identified as settled seston (Seston). Biodeposits and settled seston samples were sieved to remove epibenthic fauna and debris, drained through 100 μm mesh, then both wet and dry weight were recorded.

To control for the effect of biodeposits being dispersed outside the array of rafts, a funnel shaped 100 μm nylon mesh curtain was deployed beneath representative oyster rafts to force accumulation of biodeposits underneath a raft during the same time period as biodeposits were collected from underneath non-fenced rafts (Fig. 3.2 B). The biodeposit fence was deployed in experiments in June 2008 at both aquaculture

sites and again at St. Jerome Creek in summer 2009 to force collection of biodeposits directly beneath the raft and facilitate local *in situ* sediment processes. Sediment cores were collected from the fenced biosedimentation area after 48 hr and analyzed for DNF, AMX, and total N₂ efflux and compared to analysis of untreated reference sediment cores.

Sediment, biodeposit, and pore water nutrient analysis. Well-mixed (0-3 cm) subsamples from replicate cores and freshly collected biodeposits were analyzed for total N (TN) and total C (TC) by combustion using a Perkin-Elmer 2400 CHN elemental analyzer (Perkin Elmer Corp., Norwalk, CT). Nutrient content was determined by an EPA approved commercial laboratory using standard methods: NO_x (SM 4500-NO₃F), NH₄⁺ (EPA 350.2), TKN (EPA 351.3), BOD (5210B), COD (5520C), Chla (measured as a proxy for microphytobenthos), OM (SM 2540G). Porewater in the 0-3 cm section of cores was obtained from subsamples by centrifugation at 7000 x g for 20 min at 4°C, followed by analysis for NH₄⁺ (350.1) and NO₃⁻ (353.2). For MIMS analyses, water was filtered through Whatman GF/F filters (25 mm diameter, 0.7 µm nominal pore size) and the filtrate was analyzed with a Lachat Quick-Chem 8000 automated ion analyzer for NO₃⁻, NH₄⁺, PO₄⁻ and total organic (TON).

¹⁵N Stable Isotope Method. The ¹⁵N stable isotope enrichment analysis is a direct measure of DNF and AMX potential production rates in marine sediments by incubating sediment samples using ¹⁵N-labelled NO₃⁻ and or NH₄⁺ substrates (Thamdrup and Dalsgaard 2002). Because substrates are supplied in a small excess relative to

environmental concentrations, under a helium (He) atmosphere it is a measure of the metabolic readiness of DNF and AMX. Sediment and biodeposit samples were collected at both sites in the 2008 summer and in the fall seasons and at the St. Jerome (MD) site in spring, summer, and fall 2009. A sampling transect of sediments was conducted in the spring with samples collected in triplicate for each of the following sample types: underneath the array of oyster rafts (Oyster), 5-10m outside of the oyster array (Near Oyster), no aquaculture samples (Reference), samples from biodeposit fence experiment (Fence), and oyster biodeposits (Biodeposits). The fall seasonal sampling included triplicate samples for the following sample types: Oyster, Reference, and Biodeposits.

One gram of wet sediment (0-3 cm, homogenized surface sediment) was sampled from sediment cores. One gram of wet biodeposits was sampled from the biodeposit catchment device. The sediment and biodeposit samples were weighed into labeled glass exetainers (Labco International, Inc., Houston, TX) in eight replicates per sample for time series incubations. Four tubes were reserved for $^{15}\text{NO}_3^- / ^{14}\text{NH}_4^+$ and four tubes for $^{15}\text{NH}_4^+$ treatments for the time series incubations at times ($t=0$ min, $t=60$ min, $t=150$ min, and $t=300$ min) allowing us to distinguish between DNF and AMX based on the mass of the N_2 produced (adapted from Thamdrup & Dalsgaard 2002). Exetainers were then flushed with ultra high pure (UHP) He for 10 min, capped immediately, and stored overnight allowing residual $\text{NO}_3^- + \text{NO}_2^-$ to be consumed. The exetainers were again flushed with UHP He ($>100 \text{ ml min}^{-1}$) for 15 min prior to introducing an aliquot of injectate to each exetainer, either $^{15}\text{NO}_3^- + ^{14}\text{NH}_4^+$ or $^{15}\text{NH}_4^+$, to a final porewater nitrogen concentration of $25 \mu\text{M N}$. Following incubation for the

assigned time period, each set of incubations was stopped by addition of 200 µl of a 50% w:v saturated solution of ZnCl₂. Isotopically labeled N₂ was analyzed from the headspace on an isotope ratio mass spectrometer (IRMS; Thermo Electron Delta V). The areas of ²⁹N₂ and ³⁰N₂ peaks were integrated and mass calibrated against known N₂ air standards. AMX and DNF rates were calculated from time series regressions of the mass 29 (AMX) and mass 30 (DNF) accumulation (Thamdrup and Dalsgaard 2002).

Membrane Inlet Mass Spectrometry. Sediment core samples were collected at St. Jerome Creek in spring 2009: three underneath rafts (Oyster) and six at the reference site (Reference). Three of the six reference cores were used in biodeposit addition experiments. Core samples were collected at both sites in the summer 2009: three underneath rafts, six at the reference site, and at St. Jerome Creek three fence samples also were collected. In both instances, ambient water also was collected from each study site. Cores were acclimated and incubated as described in Piehler and Smyth (2011) in an environmental chamber set to the ambient site water temperature (19°C and 27°C, respectively) and kept in the dark. After 24 hr, 50 ml water samples were collected for nutrient analysis from the reservoir water line and each core. Fluxes were calculated as described in Piehler and Smyth (2011).

Biodeposits were collected for the biodeposit addition experiments 24 hours prior to core sample collection and stored at 4°C until addition to sample cores. In both analyses, wet weight biodeposit samples (0.16 g, 0.64 g, and 5.00 g) were added to the water overlying reference cores and allowed to settle during the acclimation period. These inoculated cores were used to test whether oyster biodeposits stimulated

increased N₂ production rates in the sediment. Nutrient flux and denitrification rates were determined using the same MIMS methods as used for the sediment cores.

Statistical analysis. Most data conformed to the expectations of normality and homogeneity of variance. Differences among samples types, sites, and seasons were assessed using One-way, Two-way, and Three-way ANOVA for total N₂ production, DNF and AMX (SPSS v.18.0). Post-hoc testing was conducted using Tukey's HSD or LSD. Regression and correlation (Pearson Product Moment Correlation) analysis was performed using SigmaPlot v.11.0 and SPSS v.18.0. When samples failed to conform for normality or constant variance, Mann-Whitney U non-parametric procedures were used to determine statistical significance (p-value ≤ 0.05).

RESULTS

Oyster biodeposition rates

Oyster biodeposition rates followed a seasonal pattern, increasing in the warmer months of late spring and summer when filtration rates are at a maximum (Fig. 3.3). On average, for a floating raft aquaculture site with ~286 oysters m⁻² (TL 76.2 mm) 11.21±1.76 g dwt m⁻² d⁻¹ biodeposits are transferred to sediments in summer, decreasing in the spring and fall to 9.07±1.67 and 2.44±0.58 dwt biodeposits m⁻² d⁻¹, respectively (Table 3.1). Averaging biodeposition rates for spring, summer, and fall seasons at both sites, one oyster deposits 0.04±0.01 g dwt d⁻¹. At St. Jerome Creek where survival and growth rates were higher than Spencer's Creek, oyster biodeposition rates were significantly higher (One-way ANOVA, p < 0.04).

Considering biodeposit nutrient content for replicate seasons at both sites, a site-season interaction was detected for TN, possibly masking site as a significant factor which was insignificant (Two-way ANOVA, $p < 0.04$). A seasonal effect was detected for both TN and TC, and site was not a significant factor in determining the TC content of oyster biodeposits (Two-way ANOVA, $p < 0.04$). Biodeposits had higher TN in the fall and summer than in the spring, considering all seasons, but overall variation was small and ranged from 0.75-1.74% TN (Two-way ANOVA, $p < 0.04$). More variation in TN content was observed in biodeposits produced at St. Jerome Creek, ranging from a low of 0.75% in early spring to a high of 1.78% TN in fall (Table 3.2). Post-hoc testing indicated biodeposit TC was higher in the spring than in fall, 11.5% versus 9.2%, respectively (Two-way ANOVA, Tukey HSD, $p < 0.01$). The sites differed in biodeposit TP, and both a seasonal effect and a site-season interaction were detected (Two-way ANOVA, $p < 0.01$). Biodeposits had higher TP in the fall than in the spring, 0.19% and 0.16%, respectively, but overall variation in TP was low ranging from 0.09-0.29% for the entire data set (Two-way ANOVA, Tukey HSD, $p < 0.01$). Biodeposits at St. Jerome Creek had significantly higher TP than Spencer's Creek, ranging from 0.10-0.29% TP versus 0.09-0.17% TP, respectively (One-way ANOVA, $p < 0.01$).

The majority of the biodeposit TN consisted of organically bound N, ranging from 81% to 95% of TN at both sites for all seasons. Very little of the TN was inorganic, with NH_4^+ content ranging from 0.07-0.25% at both sites, and NO_x^- below detection limit ($<0.0001\%$). The flux of OM to the sediments ranged from 0.36-1.21 g OM $\text{m}^{-2} \text{d}^{-1}$ at Spencer's Creek and 0.74-2.95 g OM $\text{m}^{-2} \text{d}^{-1}$ at St. Jerome Creek and ranged from 18.6-25.5% OM at both sites across all seasons sampled. Delivery of TN to sediments

for a floating raft aquaculture site with ~ 286 oysters m^{-2} ($\text{TL} \leq 76.2$ mm) was 10.88 ± 1.26 $\text{mmol N m}^{-2} \text{d}^{-1}$ when filtration rates were highest in the summer months and 7.71 ± 1.55 $\text{mmol N m}^{-2} \text{d}^{-1}$ in spring and 2.86 ± 0.73 $\text{mmol N m}^{-2} \text{d}^{-1}$ in fall when filtration rates declined (Table 3.1). We estimate that one ≤ 76 mm TL oyster deposits on average 0.06 ± 0.01 mmol N d^{-1} in the summer, and 0.03 ± 0.01 mmol N d^{-1} and 0.01 ± 0.00 in spring and fall, respectively. There is an effect of the floating raft structure based on the results of settled seston collections from control rafts which are a source of habitat heterogeneity because they become substrate for flora, fauna, and fouling organisms. These rates are relatively low, but may be considered as an essential component of the system. Rates of settled seston ranged from a low in the fall to a high in the spring at both sites: 0.34 to 3.29 $\text{g m}^{-2} \text{d}^{-1}$ at Spencer's Creek and 0.60 to 3.41 $\text{g m}^{-2} \text{d}^{-1}$ at St. Jerome Creek.

Physicochemical characteristics of sediments and oyster biodeposits

Oyster biodeposits had significantly higher Chla, BOD, COD, NH_4^+ , and organic N than any sediment types at both sites and did not differ between the sites for any of the parameters measured ($p < 0.001$). Mean values for oyster biodeposits were: 90.3 ± 6.0 $\mu\text{g cm}^{-3}$ Chla, 748 ± 160 mg/kg BOD, $16,289 \pm 10,320$ mg/kg COD, $1,338 \pm 93$ mg/kg NH_4^+ and $13,896 \pm 1,312$ mg/kg organic N (Table 3.3).

Significant differences in microphytobenthos (Chla), BOD, COD, and NH_4^+ were detected between the sites, therefore the two sites were analyzed separately (Three-way ANOVA, $p < 0.002$). Only at Spencer's Creek were differences detected among sample types where oyster and fence sediment Chla was approximately twice that

observed in the reference and near-oyster sediments, $13.3 \pm 1.7 \mu\text{g cm}^{-3}$ and $11.8 \pm 1.6 \mu\text{g cm}^{-3}$ versus $6.1 \pm 0.5 \mu\text{g cm}^{-3}$ and $5.6 \pm 0.4 \mu\text{g cm}^{-3}$, respectively (Table 3.3). Oyster and fence sediments did not differ from one another ($p > 0.05$). Forced accumulation of concentrated oyster biodeposits did appear to increase Chla levels closer to that of biodeposits, which were six times higher than either the oyster or fence sediments. No significant seasonal differences in sediment Chla was detected at either site ($p > 0.05$).

The high C site, Spencer's Creek, had sediment BOD and COD that were twice and 4-7 times higher, respectively, than sediments at St. Jerome Creek, the low C site. At Spencer's Creek, the oyster sediments had BOD 190-264 mg/kg higher than either the reference or near-oyster sediments, but no differences were detected among sediment types at St. Jerome Creek ($p < 0.01$). No significant differences in sediment COD were detected among sediment types at either site.

Oyster and fence sediment NH_4^+ increased 1-3 times and 3-6 times above reference and near-oyster sediments, respectively at Spencer's Creek ($p < 0.01$). A seasonal effect was detected at Spencer's Creek, where sediment NH_4^+ peaked in fall above spring and summer seasons, likely due to increased allochthonous inputs from the forested riparian zone and subsequent decomposition. Oyster, near-oyster, and fence sediments had NH_4^+ ~4-9 times higher at Spencer's Creek than at St. Jerome Creek, whereas reference sediment NH_4^+ was only a factor of 2 times higher. No significant differences in NH_4^+ were detected among St. Jerome Creek sediment types ($p > 0.05$). The two sites did not differ significantly in sediment organic N, but only at Spencer's Creek did oyster and fence sediments have significantly higher organic N than reference sediments, 3483 ± 157 and 4560 ± 387 , respectively, versus 2121 ± 466 (p

< 0.01). No significant correlation between sediment N_2 production and sediment BOD, COD or NH_4^+ was detected when considering both aquaculture sites or sites individually ($p > 0.05$).

Oyster and fence sediment pore water NH_4^+ concentrations were 3-6 times higher than reference and near oyster sediments, $24.73 \pm 3.45 \text{ mg L}^{-1}$ and $38.90 \pm 6.10 \text{ mg L}^{-1}$, respectively ($n = 40$, $p < 0.001$, Fig. 3.4). St. Jerome Creek had significantly higher pore water NH_4^+ concentrations than Spencer's Creek, by 9.90 mg L^{-1} , but no interactions between sample type and site or season were detected ($p < 0.03$). A significant negative correlation was detected between sediment N_2 production and sediment pore water NH_4^+ concentrations ($r = -0.47$, $p < 0.01$). Non-linear regression analysis revealed that pore water NH_4^+ concentrations increased in sediments when N_2 production was low and decreased as N_2 production increased (Fig. 3.4). No detectable effect of oyster biodeposit treatment on sediment pore water NO_x concentrations were observed, and mean levels were $0.08 \pm 0.02 \text{ mg L}^{-1}$ (Table 3.4). Site was a significant factor in determining sediment NO_x levels, which were 3 times higher at St. Jerome Creek than Spencer's Creek, a 0.08 mg L^{-1} difference (Table 3.4, $p < 0.05$). No significant correlation was detected between sediment N_2 production and sediment pore water NO_x concentrations ($p > 0.05$).

Effect of oyster biodeposition on sediment N₂ production

¹⁵N Stable Isotope sediment analysis

Sediment type (varying levels of oyster biodeposit treatment) was a significant factor in determining DNF, AMX, and N₂ production rates when considering type, site, and season, but no interactions between type and site or between site, type, and season were detected (Two-way, Three-way ANOVA, $p < 0.05$). DNF accounted for the vast majority of total N₂ production, 86.3-100%, compared to AMX which contributed 0.0-13.8%; therefore, differences in N₂ production rates detected among sediment types were due to differences in DNF (Table 3.5). Combining seasonal data, no significant differences in N₂ production were detected in oyster sediments between St. Jerome Creek and Spencer's Creek, $1.06 \pm 0.25 \text{ mmol N m}^{-2} \text{ d}^{-1}$ and $0.75 \pm 0.24 \text{ mmol N m}^{-2} \text{ d}^{-1}$, respectively; therefore, we did not find that aquaculture site significantly impacted N₂ production rates (One-way ANOVA, $p > 0.05$).

Summer oyster sediments at St. Jerome Creek had N₂ production rates 1.41-1.65 mmol N m⁻² d⁻¹ lower than both reference and near-oyster sediments which did not differ significantly from one another (Tukey's HSD, $p < 0.04$, Table 3.6). In fall and spring at St. Jerome Creek, N₂ production rates in oyster and reference sediments did not differ significantly (One-way ANOVA, $p > 0.05$). Forced biodeposit accumulation did not induce significantly higher rates as expected; rather, fenced biodeposit sediments did not differ from any other sample type and had N₂ production rates 1.62 mmol N m⁻² d⁻¹ lower than reference sediments (Table 3.5). Biodeposits had very little activity, $0.04 \pm 0.12 \text{ mmol N m}^{-2} \text{ d}^{-1}$, lower than both reference and near-oyster sediments (Tukey's HSD, $p < 0.003$, Fig. 3.5). In summer and fall at Spencer's Creek, oyster

sediment N₂ production rates were 0.85-1.09 mmol N m⁻² d⁻¹ lower than reference sediments; however, these differences were not statistically significant due to large variation and small sample sizes (One-way ANOVA, $p > 0.05$, Table 3.5). Biodeposits at Spencer's Creek also had low N₂ production, and fenced biodeposit sediments did not differ from any other sediment types or biodeposits with N₂ production rates 0.40 mmol N m⁻² d⁻¹ lower than the reference sediments (LSD, One-way ANOVA, $p > 0.05$).

MIMS analysis of sediments

At both sites in mid-summer, oyster sediments had higher N₂ production rates than reference sediments, whereas no differences were detected between oyster and reference sediments in spring (One-way ANOVA, $p < 0.005$). Oyster sediment N₂ production rates were 0.99 mmol N m⁻² d⁻¹ higher than the reference stations, which had very little activity (0.25 ± 0.08 mmol N m⁻² d⁻¹), N₂ production rates in St. Jerome Creek reference sediments were 0.34 mmol N m⁻² d⁻¹ higher than at Spencer's Creek, but oyster sediment rates were not significantly different between the two sites, and no interaction between sample type and site was detected (One-way ANOVA, $p < 0.006$).

There was an interaction between sediment type and season at St. Jerome Creek. Spring N₂ production rates were uniform across oyster and reference sediments, but summer oyster rates were 1.14 mmol N m⁻² d⁻¹ higher than reference sediments (Two-way ANOVA, $p = 0.04$). N₂ production in oyster sediments increased by 1.6-fold in summer compared to the spring when no N₂ production activity was detected (Table 3.6). This same trend was observed at Spencer's Creek where oyster

N₂ production was 0.85 mmol N m⁻² d⁻¹ higher than reference sediments (Mann-Whitney U, p = 0.05).

Sediment oxygen demand (SOD) and NH₄⁺ flux from sediment cores were strongly correlated with N₂ production rates and tended to increase as N₂ production increased from sediment cores (Fig. 3.6, Pearson, p < 0.000, R > 0.75), whereas NO_x had a weak negative correlation with N₂ production rates, decreasing as N₂ production rates increased (Pearson, p < 0.02, r = 0.452). Linear regression analysis revealed a significant relationship between N₂ production rates and both SOD and NH₄⁺, explaining 58% and 56% of variation in N₂ production rates respectively (Linear regression, p < 0.01). An interaction between SOD and NH₄⁺ also was detected (p < 0.05) as well as a strong positive correlation between SOD and NH₄⁺, which was exponential to a maximum related by the following equation (Fig. 3.7):

[Eq. 1]

$$SOD \text{ (mmol O}_2 \text{ m}^{-2} \text{ d}^{-1}) = 38.6870 * 1 - e^{-0.3030 * NH_4^+ \text{ (mmol m}^{-2} \text{ d}^{-1})} \quad (R^2 = 0.67)$$

The relationship between SOD and NH₄⁺ followed a linear pattern until higher OM loads were added. Regardless of the OM load, SOD did not increase above ~40 mmol O₂ m⁻² d⁻¹, whereas NH₄⁺ continued to increase. In the range of OM loading typically found in nature, the relationship between the two variables appears to be linear, but SOD reaches a maximum as sediment likely becomes anoxic with higher OM loads experienced at the oyster sites. Sediments treated with the highest OM loads (biodeposit additions to sediment cores and fenced biodeposit sediments), had SOD

rates similar to those found at the oyster sites, $\sim 40 \text{ mmol O}_2 \text{ m}^{-2} \text{ d}^{-1}$, but these sediments continued to release NH_4^+ , more than doubling NH_4^+ efflux rates to $\sim 12\text{-}15 \text{ mmol m}^{-2} \text{ d}^{-1}$ (Fig. 3.6).

Sediments treated with fenced biodeposits had significantly higher SOD and NH_4^+ flux rates than all other sample types, indicating that oyster biodeposition elicits higher rates of OM decomposition and promotes recycling of N in the form of NH_4^+ ($p < 0.01$). Fenced biodeposit sample SOD rates were 2-3 times higher than rates observed in sediments underneath oyster rafts, at the reference stations, or sediments treated with biodeposit additions, consuming $40.56 \text{ mmol O}_2 \text{ m}^{-2} \text{ d}^{-1}$. This effect also was observed in SOD rates underneath oyster rafts, which were 1.5 times the reference station (21.64 ± 5.82 versus 14.07 ± 3.72) and increased significantly in summer compared to spring, 4-fold underneath oyster rafts and 3-fold at the reference station ($p < 0.000$, Table 3.6). Flux rates of NH_4^+ were 4-6 times higher in fenced biodeposit sediments than in oyster, reference, or biodeposit addition sediments, and 2-4 times higher in oyster than reference sediments but these differences were not significant (Table 3.6).

We observed a stepwise increase in NH_4^+ flux rates in sediments treated with oyster biodeposits. Compared to reference sediments, NH_4^+ flux rates underneath rafts were 2-4 times higher, biodeposit addition sediments were 2-6 times higher, and fenced biodeposit sediments were 2 times higher, indicating that oyster biodeposits are recycled and released back to the water column as DIN (Table 3.6). Sediments consumed NO_x at the aquaculture sites, whereas NO_x was either released or was consumed at a lower rate at the reference sites ($p < 0.04$). No differences between

sample types or oyster biodeposit treatments were detected in TON sediment fluxes ($p > 0.05$).

No significant difference was detected between the total N_2 production rates measured by the ^{15}N and MIMS methods, and no interaction between sample type and method was detected in spring at St. Jerome Creek when comparative analysis was conducted using both methods on replicate oyster and reference sediments (Two-way ANOVA, $p > 0.05$). Overall, combining N_2 production data across seasons measured by both methods, reference and oyster sediments did not differ significantly, although reference sediments had slightly higher rates, $1.26 \pm 0.20 \text{ mmol N m}^{-2} \text{ d}^{-1}$ and $0.89 \pm 0.15 \text{ mmol N m}^{-2} \text{ d}^{-1}$, respectively (Tables 3.5, 3.6, One-way ANOVA, $P > 0.05$). Season and site were significant factors, although these differences were marginal (Three-way ANOVA, $p < 0.05$). N_2 production rates were $0.14 \text{ mmol N m}^{-2} \text{ d}^{-1}$ higher at St. Jerome Creek than Spencer's Creek, and fall rates were higher than spring or summer rates, $1.49 \pm 0.21 \text{ mmol N m}^{-2} \text{ d}^{-1}$ versus $0.56 \pm 0.19 \text{ mmol N m}^{-2} \text{ d}^{-1}$ and $1.13 \pm 0.18 \text{ mmol N m}^{-2} \text{ d}^{-1}$, respectively ($p < 0.02$).

Effect of biodeposit addition to estuarine sediments

Due to low N_2 production rates, we found no evidence that oyster biodeposits deliver to the sediments a source of immediate inocula that enhance DNF or AMX rates and drive increased N loss from the system. Although DNF and AMX activity both were detected in oyster biodeposits, the rates were low compared to sediment rates, ranging from 0.07 ± 0.07 to $0.28 \pm 0.07 \text{ mmol N m}^{-2} \text{ d}^{-1}$ (Table 3.5). Post hoc testing showed biodeposits were significantly lower than both reference and near-oyster sediments

(LSD, One-way ANOVA, $p = 0.04$). The fenced biodeposit sediments that received an *in situ* biodeposition rate of twice the measured non-fenced rate, $\sim 28.2 \text{ mmol TN m}^{-2} \text{ d}^{-1}$ at St. Jerome Creek and $\sim 12.2 \text{ mmol TN m}^{-2} \text{ d}^{-1}$ at Spencer's Creek, exhibited intermediate N_2 production rates at both sites ($1.02 \pm 0.21 \text{ mmol N m}^{-2} \text{ d}^{-1}$ and $1.26 \pm 0.42 \text{ mmol N m}^{-2} \text{ d}^{-1}$, respectively), and did not differ significantly from any other sediment types at either site. These results contrast with mid-summer fence experiments using MIMS at St. Jerome Creek when fenced sediments had N_2 production rates 1.6-fold higher than oyster sediments and 6.0-fold higher than reference sediments (Fig. 3.8, Two-way ANOVA, Tukey HSD, $p < 0.03$). These results parallel the summer findings at both aquaculture sites where significantly higher N_2 production rates were observed in oyster sediments compared to reference rates.

Considering all biodeposit addition experiments, no significant difference in N_2 production was observed among the three levels of biodeposit additions (One-way ANOVA, $P > 0.05$). However, a seasonal trend was observed between spring and summer. In the spring at St. Jerome Creek, N_2 production rates decreased with increasing additions of biodeposits. The highest N_2 production rate was observed with 0.16 g biodeposit addition ($0.97 \text{ mmol N m}^{-2} \text{ d}^{-1}$), and the rate decreased with increasing amounts of biodeposits (0.64 g and 5.00 g) to $0.52 \text{ mmol N m}^{-2} \text{ d}^{-1}$ and $0.29 \text{ mmol N m}^{-2} \text{ d}^{-1}$, respectively (Fig. 3.8). In the summer analysis, the opposite trend was observed at both sites. At St. Jerome Creek, N_2 production rates increased with each increment of addition from $0.14 \text{ mmol N m}^{-2} \text{ d}^{-1}$ for the 0.16 g level of biodeposit addition to a maximum of $2.35 \text{ mmol N m}^{-2} \text{ d}^{-1}$ when 5.0 g of biodeposits were added to a reference sediment core. At Spencer's Creek, the N_2 production rates were lower overall, but the

same trend was observed, increasing from a low of $0.38 \text{ mmol N m}^{-2} \text{ d}^{-1}$ in cores with the least amount of added biodeposit to a maximum of $1.69 \text{ mmol N m}^{-2} \text{ d}^{-1}$ at the highest level of biodeposit addition. The mean N_2 production rate for sediments to which biodeposits were added (treated as a single sample type), did not differ significantly from either the reference or oyster sediments at either site for the three seasonal experiments (One-way ANOVA, $p > 0.05$).

Relationship of N_2 production and oyster biodeposition

A slight but significant relationship was detected between N_2 production and oyster biodeposition of TN, but none was detected between N_2 production and rates of either oyster biodeposition ($\text{g dwt m}^{-2} \text{ d}^{-1}$) or OM biodeposition ($\text{g dwt m}^{-2} \text{ d}^{-1}$). This relationship may be qualitative rather than strictly quantitative, as significance was detected between N_2 production and PON biodeposition ($R^2 = 0.23$, $p < 0.02$, Pearson) but no significant relationship was detected for DIN. Linear regression analysis showed that 10% of variation in N_2 production could be explained by variation in oyster biodeposit TN (Fig. 3.9, $R^2 = 0.10$). We also observed a weak positive linear correlation between oyster TN biodeposition rates and total N_2 production, with variation in TN biodeposition explaining 21% of variation in N_2 production (Fig. 3.10, $R^2 = 0.21$). The two variables tended to increase together and had a slight but significant positive correlation (Pearson $r = 0.459$, $p < 0.024$).

DISCUSSION

Effect of oyster biodeposit TN loading

Given the accelerated rate of TN delivery to sediments *via* oyster biodeposition, it is reasonable to expect that increasing TN inputs would stimulate N_2 production, thereby enhancing the permanent removal of excess N from the system. A synthesis of data across a range of lake, coastal marine, estuarine, and continental shelf ecosystems indicates that sediment DNF increases linearly with TN inputs, explaining as much as 77% of variation in DNF (Seitzinger 1988, 2000, Seitzinger et al. 2006). However, at the scale of an oyster aquaculture site in Chesapeake Bay, we found N_2 production and oyster-based TN input rates to be weakly correlated, and neither seasonal increases in the flux of oyster biodeposits nor biodeposit OM translated into increases in N_2 production. The mass flux of TN via oyster biodeposition, and in particular the flux of organic N, have a weak positive correlation with increases in N_2 production, explaining 21% and 23% of the variation, respectively. Therefore, a model predicting the amount of N loss from the system through oyster-stimulated N_2 production based on percentage of biodeposit TN subject to N_2 production could not be developed because these variables did not provide adequate predictive power.

Based on the data gathered, we estimate that a typical floating raft oyster aquaculture site in Chesapeake Bay (200,000 oysters at 286 oysters m^{-2}) produces ~1816 g biodeposits ($dwt\ m^{-2}\ yr^{-1}$) composed of 1.3% TN, resulting in annual flux ~1544 $mmol\ N\ m^{-2}\ yr^{-1}$ (Table 3.1). Oysters deposit 1.7-14.1 $mmol\ N\ m^{-2}\ d^{-1}$ to sediments at an aquaculture site during periods of active suspension-feeding while N removal *via* sediment N_2 production ranged from 0.63 -1.56 $mmol\ N\ m^{-2}\ d^{-1}$ for sediments directly

underneath oyster rafts. Considering N_2 production as a percentage of the oyster biodeposit TN load, one might conclude that 7.04 - 40.94% of the TN in oyster biodeposits is removed through N_2 production; however, because the rates underneath oyster rafts do not generally exceed baseline rates observed from reference sediments, such a conclusion would be erroneous. We did not find that any significant percentage of oyster biodeposit N is removed through N_2 production. Rather, given high concentrations of NH_4^+ found in the biodeposits and ~2-fold increases in NH_4^+ detected in laboratory and field studies of the effects of increasing levels of biodeposits from oyster sediments, our results indicate that biodeposits are rapidly recycled and release nutrients into the water column as DIN (Table 3.3).

There is precedence for this interpretation. Previous studies of bivalve reefs and aquaculture sites have found that these systems may act as sinks for particulate organic N (PON) and sources for dissolved inorganic N (DIN), finding that nutrients are regenerated and ambient water NH_4^+ levels increase in the vicinity (Dame et al. 1984, Kaspar et al. 1985, Dame & Dankers 1988, Dame et al. 1989, Prins & Smaal 1990, Dame et al. 1992, Hatcher et al. 1994, Dame 1996). We found that SOD increases linearly with sediment NH_4^+ efflux rates, but with concentrated OM loading in the fence and biodeposit addition experiments, we observed that SOD reaches a maximum at ~30-40 $mmol\ O_2\ m^{-2}\ d^{-1}$ while NH_4^+ continues to increase (Fig. 3.7). From measures of sediment NH_4^+ we found that oyster and fence sediment levels increased 1-3 times and 3-6 times above reference and near-oyster sediments, respectively, at the high C site, indicating that biodeposits mineralized in the sediments. Evidence of oyster biodeposit ammonification also was observed in sediment pore water NH_4^+ concentrations, which

were 3-6 times higher than reference and near oyster sediments, whereas pore water NO_x concentrations were not different among biodeposit treated sediments and did not fluctuate seasonally (Fig. 3.4).

Dissimilatory nitrate reduction to ammonia (DNRA) may play an important yet currently undetermined role in recycling biodeposited N as it is understood to be favored over DNF in NO_3^- limited but labile-C rich sediments (Tiedje 1988, Burgin and Hamilton 2007, Tobias et al. 2001). There is some evidence of enhanced DNRA under finfish aquaculture cages attributed to high organic loading and extant increases in sulfate reduction (Christensen et al. 2000). It also is possible that denitrification was incomplete in the oyster sediments, and that gaseous N was removed in the form of NO or N_2O , which were not measured in this study. Oyster biodeposition transfers large quantities of nutrients and energy from the water column to the benthos and increases the abundance and diversity of epibenthic macrofauna (D'Amours et al. 2008). A significant portion of oyster biodeposit nutrients may enter the food web and contribute to supporting biodiversity, an ecosystem service not considered in this study. Our results and interpretation are supported by evidence from other studies that oysters may serve as nutrient retention rather than removal mechanisms, promoting release of DIN that in turn further promotes phytoplankton growth (Asmus & Asmus 1991, Dame & Libbes 1993, Dame 1996). This interpretation is logical in light of the N-limited systems in which suspension-feeders evolved and the fact that these systems have only geologically recently become overloaded with excess nutrients.

Effect of oyster biodeposition on sediment N₂ production rates

At two oyster aquaculture sites representing sediment physicochemical and hydrological extremes under which oysters are grown in the Bay, we did not find evidence that the capacity of the sediment microbial community to remove N is substantially expanded by the addition of biodeposits. Overall, the N removal capacity of sediments at an oyster aquaculture site was no different than if no oyster cultivation activity were taking place. Combining results from two methods at oyster and reference stations, sediment N₂ production rates were lowest in spring and increased in summer and fall. Mean N₂ production rates of oyster sediments in spring, summer, and fall were 0.37 ± 0.27 mmol N m⁻² d⁻¹, 1.07 ± 0.19 mmol N m⁻² d⁻¹, and 1.07 ± 0.27 mmol N m⁻² d⁻¹, compared to reference sediments which were 0.75 ± 0.37 mmol N m⁻² d⁻¹, 1.20 ± 0.26 mmol N m⁻² d⁻¹, and 1.90 ± 0.37 mmol N m⁻² d⁻¹, respectively.

The sediment N₂ production rates measured at the reference stations are similar to other reported estuarine rates and somewhat higher than previously reported from sites studied Chesapeake Bay (Kemp et al. 1990, Kana et al. 1998, Kana et al. 2006, Fennel et al. 2008). Although the two methods used in this study did not differ in cross-validation analysis, MIMS rates were more variable and detected no N₂ production at the oyster site in spring, whereas a rate of 0.63 ± 0.27 mmol N m⁻² d⁻¹ was detected using the ¹⁵N method, indicating that the system is likely NO₃⁻ limited (Fig. 3.5). These findings for oyster aquaculture are similar to studies of the effect of suspended mussel cultivation on sediment DNF rates. In a New Zealand bay, DNF was inhibited as sediments became anoxic from OM deposition. Likewise, in studies of clam and

suspended mussel cultivation in the Mediterranean Sea, no substantial increases above reference sediments were reported (Christensen et al. 2003, Minjeaud et al. 2009).

Our results illustrate that oyster biodeposition may serve to maintain elevated N_2 production rates in the warmest months underneath oyster rafts when baseline reference rates dropped by 88%. In early-summer, oyster sediment N_2 production rates were lower at both aquaculture sites than the corresponding reference and near-oyster sediments, indicating that N_2 production is at times inhibited by oyster biodeposition and that the area affected by biodeposits is limited to within a few meters of an oyster aquaculture site, regardless of variable flow conditions between the two sites. In contrast, N_2 production rates were higher in oyster than reference sediments in mid-summer at both sites. This difference was moderate yet significant, $1.14 \text{ mmol N m}^{-2} \text{ d}^{-1}$ higher than the reference site at St. Jerome Creek, and $0.85 \text{ mmol N m}^{-2} \text{ d}^{-1}$ higher at Spencer's Creek (Fig. 3.5). This comparative change from early to mid-summer is due to a drop in reference sediment N_2 production rates from early to mid-summer. For example, at St. Jerome Creek, reference sediment rates decreased significantly from $2.15 \pm 0.26 \text{ mmol N m}^{-2} \text{ d}^{-1}$ to $0.25 \pm 0.08 \text{ mmol N m}^{-2} \text{ d}^{-1}$, whereas oyster sediments remained unchanged, $1.07 \pm 0.21 \text{ mmol N m}^{-2} \text{ d}^{-1}$ (Tables 3.5, 3.6, $P < 0.000$).

Effect of concentrated oyster biodeposits

The field results were replicated across active oyster suspension-feeding seasons at different sites and were supported by *in situ* field and laboratory oyster biodeposit addition experiments. Oyster biodeposits do not appear to be a significant source of inocula of denitrifier or anammox microorganisms to the sediments below and

we found no evidence that biodeposits alone drive higher rates of N_2 production and thus N removal from the system by transferring significantly active microbes from pelagic to benthic zones. The summer trend detected in the biodeposit-addition core experiments was similar to the findings of Newell et al. (2002) (Fig. 3.8). However, the opposite trend was observed in spring, when N_2 production tended to decrease with increases in biodeposit additions in sediment cores at St. Jerome Creek. These trends may indeed reflect an induced effect. However, considering all three biodeposit addition experiments, no significant difference was detected between the three levels of biodeposit additions (One-way ANOVA, $P > 0.05$). A similar seasonal trend also was observed in the fenced biodeposit experiments. The artificially induced rate we observed ($2.50 \text{ mmol N m}^{-2} \text{ d}^{-1}$) can be taken to represent the maximum *in situ* capacity of the Chesapeake Bay system to remove N by controlling for the effect of flow and maximizing the concentration of TN in sediments. Even this forced accumulation of biodeposits did not increase N_2 production above sediment baseline reference or oyster rates at either site in spring, but rates were significantly increased in summer at St. Jerome Creek, $2.08 \text{ mmol N m}^{-2} \text{ d}^{-1}$ above reference sediment rate (Table 3.6).

Quantification of N loss potential

Previous modeled estimates of N removal resulting from oyster biodeposit-stimulated increases in sediment nitrification-denitrification are $0.5 \text{ g per g}^{-1} \text{ DW oyster yr}^{-1}$ (Newell et al. 2005), translating to a potential N removal rate of 250 kg N yr^{-1} ($551 \text{ lbs N yr}^{-1}$) at an oyster aquaculture site producing 5×10^5 oysters covering 1750 m^2 . We observed at two commercial-scale floating raft aquaculture sites in Chesapeake Bay

that $\sim 37.8 \text{ kg N yr}^{-1}$ are transferred to sediments through oyster biodeposition and that under these conditions, N removal *via* sediment total N_2 production at these sites ranged from $0.49\text{-}12.60 \text{ kg N yr}^{-1}$, compared to $2.27\text{-}16.72 \text{ kg N yr}^{-1}$ at a reference site of the same area. The difference between previously modeled estimates and our empirical results can be explained by our observation that there is a limited capacity of the sediments to remove N *via* N_2 production. Although we were able to artificially induce increased N_2 production rates in sediments in summer at both sites (biodeposit addition core experiments and fenced biodeposits experiment), this does not reflect the natural state at an oyster aquaculture site. We estimate $\sim 2.50 \text{ mmol N m}^{-2} \text{ d}^{-1}$ is likely the maximum capacity of this system to remove N *via* sediment N_2 production, a value which approximates the rates observed in summer with the highest biodeposit addition (5.0 g wet wt) and fenced biodeposits experiment. Oyster sediment N_2 production rates in mid-summer were $0.85\text{-}1.14 \text{ mmol N m}^{-2} \text{ d}^{-1}$ above baseline reference rates, a comparative increase in the warmest months when reference rates decreased at both sites (Table 3.6). The observed decline sediment N_2 production rates during summer concurs with the results of Kemp et al. (1990) who found that DNF rates in mesohaline sediments of Chesapeake Bay are higher in spring and fall and decrease to minimal levels during the summer.

CONCLUSIONS

This study demonstrates that oyster biodeposition does not induce significant increases in sediment N_2 production rates above baseline levels. Overall, we estimate N removal rates *via* sediment N_2 production at an aquaculture site of 5×10^5 oysters (1750 m^2) to

range from 0.49-12.60 kg N yr⁻¹, compared to 2.27-16.72 kg N yr⁻¹ at a reference site of the same area. When increased N₂ production rates were detected in one mid-summer sampling at both sites, this increase was modest (0.85-1.14 mmol N m⁻² d⁻¹ above baseline reference rates) and given reduced rates in other seasons, this increase did not translate into enhanced N removal. The N removal potential *via* sediment N₂ production is limited by sediment microbial capacity and area, making the issue of enhancing a sediment N sink a matter of scale relative to system wide inputs. We found the maximum capacity of the system to be ~2.5 mmol N m⁻² d⁻¹ as demonstrated by the fence and laboratory biodeposition addition experiments. The area impacted by oyster biodeposition is limited to within 5 m of an oyster array, regardless of variable flow conditions, making N removal minimal based on the limited capacity of sediments to reach N₂ production rates above 2-3 mmol N m⁻² d⁻¹. At the scale of an estuary, substantial portions of TN inputs may be removed through sediment N₂ production; however, at the scale of an oyster aquaculture site in Chesapeake Bay, we found that the TN inputs through oyster biodeposition and N removal *via* sediment N₂ production are only weakly correlated and limited by impacted sediment area. A possible best-case scenario for the Bay can be estimated assuming one-quarter of the Bay were exposed to similar rates of oyster biodeposition and sustained a 1.14 mmol N m⁻² d⁻¹ increase in N₂ production over a two month period. Under these assumptions and assuming no inhibition in other seasons, we estimate 4.10 X 10⁶ kg N yr⁻¹ (6.73-9.03 X 10⁶ lbs N yr⁻¹) could be removed from the system, equating to 3.2% of Bay N load estimates.

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FIGURE CAPTIONS

Figure 3.1. Tributary locations of two field sites where Eastern oysters were cultivated in Chesapeake Bay, reference stations were located ~350 m from aquaculture sites within the same tributaries.

Figure 3.2. Diagrams of biodeposit catchment device and biodeposit fence used to (A) quantify the mass flux of nutrients and (B) force accumulation of oyster biodeposition to estuarine sediments in Chesapeake Bay.

Figure 3.3. Seasonal biodeposition rates for Eastern oysters at aquaculture sites in Chesapeake Bay (mean dry weight \pm SE).

Figure 3.4. Dissolved N concentrations (mean \pm SE) of sediment pore water samples at two Eastern oyster aquaculture sites in Chesapeake Bay for samples directly underneath oyster aquaculture rafts (Oyster), control site ~250 m from oyster aquaculture site (Reference), 5-10 m outside of oyster aquaculture array (Near Oyster), forced accumulation of oyster biodeposits (Fence), and interstitial water from freshly collected oyster biodeposits (Biodeposits). (A) NH_4^+ mg L^{-1} concentrations (B) NO_x mg L^{-1} concentrations (C) Regression analysis of pore water NH_4^+ concentrations versus sediment N_2 production rates.

Figure 3.5. Direct measures of total N_2 production ($\text{mmol N m}^{-2} \text{d}^{-1}$; mean \pm SE) using ^{15}N and MIMS methods in sediments treated with various levels of oyster biodeposition at St. Jerome Creek in Chesapeake Bay: directly underneath oyster aquaculture rafts (Oyster), ~250 m from oyster aquaculture site (Reference), forced accumulation of oyster biodeposits

(Fence), 5-10 m outside of oyster aquaculture array (Near-oyster), and collected oyster biodeposits (Biodeposits).

Figure 3.6. Linear regression analysis of sediment N_2 production versus SOD and NH_4^+ efflux rates from MIMS sediment core samples collected from two oyster cultivation sites in Chesapeake Bay, respective reference sites and biodeposit addition experiments.

Figure 3.7. Regression analysis for SOD and NH_4^+ efflux from MIMS sediment cores for four oyster biodeposit treatments: Biodeposit additions to sediment cores (Biodeposit Additions), forced in situ biodeposit accumulation (Fence), underneath oyster rafts (Oyster), and baseline reference (Reference).

Figure 3.8. Oyster biodeposit addition experiment results for two aquaculture sites in Chesapeake Bay. Increasing quantities (g wet weight) of oyster biodeposits were added to reference sediment cores and N_2 production rates were measured using MIMS. In summer at both sites, a trend of increasing N_2 production rates with increasing addition was observed (B and D), but in spring the opposite trend was observed at St. Jerome Creek (B). Dashed lines are mean N_2 production rates of reference cores used in the experiments. No significant difference among the three levels of treatment was detected (C) when combining the three experiments ($P > 0.05$).

Figure 3.9. Oyster biodeposit TN and sediment N_2 production rates as compared to oyster biodeposition rates for Eastern oysters at two aquaculture sites in Chesapeake Bay.

Figure 3.10. Linear regression analysis of oyster biodeposit TN and sediment N₂ production rates for Eastern oysters at two aquaculture sites in Chesapeake Bay.

Table 3.1. Seasonal and annual oyster biodeposition rates and biodeposit nitrogen rates (TN) (mean \pm SE) measured at two aquaculture sites in Chesapeake Bay.

		TN [‡]			Biodeposits (dwt) [‡]	
		g Oyster ⁻¹ d ⁻¹	mmol Oyster ⁻¹ d ⁻¹	mmol m ⁻² d ⁻¹	g Oyster ⁻¹ d ⁻¹	g m ⁻² d ⁻¹
St. Jerome's Creek, MD						
	Summer 08	0.0008 \pm 0.0001	0.05 \pm 0.01	14.09 \pm 2.69	0.06 \pm 0.01	15.86 \pm 3.12
	Fall 08	0.0003 \pm 0.0001	0.02 \pm 0.00	4.84 \pm 1.30	0.01 \pm 0.00	3.88 \pm 1.16
	Spring 09	0.0003 \pm 0.0001	0.02 \pm 0.01	3.90 \pm 0.84	0.04 \pm 0.02	7.08 \pm 1.12
	Summer 09	0.0008 \pm 0.0002	0.06 \pm 0.01	10.88 \pm 1.26	0.06 \pm 0.01	11.21 \pm 1.76
Spencer's Creek, VA						
	Summer 08	0.0003 \pm 0.0001	0.02 \pm 0.01	6.08 \pm 1.56	0.02 \pm 0.00	4.76 \pm 1.03
	Fall 08	0.0001 \pm 0.0000	0.01 \pm 0.00	1.68 \pm 0.24	0.01 \pm 0.00	1.58 \pm 0.23
Seasonal Mean						
	Spring	0.0003 \pm 0.0001	0.02 \pm 0.01	3.90 \pm 0.84	0.04 \pm 0.02	7.08 \pm 1.12
	Summer	0.0007 \pm 0.0001	0.05 \pm 0.01	10.39 \pm 1.34	0.05 \pm 0.01	10.66 \pm 1.68
	Fall	0.0002 \pm 0.0000	0.01 \pm 0.00	2.86 \pm 0.73	0.01 \pm 0.00	2.44 \pm 0.58
Annual Mean		0.0004 \pm 0.0001	0.03 \pm 0.01	6.83 \pm 1.00	0.04 \pm 0.01	7.44 \pm 1.12
‡ Biodeposition Rate yr ⁻¹		0.1080	7.20	1543.50	9.00	1816.20

[‡] Area estimates assume 0.3 m between oyster rafts and 10% annual mortality.

[‡] Estimate calculation is sum of mean seasonal flux * 90 days.

Table 3.2. Seasonal nutrient content of oyster biodeposits (mean \pm SE) measured at two aquaculture sites in Chesapeake Bay. NO_x (data not shown) were below detection limit ($< 0.0001 \text{ mg L}^{-1}$).

Oyster Biodeposit Nutrient Content									
Nutrient	Spencer's Creek		Summer 2008	St. Jerome's Creek			Spring	Seasonal Mean	
	Summer 2008	Fall 2008		Fall 2008	Spring 2009	Summer 2009		Summer	Fall
TN (%)	1.74 \pm 0.09	1.49 \pm 0.00	1.26 \pm 0.07	1.78 \pm 0.05	0.75 \pm 0.06	1.40 \pm 0.08	0.75 \pm 0.06	1.46 \pm 0.07	1.60 \pm 0.06
Organic N (%)	1.66 \pm 0.09	1.37 \pm 0.00	1.12 \pm 0.04	1.63 \pm 0.09	0.68 \pm 0.05	1.14 \pm 0.08	0.68 \pm 0.05	1.29 \pm 0.08	1.47 \pm 0.06
NH ₄ ⁺ (%)	0.08 \pm 0.00	0.12 \pm 0.00	0.14 \pm 0.03	0.15 \pm 0.04	0.07 \pm 0.01	0.25 \pm 0.04	0.07 \pm 0.01	0.16 \pm 0.03	0.13 \pm 0.01
TP (%)	0.17 \pm 0.01	0.09 \pm 0.00	0.16 \pm 0.01	0.29 \pm 0.02	0.10 \pm 0.00	0.18 \pm 0.01	0.10 \pm 0.00	0.17 \pm 0.01	0.17 \pm 0.04
TC (%)	12.92 \pm 1.20	9.92 \pm 0.31	9.99 \pm 0.72	8.47 \pm 0.21	5.56 \pm 0.16	8.43 \pm 0.96	5.56 \pm 0.16	10.29 \pm 0.75	9.38 \pm 0.33
OM (%)	25.51 \pm 1.61	23.07 \pm 0.00	18.61 \pm 0.21	19.13 \pm 0.05	19.60 \pm 0.27	19.63 \pm 0.81	19.60 \pm 0.27	21.12 \pm 1.01	21.59 \pm 0.72
C:N	8.79 \pm 1.08	7.76 \pm 0.24	9.38 \pm 1.01	5.57 \pm 0.11	8.81 \pm 0.57	7.16 \pm 0.94	8.81 \pm 0.57	8.35 \pm 0.60	6.94 \pm 0.43

Table 3.3. Sediment and oyster biodeposit Chla, BOD, COD, NH₄⁺ and organic N levels for various levels of biodeposit treatments at two Eastern oyster aquaculture sites in Chesapeake Bay.

		Chla µg cm ⁻³	BOD mg/kg	COD mg/kg	NH₄⁺ mg/kg	Org N mg/kg
Reference	St. Jerome	19.4 ± 6.2	200 ± 22	7622 ± 524	132 ± 52	1387 ± 786
	Spencer	6.1 ± 0.5	405 ± 43	34436 ± 3438	57 ± 50	2121 ± 466
	Total	*12.2 ± 3.7	*354 ± 69	*21436 ± 3112	93 ± 37	1869 ± 578
Near Oyster	St. Jerome	10.2 ± 1.5	232 ± 42	7230 ± 887	33 ± 4	568 ± 45
	Spencer	5.6 ± 0.4	480 ± 37	41148 ± 4029	133 ± 16	2292 ± 245
	Total	*7.5 ± 4.2	*378 ± 59	*22435 ± 3319	*90 ± 51	*1518 ± 676
Oyster	St. Jerome	11.9 ± 1.2	398 ± 125	5319 ± 487	22 ± 3	2922 ± 2330
	Spencer	13.3 ± 1.7	669 ± 52	37934 ± 2142	198 ± 28	3483 ± 157
	Total	12.7 ± 3.9	*557 ± 47	*23438 ± 2979	*116 ± 34	3296 ± 526
Fence	St. Jerome	24.3 ± 7.3	241 ± 81	7564 ± 2357	43 ± 10	700 ± 106
	Spencer	11.8 ± 1.6	545 ± 18	27567 ± 12351	367 ± 61	4560 ± 387
	Total	18.6 ± 7.3	362 ± 124	15565 ± 7994	*190 ± 102	*2455 ± 1426
Biodeposits	St. Jerome	106.4 ± 21.5	643 ± 54	14694 ± 395	1470 ± 228	12733 ± 1082
	Spencer	74.2 ± 23.3	959 ± NA	19479 ± NA	1225 ± 289	14893 ± 962
	Total	90.3 ± 6.0	748 ± 160	16289 ± 10320	1338 ± 93	13896 ± 1312

*Indicates significant differences between aquaculture sites with respect to sample type (One-way ANOVA, P < 0.05)

Table 3.4. Dissolved N concentrations (mean \pm SE) of sediment pore water samples at two Chesapeake Bay Eastern oyster aquaculture sites treated with varying levels of oyster biodeposition: directly underneath oyster aquaculture rafts (Oyster), control site ~250 m from oyster aquaculture site (Reference), 5-10 m outside of oyster aquaculture array (Near Oyster), forced accumulation of oyster biodeposits (Fence), and interstitial water from freshly collected oyster biodeposits (Biodeposits).

	St. Jerome Creek		Spencer's Creek		Total	
	NH ₄ ⁺ mg L ⁻¹	NO _x mg L ⁻¹	NH ₄ ⁺ mg L ⁻¹	NO _x mg L ⁻¹	NH ₄ ⁺ mg L ⁻¹	NO _x mg L ⁻¹
Oyster	28.61 \pm 4.45	0.16 \pm 0.04	18.91 \pm 5.46	0.04 \pm 0.05	24.73 \pm 3.45	0.11 \pm 0.03
Reference	9.83 \pm 4.81	0.06 \pm 0.04	4.71 \pm 5.46	0.02 \pm 0.05	7.78 \pm 3.62	0.04 \pm 0.03
Near Oyster	6.07 \pm 7.71	0.20 \pm 0.06	6.79 \pm 7.71	0.04 \pm 0.06	6.43 \pm 5.46	0.12 \pm 0.05
Fence	57.26 \pm 9.45	0.07 \pm 0.08	20.54 \pm 7.71	0.06 \pm 0.06	38.90 \pm 6.10	0.07 \pm 0.05
Pore Water Total	22.33 \pm 2.89	0.12 \pm 0.02	12.43 \pm 3.15	0.04 \pm 0.03	18.09 \pm 2.13	0.08 \pm 0.02
Biodeposits	49.19 \pm 10.62	0.03 \pm 0.17	86.01 \pm 10.62	0.64 \pm 0.17	73.74 \pm 7.92	0.43 \pm 0.13

Table 3.5. Denitrification (DNF) and Anammox (AMX) rates (mean \pm SE) measured using ^{15}N in sediments at two Chesapeake Bay sites treated with varying levels of oyster biodeposition: directly underneath oyster aquaculture rafts (Oyster), ~250 m from oyster aquaculture site (Reference Station), forced accumulation of oyster biodeposits (Biodeposits Fenced), 5-10 m outside of oyster aquaculture array (Near-oyster Station), and collected oyster biodeposits (Biodeposits Only). When replicate seasonal measures were taken, sample type means are shown.

St. Jerome's Creek, MD					Spencer's Creek, VA		
Sample Location	Sample Season	DNF mmol N m ⁻² d ⁻¹	AMX mmol N m ⁻² d ⁻¹	Total N ₂ mmol N m ⁻² d ⁻¹	DNF mmol N m ⁻² d ⁻¹	AMX mmol N m ⁻² d ⁻¹	Total N ₂ mmol N m ⁻² d ⁻¹
<i>Oyster</i>							
	Summer 2008	0.93 ± 0.55	0.07 ± 0.02	0.99 ± 0.58	0.73 ± 0.37	0.07 ± 0.04	0.81 ± 0.41
	Fall 2008	1.31 ± 0.45	0.14 ± 0.02	1.46 ± 0.46	0.65 ± 0.31	0.04 ± 0.01	0.69 ± 0.32
	Spring 2009	0.63 ± 0.27	0.10 ± 0.04	0.73 ± 0.31			
	Mean	0.96 ± 0.24	0.10 ± 0.02	1.06 ± 0.25	0.69 ± 0.22	0.05 ± 0.02	0.75 ± 0.24
<i>Reference</i>							
	Summer 2008	2.49 ± 0.25	0.15 ± 0.02	2.64 ± 0.26	1.58 ± 0.20	0.08 ± 0.01	1.66 ± 0.21
	Fall 2008	1.93 ± 0.18	0.10 ± 0.02	2.02 ± 0.20	1.69 ± 0.35	0.09 ± 0.03	1.78 ± 0.38
	Spring 2009	0.69 ± 0.10	0.11 ± 0.01	0.80 ± 0.10			
	Mean	1.70 ± 0.28	0.12 ± 0.01	1.82 ± 0.29	1.64 ± 0.18	0.08 ± 0.01	1.72 ± 0.19
<i>Biodeposits Fenced</i>							
	Summer 2008	0.98 ± 0.19	0.04 ± 0.02	1.02 ± 0.21	1.21 ± 0.40	0.04 ± 0.01	1.26 ± 0.42
<i>Near Oyster</i>							
	Summer 2008	2.29 ± 0.25	0.11 ± 0.02	2.40 ± 0.25	1.61 ± 0.28	0.06 ± 0.02	1.67 ± 0.30
<i>Biodeposits Only</i>							
	Summer 2008	0.04 ± 0.12	0.00 ± 0.00	0.04 ± 0.12	0.26 ± 0.07	0.02 ± 0.01	0.28 ± 0.07
	Fall 2008	0.09 ± 0.04	0.01 ± 0.00	0.10 ± 0.05			
	Mean	0.06 ± 0.07	0.00 ± 0.00	0.07 ± 0.07			

Table 3.6. Total N₂ production/denitrification rates (DNF) measured using MIMS (mean ± SE) in sediments at two Chesapeake Bay sites treated with varying levels of oyster biodeposition: directly underneath oyster aquaculture rafts (Oyster), ~250 m from oyster aquaculture site (Reference Station), sediment cores treated with three levels of oyster biodeposits (Biodeposit Addition), and forced accumulation of oyster biodeposits (Biodeposits Fenced). When replicate seasonal measures were taken, sample type means are shown.

Sample Location	Sample Season	Total N ₂ mmol N m ⁻² d ⁻¹	OM %	SOD mmol O ₂ m ⁻² d ⁻¹	NO _x mmol NO _x m ⁻² d ⁻¹	NH ₄ ⁺ mmol NH ₄ ⁺ m ⁻² d ⁻¹	TON mmol TON m ⁻² d ⁻¹
St. Jerome's Creek							
<i>Oyster</i>	Spring 2009	0.00 ± 0.00	2.46 ± 0.06	8.72 ± 0.75	-0.04 ± 0.07	2.01 ± 0.60	-0.09 ± 0.23
	Summer 2009	1.56 ± 0.37	2.00 ± 0.47	34.57 ± 1.40	-0.12 ± 0.00	3.58 ± 1.31	0.54 ± 0.58
	Mean	0.78 ± 0.39	2.23 ± 0.40	21.64 ± 5.82	-0.09 ± 0.03	2.95 ± 0.84	0.29 ± 0.36
<i>Reference</i>	Spring 2009	0.71 ± 0.69	2.10 ± 0.36	7.16 ± 2.99	0.01 ± 0.01	1.04 ± 0.26	-0.37 ± 0.08
	Summer 2009	0.42 ± 0.05	2.27 ± 0.38	20.97 ± 3.56	0.08 ± 0.05	2.78 ± 0.61	0.79 ± 0.65
	Mean	0.56 ± 0.31	2.19 ± 0.34	14.07 ± 3.72	0.05 ± 0.03	1.91 ± 0.49	0.21 ± 0.39
<i>Biodeposit Addition</i>	Spring 2009	0.39 ± 0.17	1.62 ± 0.82	9.50 ± 0.87	-0.04 ± 0.03	1.21 ± 0.23	-0.26 ± 0.31
	Summer 2009	1.09 ± 0.66	2.30 ± 0.13	27.58 ± 5.76	0.00 ± 0.06	6.17 ± 3.06	1.81 ± 0.28
	Mean	0.65 ± 0.27	1.88 ± 0.72	16.28 ± 3.84	-0.03 ± 0.03	3.07 ± 1.36	0.51 ± 0.43
<i>Biodeposits Fenced</i>	Summer 2009	2.50 ± 0.11	7.48 ± 1.55	40.56 ± 0.73	-0.13 ± 0.00	11.91 ± 0.30	1.81 ± 1.03
Spencer's Creek							
<i>Oyster</i>	Summer 2009	0.93 ± 0.34	11.05 ± 0.50	34.55 ± 3.90	-0.17 ± 0.05	3.90 ± 1.18	-0.11 ± 0.45
<i>Reference</i>	Summer 2009	0.08 ± 0.04	7.97 ± 2.27	7.99 ± 0.80	-0.03 ± 0.02	1.07 ± 0.04	-0.16 ± 0.88
<i>Biodeposit</i>	Summer 2009	0.73 ± 0.49	7.69 ± 2.25	17.88 ± 4.96	-0.03 ± 0.01	6.49 ± 4.15	-1.72 ± 1.42

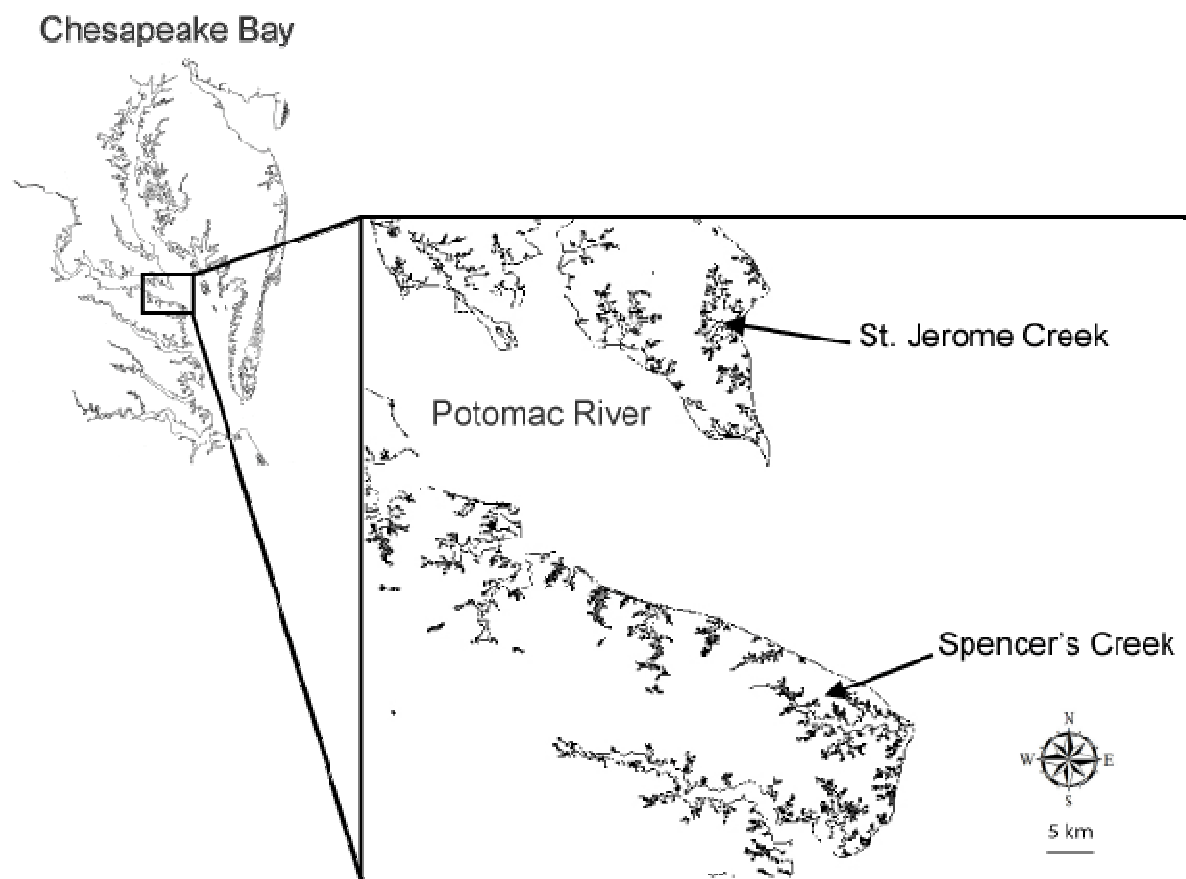


Figure 3.1.

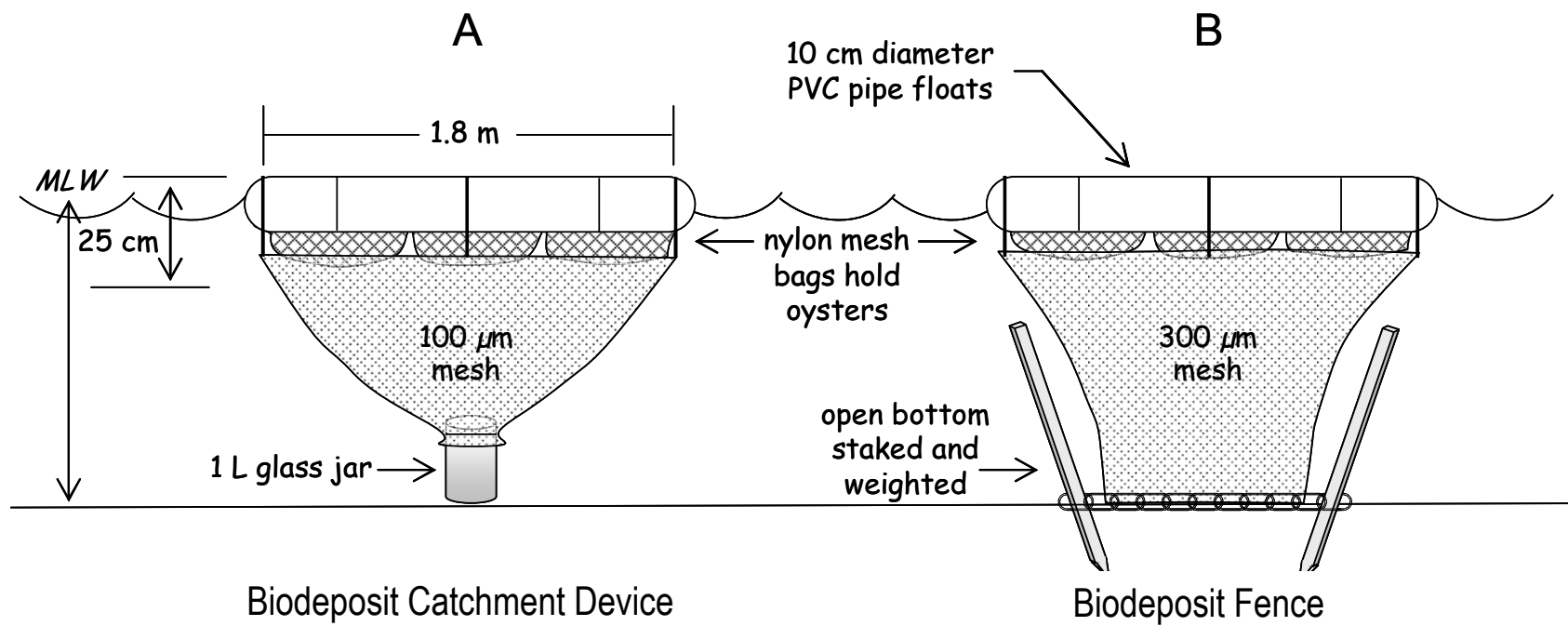


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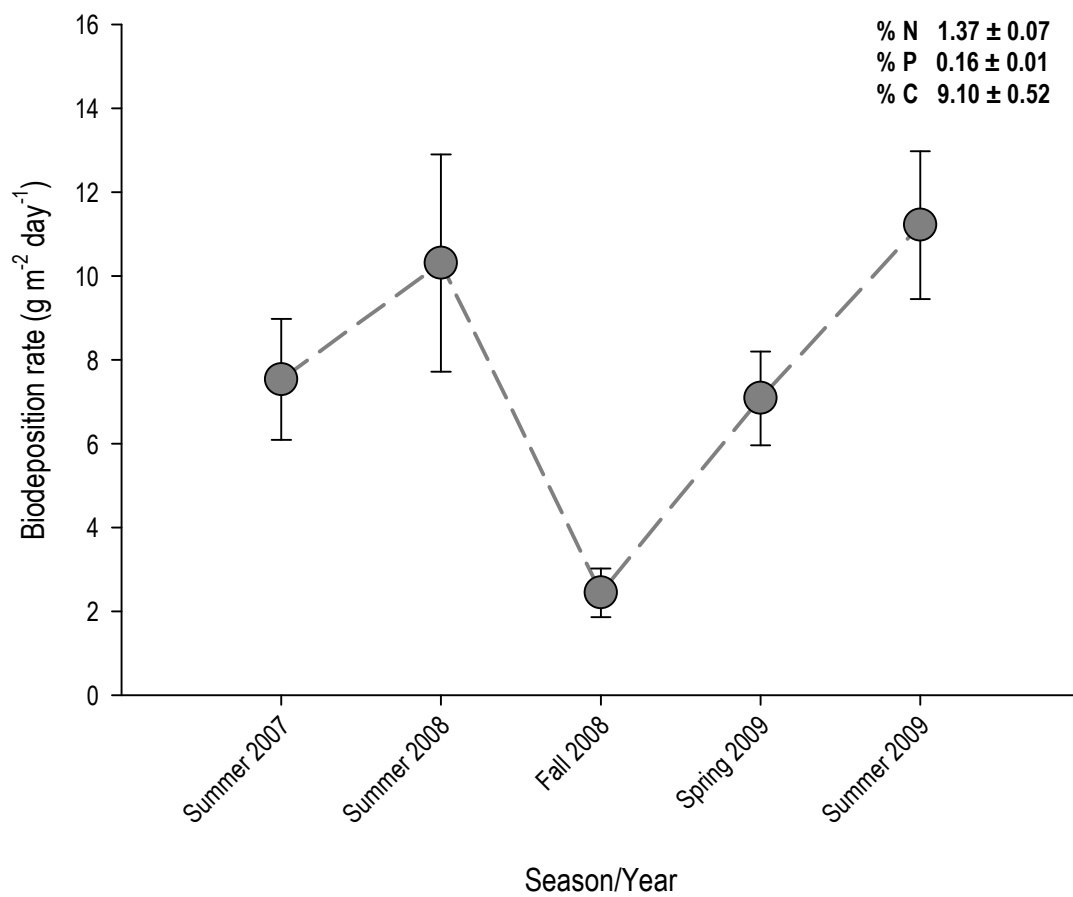


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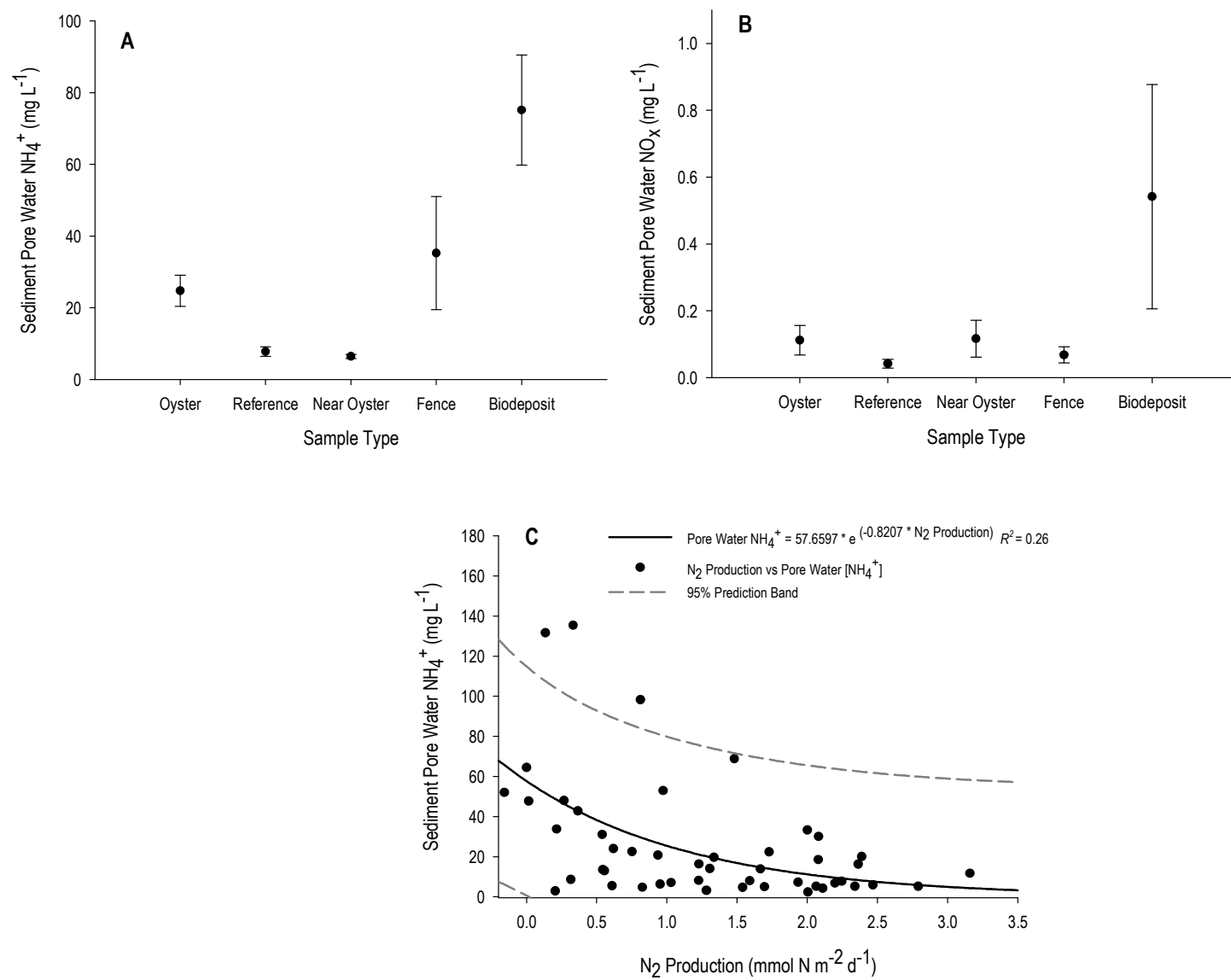
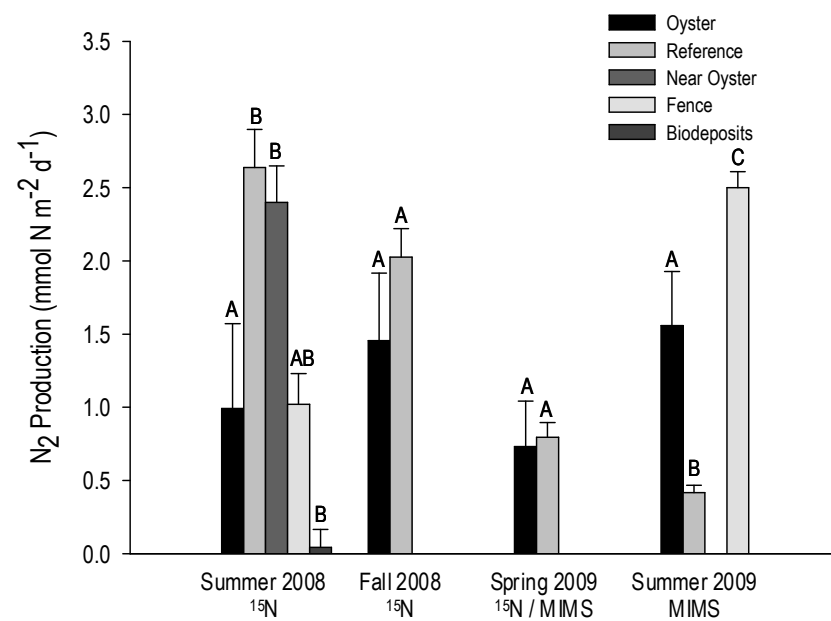
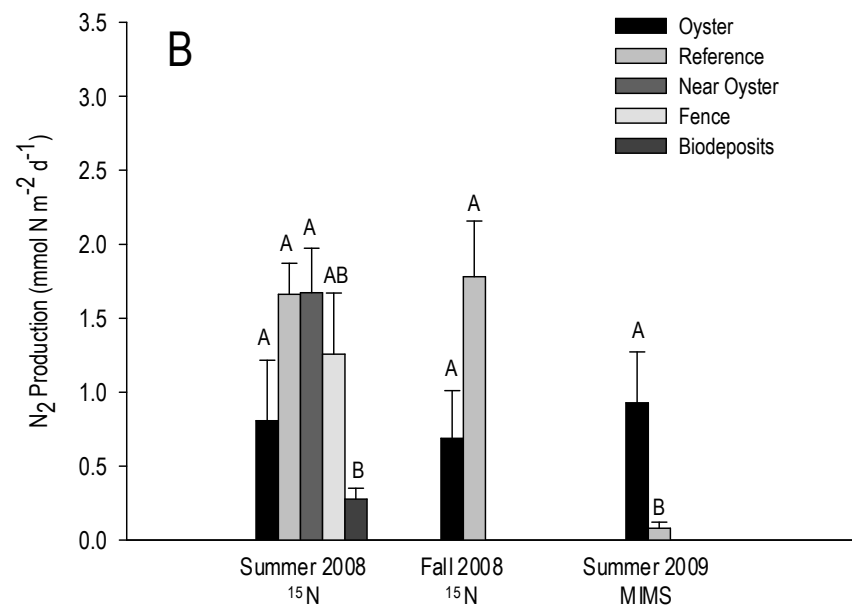


Figure 3.4.



St. Jerome Creek



Spencer's Creek

Figure 3.5.

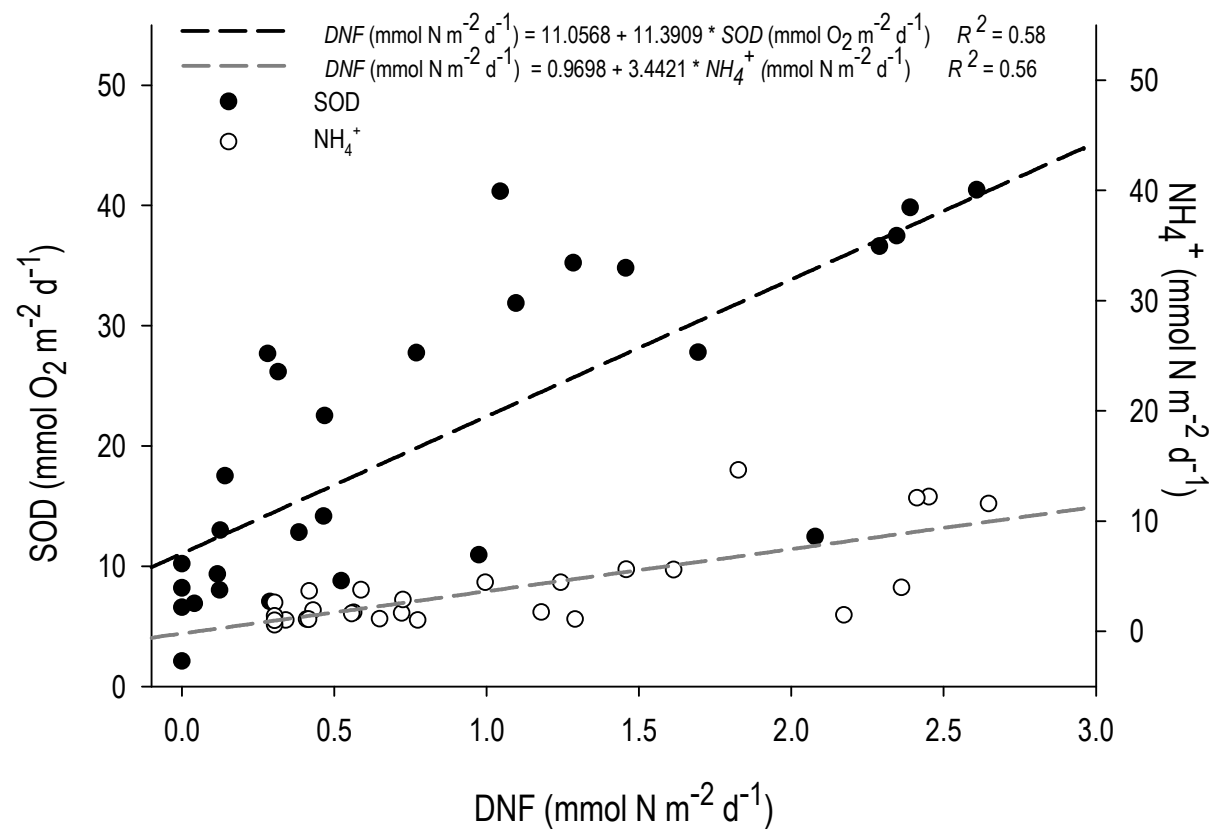


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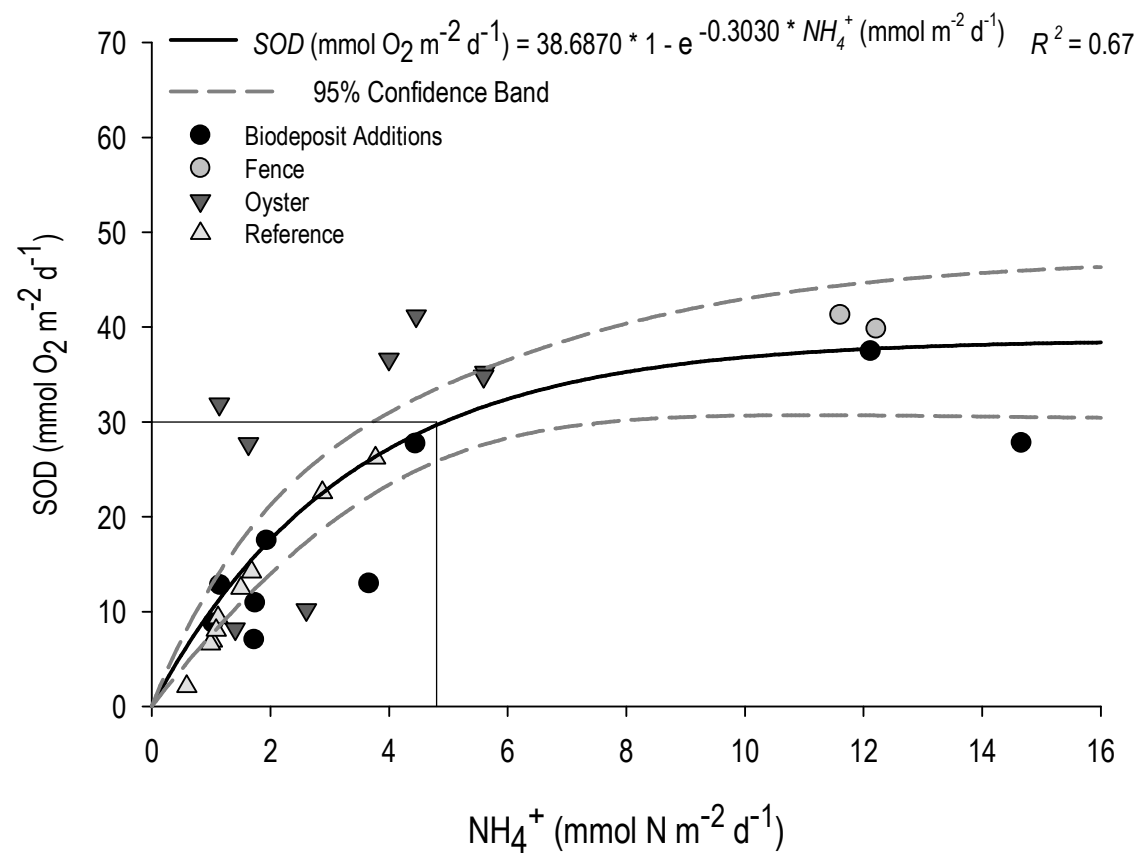


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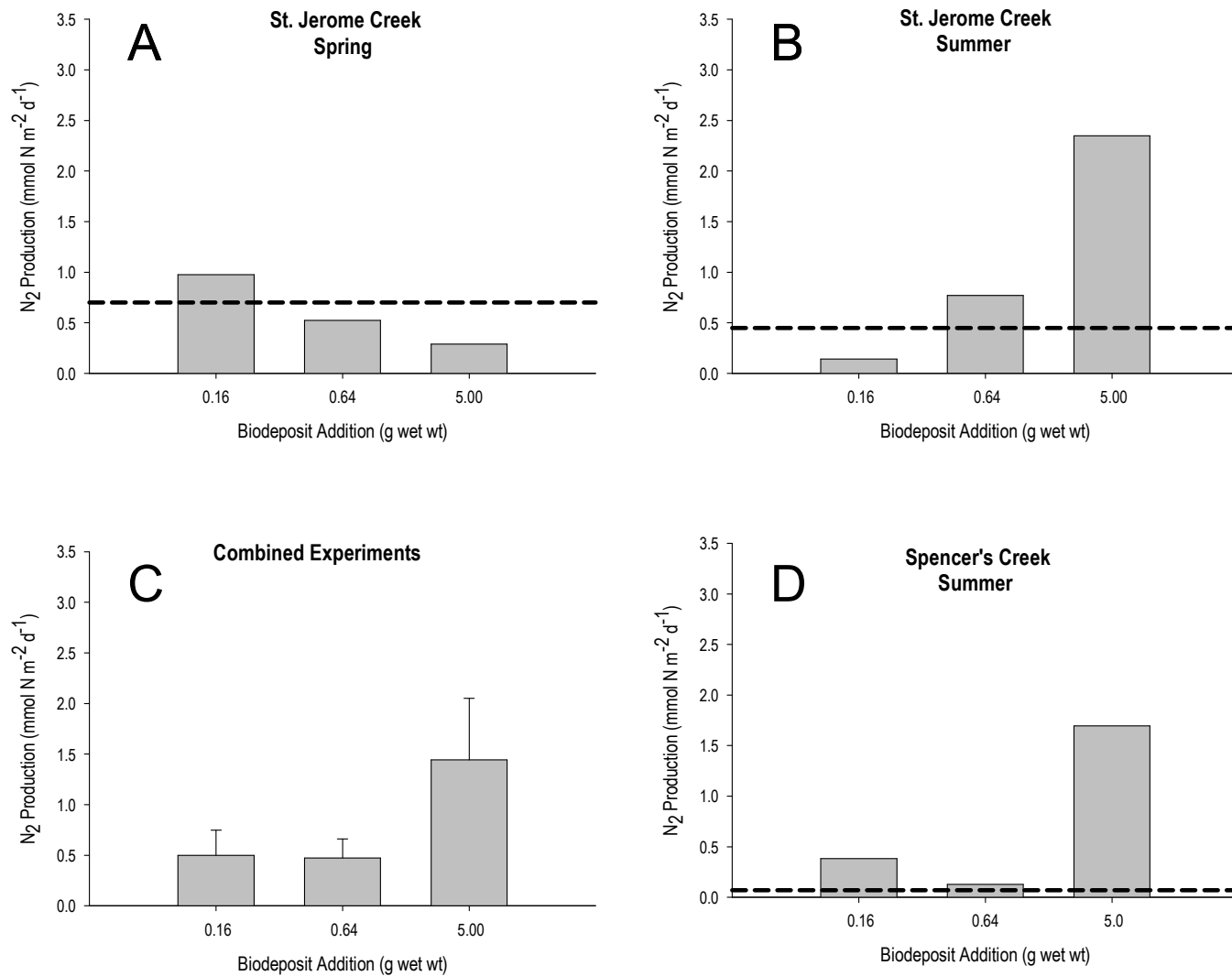


Figure 3.8.

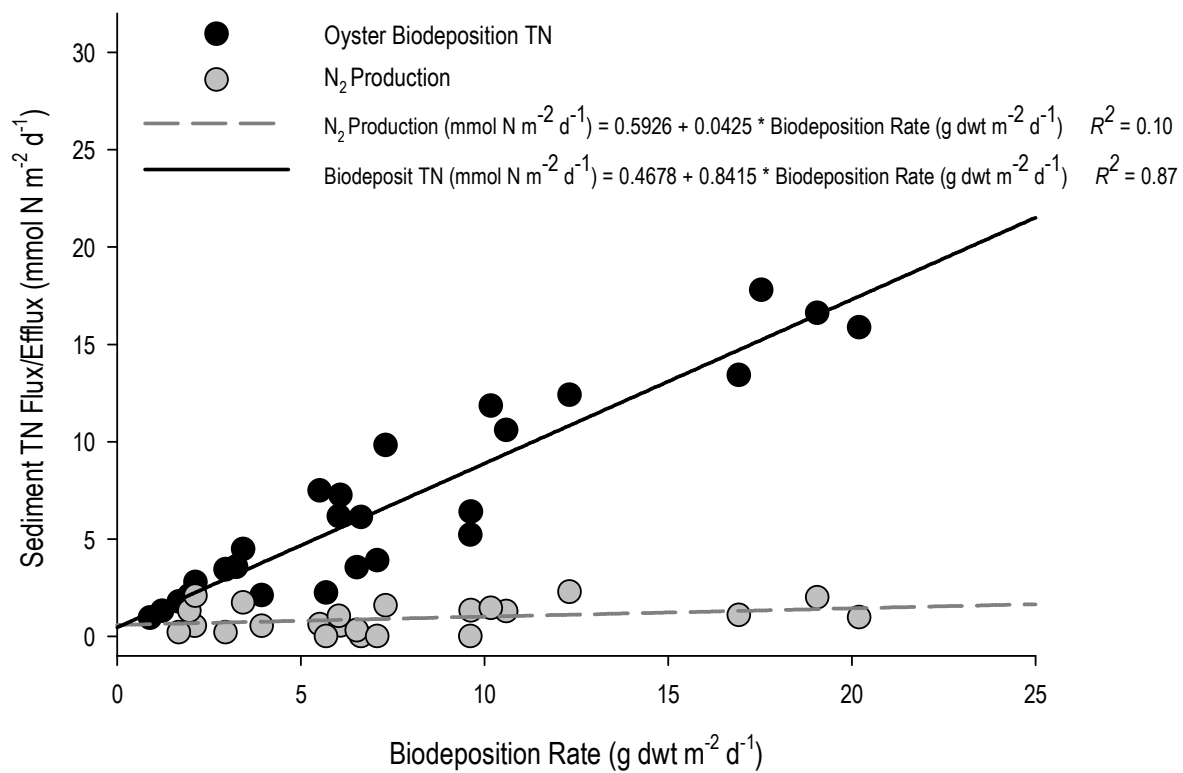


Figure 3.9.

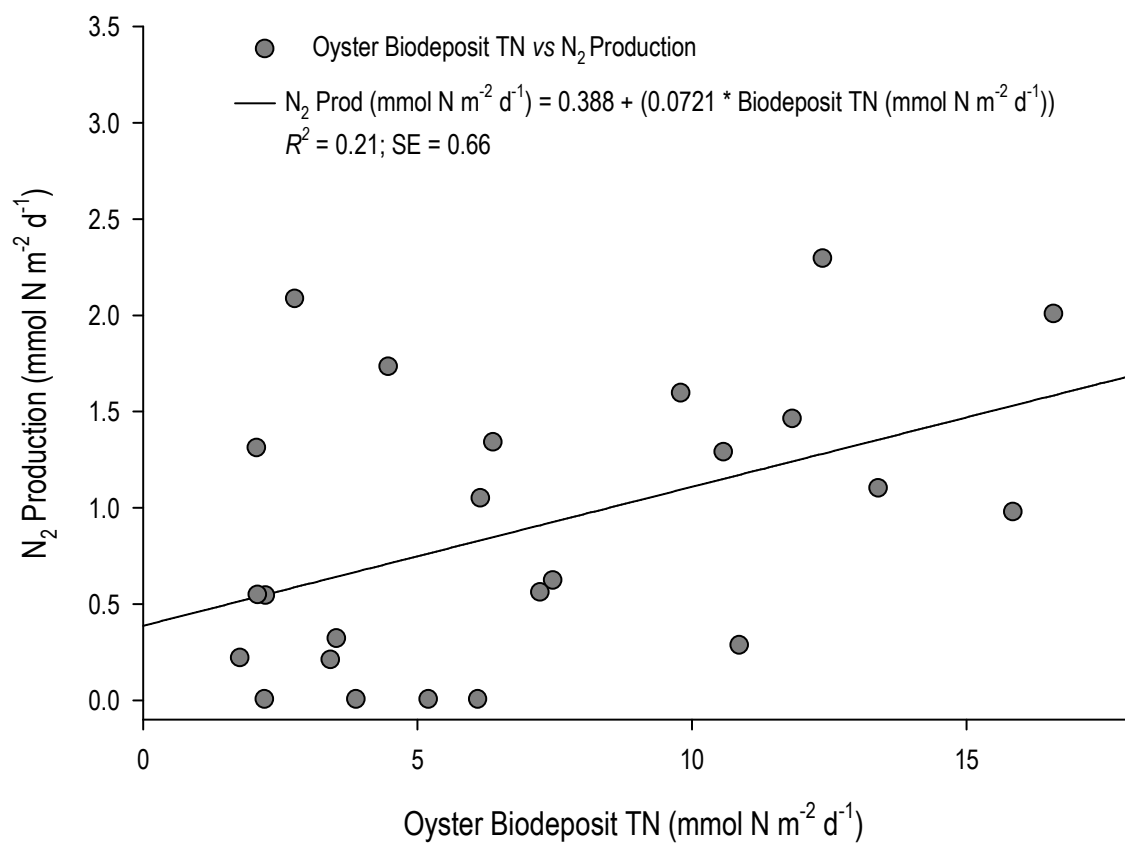


Figure 3.10.

Chapter 4

Microbial consortia associated with sediment nitrogen removal at oyster aquaculture
sites in Chesapeake Bay

**Microbial consortia associated with sediment nitrogen removal at oyster
aquaculture sites in Chesapeake Bay**

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Abstract

Expanding nitrogen (N) sinks within the ecosystem is one management approach aimed at improving water quality due to N pollution in Chesapeake Bay. The native Eastern oyster, *Crassostrea virginica*, may facilitate N removal by stimulating increased rates of sediment coupled nitrification-denitrification *via* oyster biodeposition. In this study, two oyster aquaculture sites and two nearby reference sites (no aquaculture) in Chesapeake Bay were examined to evaluate the impact of oyster biodeposition on composition of the sediment microbial community and sediment N₂ production due to coupled nitrification-denitrification. Total bacterial abundance using 4',6-diamidino-2-phenylindole (DAPI) staining and Fluorescent *in situ* Hybridization (FISH) were used to compare abundance of total bacteria (eubacteria and archaeobacteria), eubacteria, and nitrifying bacteria (ammonium-oxidizing bacteria and nitrite-oxidizing bacteria). Spatial and temporal heterogeneity of sediment eubacteria and denitrifying bacteria communities were evaluated using a universal eubacterial marker and the *nosZ* (nitrous oxide reductase) gene. Bacterial abundance measures were site dependent and neither oyster cultivation nor season played a significant role. Oyster biodeposition did not induce increases in sediment nitrifying bacterial abundance or relative abundance, indicating that oyster aquaculture is not likely to increase coupled nitrification-denitrification by increasing the number or proportion of nitrifiers. Oyster biodeposition significantly impacted the composition of the denitrifying community at both sites and there was further evidence that the area impacted by oyster aquaculture is limited to within 5 m of the oyster array. Changes in bacterial community composition were

observed at one site but not the other, indicating the influence of oyster cultivation is not uniform for all sites in the Bay.

Introduction

Nutrient pollution from various point and non-point sources has severely degraded water quality in Chesapeake Bay. Nitrogen (N) has been identified as a primary contributor to eutrophication in the Bay as well as other estuarine and coastal marine systems (Vitousek et al. 1997, Kemp et al. 2005, Howarth and Marino 2006). In excess, this nutrient stimulates growth of phytoplankton, leading to a variety of water quality problems that impact the overall health and functioning of marine environments. Controlling N sources has proven difficult in the Bay because N originates from numerous disparate sources. Finding ways to capture and remove N within the system is a potential strategy to mitigate N pollution.

The grazing behavior of suspension-feeding bivalves removes substantial amounts of phytoplankton biomass and suspended material from the water column and delivers labile carbon (C) and organic N to the surrounding sediments *via* biodeposition (Dame 1996). Cultivation of the native eastern oyster, *Crassostrea virginica*, may provide an ecosystem service in Chesapeake Bay by removing N from the water column and stimulating direct denitrification and/or coupled nitrification-denitrification in sediments through biodeposition of feces and pseudofeces (Newell et al. 2002, 2005). These microbially-mediated biogeochemical processes permanently remove N from the system in the form of inert N₂ gas. Ecosystem scale studies have shown that as much as 20-50% of organic N cycled through coastal marine sediments is denitrified, making

denitrification an important N sink in these systems (Seitzinger and Nixon 1984, Seitzinger 1988).

Denitrification is carried out by heterotrophic facultative or obligate anaerobic bacteria that convert NO_3^- to the gaseous end products NO, N_2O , and N_2 through a series of reduction reactions in which NO_3^- serves as the terminal electron acceptor when O_2 is unavailable. The overlying water column may provide NO_3^- to the denitrification pathway allowing for direct denitrification, but many studies in coastal ecosystems around the world have shown that the major source NO_3^- comes from coupled nitrification-denitrification within the top few cm of sediments (Seitzinger 1987). Nitrification is carried out by obligate aerobic bacteria that use O_2 to convert NH_4^+ to NO_2^- and NO_3^- . Ammonium-oxidizing bacteria (AOB) first oxidize NH_4^+ to NO_2^- and nitrite-oxidizing bacteria (NOB) oxidize NO_2^- to NO_3^- . Both AOB and NOB are chemolithoautotrophs, obtaining energy and reductant substrates for growth and maintenance by oxidizing NH_4^+ or NO_2^- (Fig. 4.1). Sediment organic matter loading has been identified as an important factor in controlling denitrification rates in aquatic sediments, increasing direct denitrification but decreasing coupled nitrification-denitrification in some studies, and demonstrated to be quantity and quality dependent (Caffrey et al. 1993, Sloth et al. 1995, Laursen & Seitzinger 2002, Eyre and Ferguson 2009).

Functional genes coding for four reductase enzymes of the denitrification pathway have been identified (*narG*, *nirK*, *nirS*, and *nosZ*) and applied in studies of denitrification in many environments (Braker et al. 2000, Scala and Kerkhof 2000). Because denitrification is carried out by a very diverse group of bacteria, as well as archaea and fungi, molecular approaches have focused on targeting the diversity and abundance of

functional genes in the denitrification pathway (Groffman et al. 2006). Terminal restriction fragment length polymorphism (T-RFLP) analysis is a semi-quantitative measure of community-level composition of bacterial domains or of functional gene populations that reveals community dynamics and diversity changes related to environmental variables. Nitrifying bacteria are a more closely related group of organisms than denitrifiers, therefore rRNA-targeted oligonucleotide probes for AOB and NOB have been developed and applied in Fluorescent *in situ* hybridization (FISH) techniques to enumeration nitrifying bacteria in environmental samples (Mobarry et al. 1996, Daims et al 2001).

In this study, a variety of established molecular approaches were used to determine if oyster biodeposition alters the sediment microbial community structure and N processing by nitrifying and denitrifying bacteria at two sites in Chesapeake Bay, each with a commercial-scale floating-raft oyster aquaculture facility and a reference site with no aquaculture activity. Sediment bacterial enumeration using 4',6-diamidino-2-phenylindole (DAPI) staining and FISH have been applied in microbial ecology studies in various environments including marine sediments (Llobet-Brossa et al. 1998, Battin et al. 2001, Ravensschlag et al. 2001). To measure effects on bacterial abundance, sediment bacteria were enumerated on bioadhesive slides using DAPI staining and FISH to quantify abundance of the total bacterial community, eubacteria, and nitrifying bacteria. To assess the impact of oyster cultivation on the bacterial community composition of sediments, T-RFLP analysis of eubacteria was performed. To investigate if oyster biodeposition induces changes to the denitrifying bacterial community, T-RFLP analysis

was performed using *nosZ*, the gene that codes for the nitrous oxide reductase enzyme which carries out the final step in the denitrification pathway producing N₂ gas.

Oyster and reference sediments were compared to quantify (1) abundance of the total bacterial community using DAPI staining of bacteria, (2) abundance and community composition of eubacteria using FISH and T-RFLP analysis, (3) relative abundance of AOB and NOB nitrifying bacteria using FISH and (4) community composition of denitrifying bacteria using T-RFLP analysis of the *nosZ* gene in a transect of four sediment types of various biodeposit treatments as well as biodeposits and seston.

Methods

Site descriptions and sediment sampling

Sediments were examined from two oyster aquaculture sites located in separate sub-watersheds of Chesapeake Bay: St. Jerome Creek in Ridge, MD (38° 07'13"N, 76° 20'53"W) and Spencer's Creek in Ophelia, VA (37° 54'22"N, 76° 17'27"W). The sites represent two distinct estuarine tributary environments where oysters are typically cultivated in the Bay and are subject to different channel dynamics, hydrological regimes, and sediment geomorphology. St. Jerome Creek is a high wave energy site, ~60 m wide with sandy low carbon bottom sediment and Spencer's Creek is a low wave energy site with poor hydrodynamic exchange, ~15 m wide with high carbon soft silt bottom sediment. Oysters were cultivated in an array of 80,000 – 100,000 oysters from 2007-2009 using floating raft methods previously described by Higgins et al. (2011).

Within each study site, sediment samples from selected areas were collected and analyzed in triplicate to characterize the bacterial community response to high organic

matter (OM) loading resulting from oyster biodeposition. Sediments were categorized into four types representing varying levels of oyster biodeposit treatments: directly underneath oyster aquaculture rafts (Oyster), 5-10 m outside of oyster aquaculture array (Near Oyster), forced *in situ* accumulation of oyster biodeposits (Fence), control sediments with no aquaculture (Reference), and oyster biodeposits (Biodeposits). Oyster biodeposits were collected using a biodeposit catchment device and forced accumulation of biodeposits in sediments (fence) was performed using a biodeposit fence (Fig. 4.2). Seasonal sediment samples were collected using a device that yielded sediment cores 8 cm diameter and 20 cm deep during June 2008 (Summer), October 2008 (Fall), February 2009 (Winter), May 2009 (Spring), and August 2009 (Summer) at both aquaculture sites. Cores were held on ice until processing (2-6 hr) at which time the top 0-3 cm section was removed and homogenized. Subsamples were weighed (0.25-0.50 g wet weight) and DNA was extracted using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA), according to the manufacturer's instructions and nucleic acids were stored at -20°C until analysis. Prior to PCR, DNA concentrations were determined using a Nanodrop 8000 Spectrophotometer (Thermo Scientific) and standardized to 5 ng μl^{-1} .

Bacterial community composition

Eubacterial community composition profile analysis was performed for oyster and reference sediments at Spencer's Creek (n=32) and St. Jerome Creek (n=24) using T-RFLP analysis of a 16S rRNA region for the top 0-3 cm section of sediment cores. PCR amplification was performed using 27F (FAM labeled: 5'-AGAGTTTGATCCTGGCTCAG-

3') and 1492R (5'-GGYTACCTTGTTACGACTT-3') primers obtained from Integrated DNA Technologies (Coralville, Iowa) (Lane 1991). Total volume of each PCR reaction was 50 µl containing 25 ng DNA template, 50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl₂, 5 U AmpliTaq® DNA Polymerase, 0.4 mg ml⁻¹ BSA, 1 mM each dNTP, and 0.3 µM each primer. Reagents were obtained from Applied Biosystems (Carlsbad, California) except BSA (New England Biolabs). Thermal cycling was performed in a DNA Engine thermal cycler (BioRad, Hercules, CA) using the thermal profile of 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 49°C for 1 min, 72°C for 2 min, and a final 8 min extension at 72°C. PCR products were purified using a MinElute 96 UF™ PCR purification kit (Qiagen, Valencia, CA), eluted into 18 µl of sterile molecular-grade water, of which 7 µl was then digested using *MspI* (New England Biolabs) at 37°C for 6 hr followed by deactivation at 65°C for 20 min. Digestion products were purified using the MinElute 96 UF™ PCR purification kit (Qiagen), recovered in 10 µL of sterile molecular-grade water, and resolved using capillary electrophoresis on a MegaBACE 1000 DNA Analysis System (GE Healthcare, Piscataway, NJ), injected at 3000V for 100 seconds, and run for 100 min at 10,000V. Each sample lane contained MapMarker 400™ Rox ladder (Bioventures). Following electrophoresis, T-RFLP peaks were viewed and fragments 50-400 bp were scored as present/absent using Fragment Profiler (Ver 1.2).

Denitrification diversity and quantification

T-RFLP analysis of the *nosZ* gene was conducted for four sediments types, biodeposits and seston at St. Jerome Creek (n=94) and Spencer's Creek (n=71). The *nosZ* gene was amplified with the primers 661F (FAM labeled: 5'-CGGCTGGGGGCTGACCAA-3')

and 1773R (5'-ATRTCGATCARCTGBTCGTT-3') obtained from Integrated DNA Technologies (Scala and Kerkhof 2000). Total volume of each PCR reaction was 18 μ l containing 3 μ l of DNA template, 9 μ l of REDTaq[®] DNA Polymerase (Sigma-Aldrich), 1.8 μ l of 10 mg/ml BSA (New England Biolabs), and 1.8 μ l 2mM primer mixture. Thermal cycling was performed in a DNA Engine thermal cycler (BioRad, Hercules, CA) using the thermal profile of 95°C for 5 min, followed by 36 cycles of 95°C for 30 sec, 59.7°C for 30 sec, 72°C for 1.5 min, and a final 8 min extension at 72°C. The PCR products were digested with the enzyme HinP1I (New England BioLabs) using 18 μ l of PCR product and incubated at 37°C for 16 hr followed by 65°C deactivation for 20 min. Digested amplicons were purified and analyzed as described for the eubacterial community. The two study sites were analyzed separately, therefore between-site comparisons were not performed.

Fluorescent in situ Hybridization (FISH) analysis

Sediment samples were collected and prepared for DNA extraction were also subsampled for FISH analysis. In each season, samples were analyzed in triplicate for oyster and reference sediments at St. Jerome Creek (n=30) and Spencer's Creek (n=30). To collect a representative subsample of bacterial cells, ~1 g of wet sediment (or biodeposits) was weighed into a sterile 15 ml tube and 8 ml of 25% MeOH was added. The samples were vortexed at 3000 \times g for 15 min with 100 μ m acid-washed silica beads and centrifuged at 4500 g for 2.5 min. The supernatant was discarded and the pellet reconstituted in 500 μ l of PBS, mixed well and centrifuged at 4500 \times g for 2 min. An underlayment of 50% Histodenz[™] density gradient media was used to separate

sediment particles and bacterial cells according to the manufacturer's instructions (Sigma-Aldrich). The cells that collected at the interface were aspirated and resuspended in 100 μ l of ice cold 96% EtOH then stored at -20°C until analysis. Sets of 24 samples (5 μ l each) were affixed to slides with hydrophobic septa yielding 5 mm diameter bioadhesive wells (19.6 mm², Thermo Scientific # ES-230B), then dehydrated for 3 min in 50% EtOH, followed by 80% EtOH, and finally 96% EtOH. FISH was performed using a mixture of 16S rRNA hybridization probes to identify eubacteria, ammonia-oxidizing (AOB), and nitrite-oxidizing (NOB) nitrifying bacteria (Table 4.1). To each slide well, 9 μ l of 35% formamide hybridization solution containing 50 ng of each probe was applied and incubated for 90 min at 46°C in an isotonic-equilibrated humid chamber. Probes were washed from a slide using pre-warmed (48°C) wash buffer after which the slide was immediately immersed in 35 ml of pre-warmed washing solution and incubated for 15 min. The slides were rinsed briefly with ice-cold sterile H₂O and dried.

After fluorescent *in situ* hybridization, the cells were stained with DAPI to enumerate eubacteria and archaeobacteria (hereafter referred to as total bacterial abundance) using a solution of 0.33 μ g ml⁻¹ DAPI in H₂O for 5 min and the excess DAPI was removed by rinsing the slides with ice-cold sterile H₂O. Antifade oil solution (Millipore #S7114) was applied atop slides prior to the cover slip. Enumeration was performed at 1000X under oil immersion using epifluorescence microscopy (Olympus BX-41) using DAPI, FITC, and TRITC fluorescence filters. Cells were counted from an accurately ruled eyepiece graticule (0.004 mm² total grid area) subdivided into 100 smaller squares of equal area. Several view fields were counted until a running total of

at least 200 cells was reached (or a minimum of five fields). The total cell count per g of wet sediment in each sample was then calculated as:

$$\text{[Eq. 1]} \quad \frac{(\text{average cell count per grid}) \times (\text{well area})}{(\text{grid area}) \times (\text{volume of aliquot})} \times \frac{(\text{final extraction volume})}{(\text{g of wet sediment})}$$

Sediment environmental variables

Sediment N₂ production rates were measured using ¹⁵N and N₂/Ar methods as reported in Chapter 2. Sediment measures of biological oxygen demand (BOD), chemical oxygen demand (COD), carbon (%C), nitrogen (%N), pore water nitrite (NO₂⁻), nitrate (NO₃⁻), and ammonium (NH₄⁺) were performed as previously described in Chapter 2.

Data Analysis

The raw data for the T-RFLP community-fingerprinting assay and T-RFLP *nosZ* functional gene assay consisted of a binary matrix based on presence or absence of T-RFLP peaks in each sample. Peak data were analyzed using non-metric multidimensional scaling (NMDS) with the Jaccard similarity coefficient to produce ordination analysis of the bacterial and denitrifier (*nosZ*) communities to determine whether communities varied with respect to sample type, aquaculture site, and/or season. An analysis of similarity (ANOSIM) was performed using the Jaccard similarity coefficient to identify statistically significant differences among treatment groups (Clarke 1993). All analyses were performed using PAST software (version 1.97, Hammer et al. 2001). Tests for correlation were performed using Pearson Product Moment and regression analysis using PASW Statistics (v.18) and SigmaPlot (v.11). All significance testing was evaluated at $\alpha = 0.05$.

Results

Abundance of total bacteria and nitrifying bacteria in sediments

Site played a significant role in determining the abundance of total bacterial abundance, which were 2.3 times higher at Spencer's Creek than at St. Jerome Creek, $79.1 \pm 10.3 \times 10^4$ cells g^{-1} sediment versus $34.1 \pm 4.6 \times 10^4$ cells g^{-1} sediment, respectively (Fig. 4.3, ANOVA, $p < 0.001$). However, neither oyster biodeposit treatment (oyster versus reference) nor season were significant factors in determining microbe abundance among or within the two study sites, and no interactions among sample type, site or season were detected ($p > 0.05$). Although not statistically significant, seasonal fluctuations in bacterial abundance were observed at both sites, increasing in winter and summer and decreasing in fall and spring, with the exception of reference sediments at St. Jerome Creek which showed little seasonal variation (Fig. 4.4).

Abundance of sediment total bacteria and eubacteria covaried seasonally. Many of the same site differences and seasonal fluctuation patterns observed in total bacterial abundance also were observed in eubacterial abundance because 50-78% of the sediment bacterial community was consistently comprised of eubacteria (Fig. 4.4). Sediment eubacterial abundance was 1.8 times higher at Spencer's Creek than at St. Jerome Creek, $47.7 \pm 7.7 \times 10^4$ cells g^{-1} sediment versus $25.8 \pm 3.4 \times 10^4$ cells g^{-1} sediment, respectively (Fig. 4.3). As with total bacterial abundance, study site was the dominant factor determining sediment eubacterial abundance. Neither oyster biodeposition nor season significantly impacted sediment eubacterial abundance, and no interactions among site, type, or season were detected (Three-way ANOVA, $p > 0.05$). Within a sediment type (oyster or reference) at each site, no significant changes in total

bacterial or eubacterial abundance were observed over time (ANOVA, $p > 0.05$), with the exception of oyster sediments at St. Jerome Creek in the summer 2008 when eubacterial abundance in oyster sediments was significantly higher than in all other seasons (Fig. 4.4, ANOVA, $p < 0.004$).

The abundance of nitrifying bacteria in sediments also was site dependent, significantly higher at Spencer's Creek than at St. Jerome Creek by 1.7 times, $10.0 \pm 1.1 \times 10^4$ cells g^{-1} versus $6.0 \pm 1.2 \times 10^4$ cells g^{-1} , respectively (ANOVA, $p = 0.02$). No significant difference between oyster and reference bacterial abundance or among seasons was detected (Fig. 4.5, Three-way ANOVA, $p > 0.05$). Across all seasons at Spencer's Creek, no difference in nitrifier abundance between oyster and reference sediments or among seasons was detected, and no interaction between sample type and season was detected (Three-way ANOVA, $p > 0.05$). Comparisons within site and season revealed only two seasons in which significant differences between oyster and reference were detected, but these were not consistent differences. In fall at Spencer's Creek, reference sediment nitrifier abundance was 1.9 times higher than oyster sediments, and in spring at St. Jerome Creek oyster nitrifier abundance was 2 times higher than reference sediments (Fig. 4.3, ANOVA, $p < 0.02$). At St. Jerome Creek, seasonal oscillations in nitrifier abundance appeared to follow a similar pattern in both oyster and reference sediments, increasing in fall and winter and decreasing in spring and summer. However, at Spencer's Creek sediment nitrifier abundance appeared to follow an opposite seasonal oscillation trend where oyster sediment nitrifier abundance decreased in fall/spring and increased in winter/summer, compared to reference sediments in which abundance increased in fall/spring (Fig. 4.5).

The relative abundance of nitrifiers was consistently ~20-30% of the sediment eubacterial population at both sites and was similar across sites (Fig. 4.3, $p > 0.05$). At Spencer's Creek, nitrifiers were less abundant in oyster sediments than in reference sediments ($20.4 \pm 2.9\%$ of eubacteria compared to $24.7 \pm 2.8\%$, respectively) and at St. Jerome Creek, nitrifiers accounted for a higher proportion of eubacteria in oyster sediments than reference sediments ($30.1 \pm 2.9\%$ versus $23.9 \pm 2.9\%$, respectively, Fig. 4.3). At Spencer's Creek, no significant difference in nitrifier relative abundance was detected between oyster and reference sediments in any season, nor was significant seasonal variation detected in nitrifier relative abundance (Fig. 4.6). Although season was not significant, nitrifier relative abundance appeared to increase over time in oyster sediments at St. Jerome Creek, and was highest in fall, spring, and winter when oyster biodeposition rates are lowest.

Although the sediment conditions are different at the two sites, the organic matter fraction of the sediments did not differ significantly. In general, organic matter was higher in oyster sediments than the corresponding reference sites but this difference was significant only at Spencer's Creek (Table 4.2, ANOVA, $p = 0.001$). At Spencer's Creek, nitrifier abundance was negatively correlated with organic matter ($p = 0.034$, $r = -0.514$) whereas in St. Jerome Creek sediments, nitrifier abundance was positively correlated with organic matter ($p = 0.034$, $r = 0.425$). Sediment COD was 5.5 times higher and %C was 8.3 times higher at Spencer's Creek than St. Jerome Creek (Table 4.2). Both COD ($r = 0.351$, $p = 0.008$) and %C ($r = 0.272$, $p = 0.025$) were positively correlated with higher bacterial abundance at the two sites. Overall, Spencer's Creek sediments were higher in C, N, and oxygen consumption than St. Jerome Creek. Oyster impacts were

detected in OM, %N, %C, and BOD at Spencer's Creek. At St. Jerome Creek, %C and BOD were significantly higher in oyster sediments, but COD was lower in oyster than reference sediments.

Eubacteria and denitrifier community diversity

Although the two study sites were different in terms of bacterial abundance, when comparing reference sediments, T-RFLP analysis revealed that the eubacterial community composition was not different between the two sites (ANOSIM, $p > 0.05$). The effect of oyster cultivation was detected at Spencer's Creek as significant differences in community structure at oyster and reference sites, but no effect was detected at St. Jerome Creek (Fig. 4.7, ANOSIM, $p = 0.0013$). At Spencer's Creek, oyster biodeposition lowered the richness of the eubacterial community (number of T-RFLP bands, T-RFs) as compared to the reference sediments, 13.7 ± 3.1 T-RFs versus 25.3 ± 2.9 T-RFs (Fig. 4.8).

At both sites, NMDS ordination of *nosZ* T-RFLP data revealed significant variation in the denitrifying bacterial communities among sediment types, oyster biodeposits and seston (measured only at St. Jerome Creek, Fig. 4.9). Combined, the first two axes explained 81% of the variance in bacterial community structure at Spencer's Creek and 84% of the variance at St. Jerome Creek. At both sites, significant differences were detected among the sample types considered across all seasons sampled: oyster, reference, near oyster, fence, and biodeposits (Fig. 4.9, $p = 0.001$). Pair-wise comparison tests (ANOSIM) showed that *nosZ* diversity of oyster sediments was significantly different from all other sample types except fence sediments at Spencer's Creek. Both reference and near oyster sediments were significantly different from oyster

sediments, biodeposits and seston. Biodeposits and seston differed from all other sample types with the exception of fence sediments, which were exposed to high biodeposit accumulation rates.

Within the sediment denitrifier populations, significant seasonal differences were detected at both sites. At Spencer's Creek, summer oyster sediment *nosZ* community structure differed significantly from fall and winter and the two summer seasons (June and August) differed from one another, but spring oyster sediments did not differ from any other season (ANOSIM, $p < 0.0001$). Reference and near oyster sediments in summer differed significantly from spring, fall and winter and the two summer sampling events differed significantly from one another (ANOSIM, $p < 0.04$). At St. Jerome Creek, the summer oyster sediment *nosZ* community differed significantly from fall and winter and the two summer seasons (June and August) differed from one another, but spring did not differ from other seasons (ANOSIM, $p < 0.01$). The reference and near oyster sediment communities differed significantly in summer from spring, summer and fall and the two summer seasons differed from one another (ANOSIM, $p < 0.02$).

Richness of the *nosZ* gene at each site did not differ significantly among sample types ($p > 0.05$, Three-way ANOVA). At Spencer's Creek, season was a significant factor in determining *nosZ* richness ($p < 0.000$, Three-way ANOVA). The number of T-RFs was lowest in fall and spring, 20.3 ± 4.7 bands and 31.1 ± 5.1 bands, respectively, and highest in summer and winter, 54.0 ± 3.5 bands and 49.3 ± 5.1 , respectively (Fig. 4.10). At St. Jerome Creek, no seasonal differences in *nosZ* richness was detected ($p > 0.05$, Two-way ANOVA). Although not statistically significant, summer had lower *nosZ*

richness at St. Jerome Creek, 37.7 ± 3.5 versus 51.5-60.7 in spring, fall and winter, an opposite trend than was observed at Spencer's Creek.

Because the zone of coupled nitrification-denitrification occurs within the top few cm of sediment, 0-3 cm and 4-10 cm sediment core sections were compared to study the effect of sediment depth on the denitrifying community and 4-10 cm core sections were analyzed only for this comparison. The denitrifying community composition of the upper and lower sediment layers did not differ significantly at either site (ANOSIM, $p > 0.05$). Richness of the *nosZ* gene did not differ among the six sample types ($p = 0.076$) or between 0-3 cm and 4-10 cm sediment core sections at either site (Fig. 4.11, Two-way ANOVA, $p = 0.459$).

Relationship of abundance and denitrifier composition to N_2 production

At Spencer's Creek and St. Jerome Creek, total bacterial abundance explained 33-37% of variation ($R^2 = 0.37$, $R^2 = 0.33$, respectively) and eubacterial abundance explained 30-33% of variation in N_2 production ($R^2 = 0.33$, $R^2 = 0.30$, respectively). Both total bacterial and eubacterial abundance were positively correlated with N_2 production at Spencer's Creek, but neither was correlated at St. Jerome Creek ($p < 0.02$, $r = 0.590$ and 0.578 , respectively). At both sites, nitrifier abundance was positively correlated with N_2 production (Spencer's Creek, $p = 0.012$, $r = 0.595$; St. Jerome Creek, $p = 0.030$, $r = 0.425$). Non-linear regression analysis of sediments revealed that nitrifier abundance explained 43% of variation in N_2 production at Spencer's Creek, but explained only 18% of variation at St. Jerome Creek ($R^2 = 0.43$, $R^2 = 0.18$). Both total bacterial and eubacterial abundance provided similar results at both sites when analyzed separately,

but when analyzed together, the abundance measures explained only 7.4% of variation in N₂ production ($R^2 = 0.07$) indicating that the relationships are specific to the unique sediment conditions at each site.

There was no significant correlation between sediment *nosZ* T-RFLP profiles and direct measures of N₂ production at either Spencer's Creek (Mantel Test, $r = 0.09$, $p = 0.23$) or St. Jerome Creek ($r = 0.03$, $p = 0.25$). However, with respect to qualifying a relationship between direct measures of N₂ production and molecular approaches, *nosZ* T-RFLP results reflected observed changes in N₂ production even though Mantel tests were not significant. In the two summer seasons when sediment N₂ production rates were directly measured, oyster sediments were significantly lower than reference in early summer (Jun 2008) but significantly higher in mid-summer (Aug 2009) at both sites (Fig. 2.5). Within type comparisons of both oyster and reference sediment *nosZ* community composition at each site between these two summer sampling events showed that the difference in N₂ production was reflected in significant differences in the *nosZ* community (ANOSIM, $p < 0.000$).

Discussion

During periods of active suspension-feeding, floating-raft oyster aquaculture delivers high rates of organic matter rich (~21% OM) biodeposits to the underlying sediments. In spite of increased OM inputs, no increase in sediment bacterial abundance above baseline reference abundance was observed. Rather, site controlled the abundance of total bacteria, eubacteria, and nitrifiers indicating that the sediment microbial community does not respond to addition of oyster biodeposits by increasing

sediment N₂ production. The abundance of bacteria and nitrifying bacteria were ~2 times greater at Spencer's Creek than at St. Jerome Creek. Spencer's Creek is a low energy site with deep silt sediment whereas St. Jerome Creek is a more open high energy system with coarse sand sediment that has a lower proportional surface area than Spencer's Creek silted sediments. This difference in colonizable surface area may explain the observed differences in microbial abundance. In addition, COD was ~ 6 times higher and %C ~8 times higher at Spencer's Creek than at St. Jerome Creek, regardless of the addition of 80,000-100,000 oysters (Table 4.2). The site sediment conditions of %C and COD had strong positive correlations with all three bacterial abundance measures and describe the strong reducing sediment conditions at Spencer's Creek.

Oyster biodeposition fluctuates seasonally, and if biodeposition-enhanced coupled nitrification-denitrification were to induce an expansion of the nitrifying bacterial community, an increase in nitrifier relative abundance in oyster sediments and a reduction during seasons when biodeposition is low would be expected. However, no differences in nitrifier abundance was observed between oyster and reference sediments and no interaction between season and type was detected. No evidence was found that oyster biodeposition significantly impacts the abundance or relative abundance of nitrifying bacteria in estuarine sediments. The introduction of oysters to the ecosystem did not increase abundance of sediment nitrifiers above baseline reference sediments at either site. Abundance of nitrifying bacteria followed similar trends as were observed for the general bacterial community, being site dependent and not affected by oyster biodeposition or season. Nitrifier relative abundance was consistently ~20-30% of the

eubacterial population across both sites and was not dependent on or correlated to any environmental variables measured in this study, including sediment nutrient content, BOD, COD, or dissolved N concentrations in pore water.

Ultimately, no evidence was found that oyster biodeposition promotes increased abundance or relative abundance of nitrifying bacteria in sediments below oyster rafts and therefore, if coupled nitrification-denitrification is controlled by nitrifier abundance in a significant way, oyster biodeposition was not found to enhance N removal *via* this pathway. However, nitrifier abundance did explain some variation in N₂ production at one site. At the high C site, Spencer's Creek, 43% of variation in sediment N₂ production could be explained by sediment nitrifying bacterial abundance whereas at St. Jerome Creek, the low C site, sediment nitrifying bacterial abundance did not explain variation in sediment N₂ production. Overall, across both sites, total bacterial or eubacterial abundance explained 30-37% of variation in sediment N₂ production, indicating that abundance is a potentially important factor in determining sediment N removal.

In the few instances where significant differences were detected in nitrifier abundance between oyster and reference sediments within a season at a site, these differences did not translate into observed differences in sediment N₂ production. Nitrifier abundance was obtained for all samples in which N₂ production rates were measured, and no significant correlation between N₂ production and nitrifier abundance among sites or within sites was detected. The reciprocal argument also failed to show a relationship; i.e., in seasons when significant differences in N₂ production were found, the abundance and relative abundance of nitrifiers in oyster and reference sediments

was not significantly different, implying that enumeration of nitrifiers is not a reliable predictor variable of N₂ production rates.

Oyster biodeposition was associated with taxonomic changes to the eubacterial consortium at Spencer's Creek, but not at St. Jerome Creek. Therefore oyster biodeposition may induce changes to sediment microbial processes that are not uniform across the number of different environments in which oysters are cultivated in Chesapeake Bay. Furthermore, strong evidence was found that oyster biodeposition changes the sediment denitrifying community as measured by T-RFLP analysis of the *nosZ* gene, which was consistent at both sites regardless of their different sediment conditions. At both sites, the denitrifying consortium in oyster sediments was significantly different than reference and near-oyster sediments. The denitrifying community in reference and near-oyster sediments (5-10 m away from the oyster arrays), were indistinguishable from one another, and formed a distinct group separate from oyster sediments, fence sediments, captured biodeposits, and seston. This is further evidence that the denitrification zone of sediment impacted by oyster biodeposition is limited to within a few m of an oyster array, supporting the conclusions found using direct N₂ production methods.

The denitrifying community composition of biodeposits was indistinguishable from water column seston, indicating that oyster filtration of seston does not significantly alter the denitrifying consortia in biodeposits before being delivered to the sediments. Because oyster sediments represent a unique denitrifying community, it appears that the interaction of biodeposits with sediments alters the community composition. Forced *in situ* accumulation of biodeposits in oyster sediments (fence), resulted in a denitrifying

community that did not differ significantly from biodeposits and seston, nor from reference/near oyster sediments at both sites and oyster sediments at Spencer's Creek. This suggests that the denitrifying community is dynamic in responding to oyster biodeposition and adjusts to changing conditions at both sites.

Conclusions

Sediment bacterial abundance was determined by site, and neither intensive oyster biodeposition nor season played a significant role in determining or changing these bacterial abundances. If coupled nitrification-denitrification is dependent upon sediment nitrifier abundance in some meaningful way, then no evidence was found to support the hypothesis that oyster biodeposition stimulates increased N removal *via* this pathway because nitrifier abundance remained uniform at each site regardless of the presence of oysters and the relative abundance of nitrifying bacteria was ~20-30% of the bacterial population at both sites. The baseline sediment bacterial consortia of these two sites did not differ, and evidence of changes to the broad bacterial community due to oyster aquaculture were found at the low energy silty-sediment site, but not at the high energy site with sandy sediment. This indicates that the impact of oyster cultivation on sediment microbial processes is not uniform across the various environments in which oysters are cultivated in the Bay. Oyster biodeposition significantly altered the denitrifying community at both sites and this effect was limited in area, within 5 m of the oyster arrays at both sites.

This study represents an important step in relating a treatment (oyster biodeposition) to a quantifiable change in the community of denitrifying bacteria and

measurable changes in N_2 production, i.e. between community structure and function. Molecular approaches have improved our understanding of the microbial community response to native oyster biodeposition, a process that has been greatly diminished due to loss of much of the wild oyster population. It provides new insights into the microbial communities responsible for nitrification and denitrification, and a framework from which to build on uncovering the fate of nutrients processed by suspension-feeding bivalves.

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Figure Captions

Figure 4.1. Diagram of direct denitrification and coupled nitrification-denitrification biogeochemical pathways in estuarine and marine sediments.

Figure 4.2. Diagrams of biodeposit catchment device and biodeposit fence used to (A) quantify the mass flux of nutrients and (B) force accumulation of oyster biodeposition to estuarine sediments in Chesapeake Bay.

Figure 4.3. Abundance (mean \pm SE) of total bacterial (Total), eubacteria (EUB), and nitrifying (AOB/NOB) bacteria in oyster, reference, and fence (for St. Jerome) sediments (0-3 cm) at two oyster aquaculture sites in Chesapeake Bay, St. Jerome Creek (A) and Spencer's Creek (B). No significant differences between oyster and reference were observed at either site, but Spencer's Creek had significantly higher abundance of all bacteria (ANOVA, $p < 0.05$).

Figure 4.4. Seasonal abundance (mean \pm SE) of total bacterial (Total) and eubacteria (EUB) in oyster and reference sediments (0-3 cm) at two oyster aquaculture sites in Chesapeake Bay, St. Jerome Creek (A) and Spencer's Creek (B).

Figure 4.5. Seasonal abundance nitrifying bacteria (mean \pm SE) identified using FISH in oyster and reference sediments (0-3 cm) at two oyster aquaculture sites in Chesapeake Bay, St. Jerome Creek (A) and Spencer's Creek (B).

Figure 4.6. Seasonal relative abundance (mean \pm SE) of nitrifying bacteria identified using FISH in oyster and reference sediments (0-3 cm) at two oyster aquaculture sites in Chesapeake Bay, St. Jerome Creek (A) and Spencer's Creek (B).

Figure 4.7. NMDS analysis of sediment (0-3 cm) eubacterial T-RFLP profiles from two oyster aquaculture sites (Oyster) and two reference (Reference) sites in Chesapeake Bay. ANOSIM analysis showed that the bacterial community composition of oyster and reference at Spencer's Creek (B) were significantly different ($p = 0.001$) but no differences at St. Jerome Creek (A) were observed. St. Jerome Creek stress of the plot = 0.1232, axis 1 = 0.7687, axis 2 = 0.1888; Spencer's Creek (B) stress of the plot = 0.1218, axis 1 = 0.7023 axis 2 = 0.1517. Letters indicate significant differences detected by One-way ANOSIM analysis ($p < 0.05$).

Figure 4.8. Sediment eubacterial richness calculated as number of terminal restriction fragments (mean \pm SE) from T-RFLP analysis (0-3 cm) at two sites in Chesapeake Bay comparing oyster aquaculture and reference sediments, St. Jerome Creek (A) and Spencer's Creek (B).

Figure 4.9. NMDS analysis of *nosZ* T-RFLP profiles of sediment (0-3 cm), biodeposit and seston samples across multiple seasons at two oyster aquaculture sites in Chesapeake Bay, St. Jerome Creek (A) and Spencer's Creek (B). Non-metric multidimensional scaling 2-D plots (Jaccard) of *nosZ* T-RFLP data consisting of the presence/absence of terminal restriction fragments. Sediments underneath oyster aquaculture rafts (Oyster), sediments 5-10 m outside of oyster array (Near Oyster), and forced accumulation of oyster biodeposits (Fence) are sediment sample types treated with varying levels of oyster biodeposits and compared to no-aquaculture reference sediments (Reference), oyster biodeposits (Biodeposits) and water column seston (Seston, reported for St. Jerome Creek). St. Jerome Creek stress of the plot = 0.1683, axis 1 = 0.5717, axis 2 = 0.2720; Spencer's Creek stress of the plot = 0.1931, axis 1 = 0.4973 axis 2 = 0.3156. Letters indicate significant differences detected by One-way ANOSIM analysis ($p < 0.05$).

Figure 4.10. Seasonal comparisons of *nosZ* T-RFLP analysis of sediment (0-3 cm), biodeposit and seston samples at two oyster aquaculture sites in Chesapeake Bay, St. Jerome Creek (A) and Spencer's Creek (B). Data consist of the presence/absence of terminal restriction fragments and the number of T-RFs (mean \pm SE) are reported as richness. At Spencer's Creek summer and winter are generally higher than fall and spring and no seasonal trends were observed at St. Jerome Creek.

Figure 4.11. The *nosZ* number of T-RFs are reported as richness (mean \pm SE). T-RFLP *nosZ* results for top (0-3 cm) and bottom (4-10 cm) sections of sediment cores and biodeposit samples across all seasons at two oyster aquaculture sites in Chesapeake Bay, St. Jerome Creek (A) and Spencer's Creek (B). Neither sample type nor cm section were significant factors in determining *nosZ* diversity.

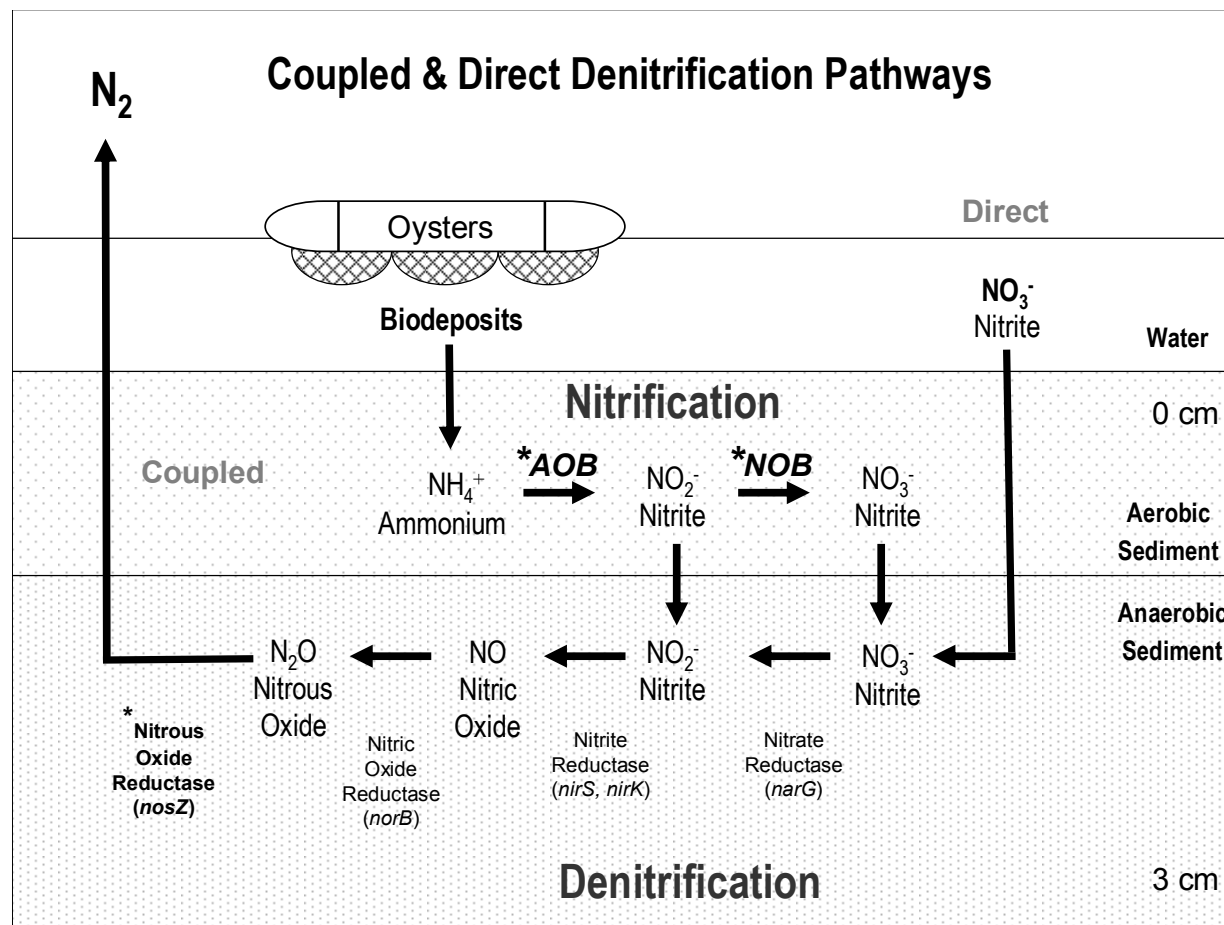
Table 4.1. Fluorescent *in situ* hybridization rRNA targeted oligonucleotide probes used for enumeration of eubacteria and nitrifying bacteria in sediments.

Target Organism	16S rRNA targeted Oligonucleotide Probe	Flourescent Label	16S rRNA Target Sequence	Reference
Domain <i>Bacteria</i>	EUB338	FAM	5'- GCTGCCTCCCGTAGGAGT-3'	Gieseke et al. 2001
Domain <i>Bacteria</i>	EUB338II	FAM	5'- GCAGCCTCCCGTAGGTGT-3'	Daims et al. 1999
Domain <i>Bacteria</i>	EUBIII	FAM	5'- GCTGCCTCCCGTAGGAGT-3'	Daims et al. 1999
AOB	NIT3	TET	5'-CCTGTGCTCATGCTCCG-3'	Mobarry et al 1996
AOB	NSO1225	TET	5'-CGCGATTGTATTACGTGTGA-3'	Mobarry et al 1996
NOB	S-G-Ntspa-0662-a-A-18	TET	5' -GGAATTCCGCGCTCCTCT -3'	Daims et al. 2001

Table 4.2. Sediment organic matter (OM, carbon (%C), nitrogen (%N), chemical oxygen demand (COD), and biological oxygen demand (BOD) at two oyster aquaculture sites and reference sites in Chesapeake Bay, St. Jerome Creek and Spencer's Creek.

Site	Type	OM (%)	C (%)	N (%)	C:N	COD (mg/kg)	BOD (mg/kg)
St. Jerome Creek	Oyster	11.1±1.1	*0.54 ± 0.19	0.07±0.01	9.2±0.5	*4934±441	*402±83
	Reference	8.9±1.1	0.38 ± 0.20	0.07±0.01	7.8±0.5	7543±441	160±83
	Total	10.0±0.7	*0.46 ± 0.14	*0.07±0.01	*8.5±0.4	*6238±312	*281±59
Spencer's Creek	Oyster	*14.7±0.8	*5.04 ± 0.21	*0.42±0.03	14.6±0.6	38345±1943	*606±47
	Reference	8.8±0.3	2.56 ± 0.21	0.19±0.03	15.4±0.6	30730±1943	316±47
	Total	11.8±0.6	3.80 ± 0.15	0.31±0.02	15.0±0.5	34537±1374	461±33

* Indicates significant differences detected between sites or within a site between oyster and reference (ANOVA, p<0.05).



* Indicates pathway steps in which molecular approaches were applied in this study.

Figure 4.1.

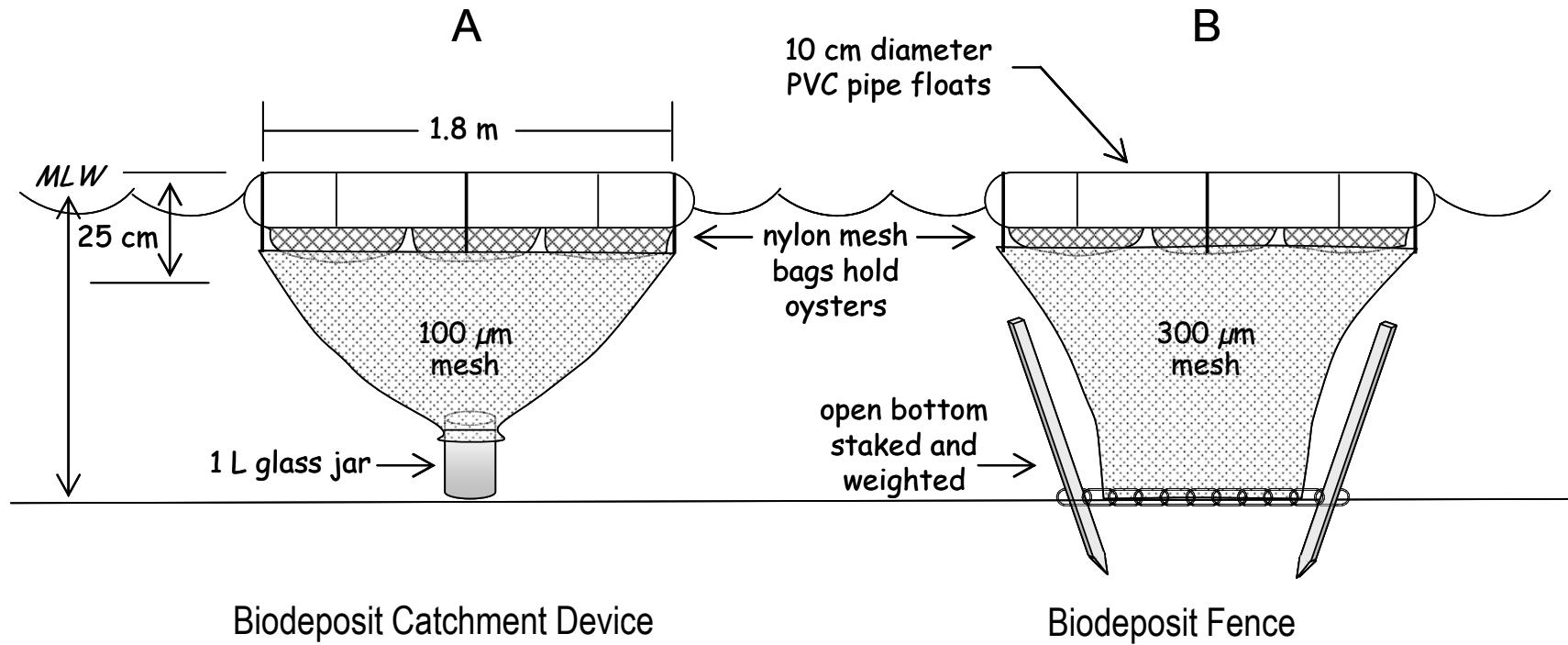
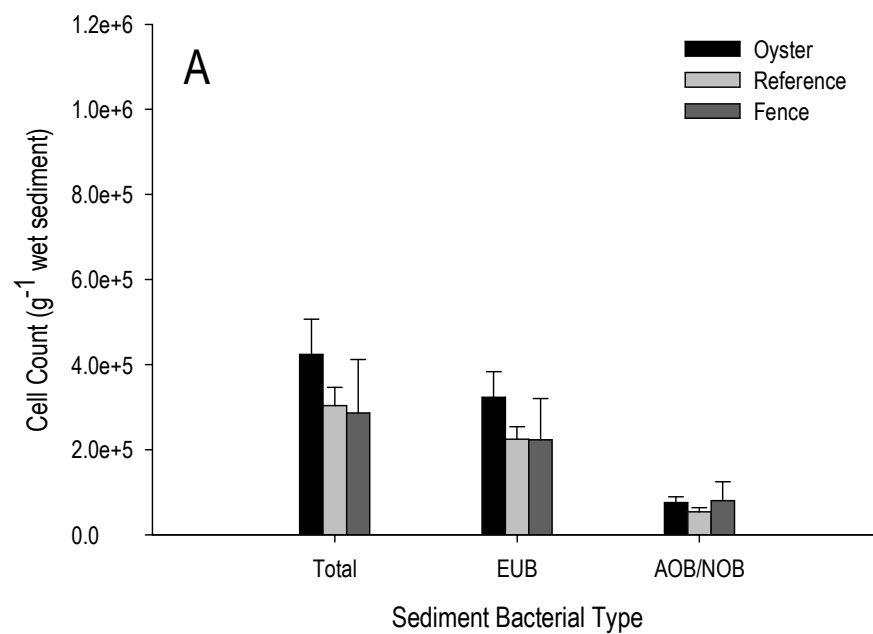
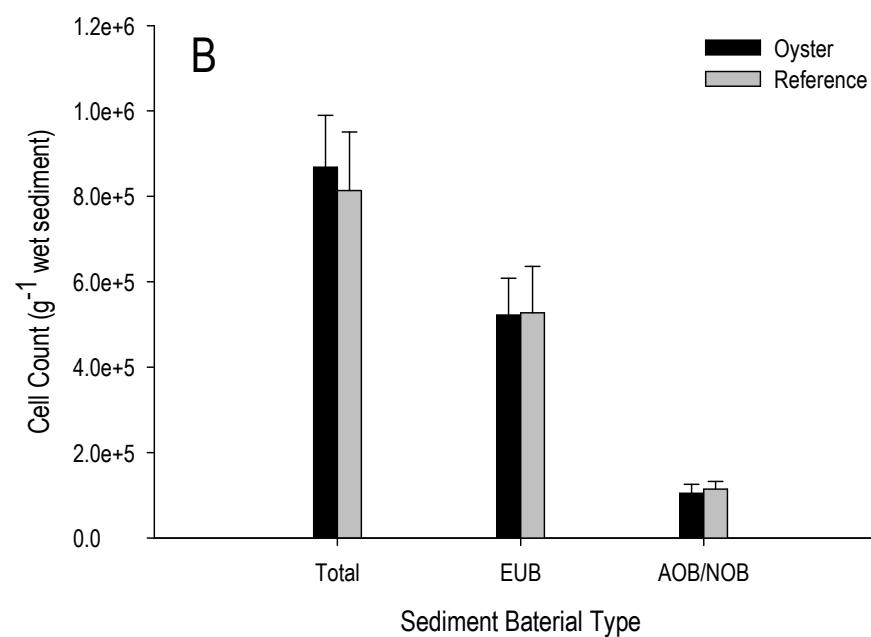


Figure 4.2.



St. Jerome Creek



Spencer's Creek

Figure 4.3.

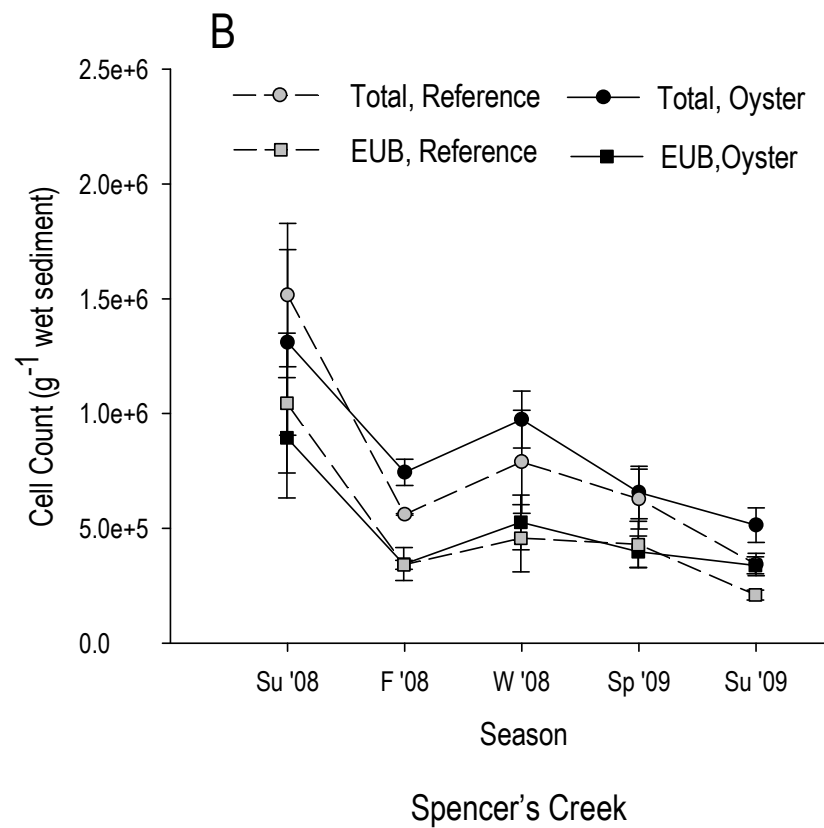
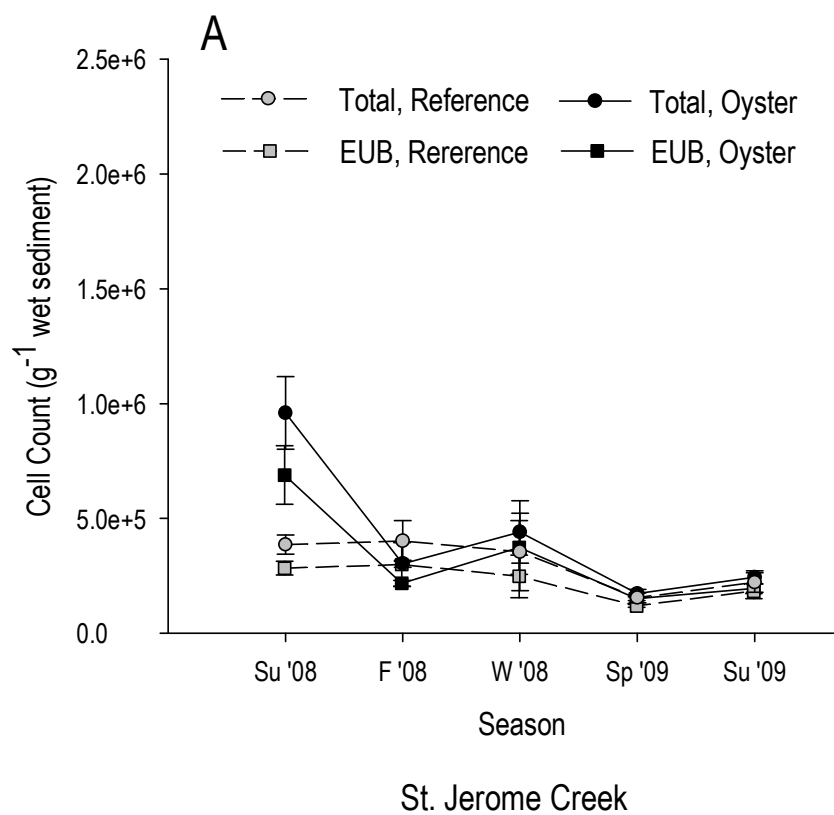


Figure 4.4.

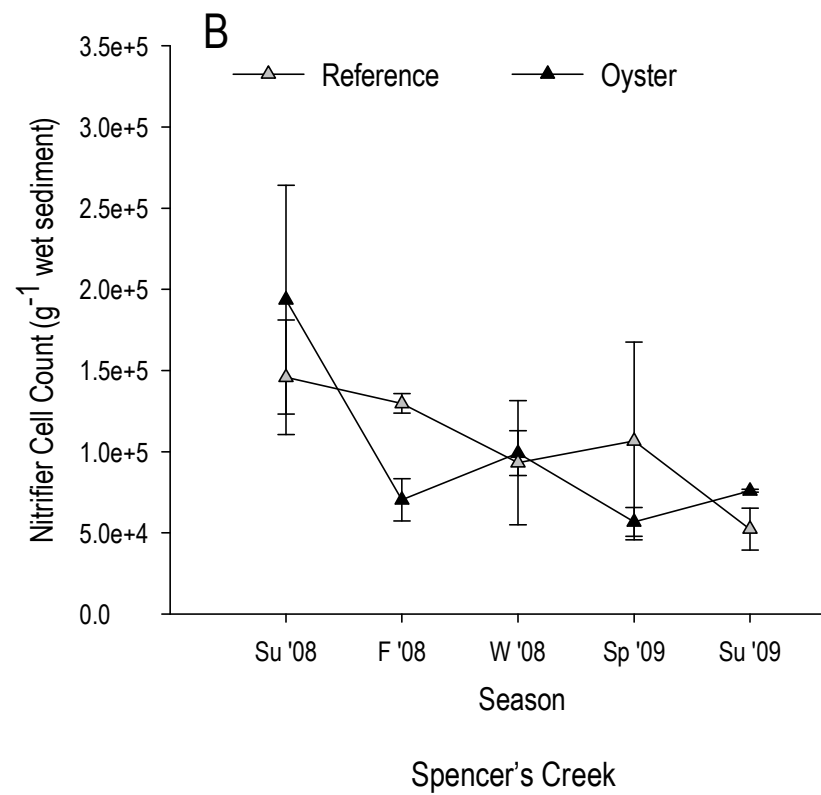
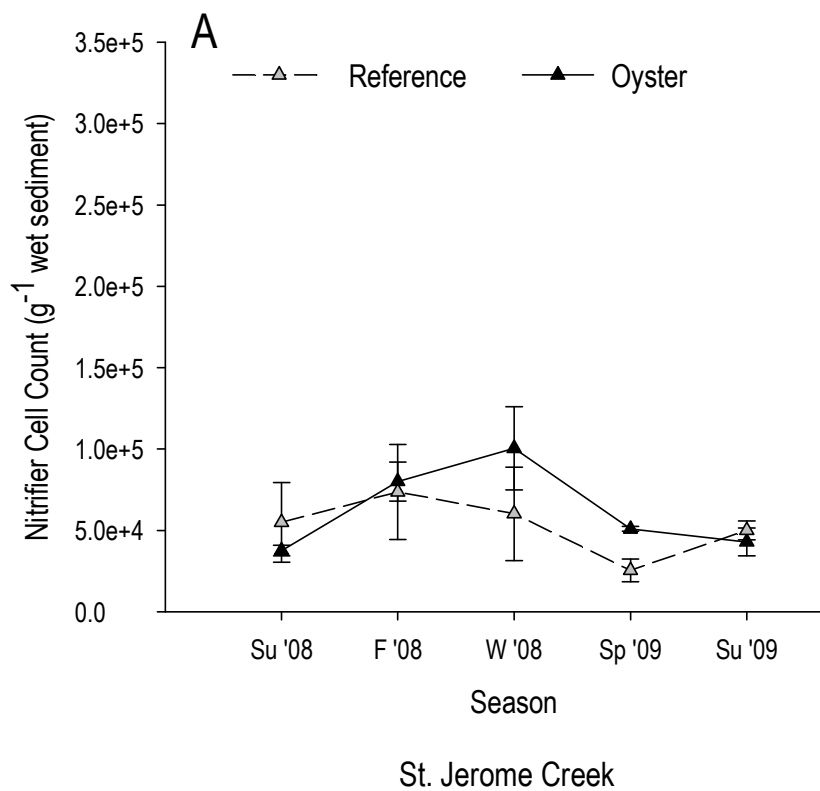


Figure 4.5.

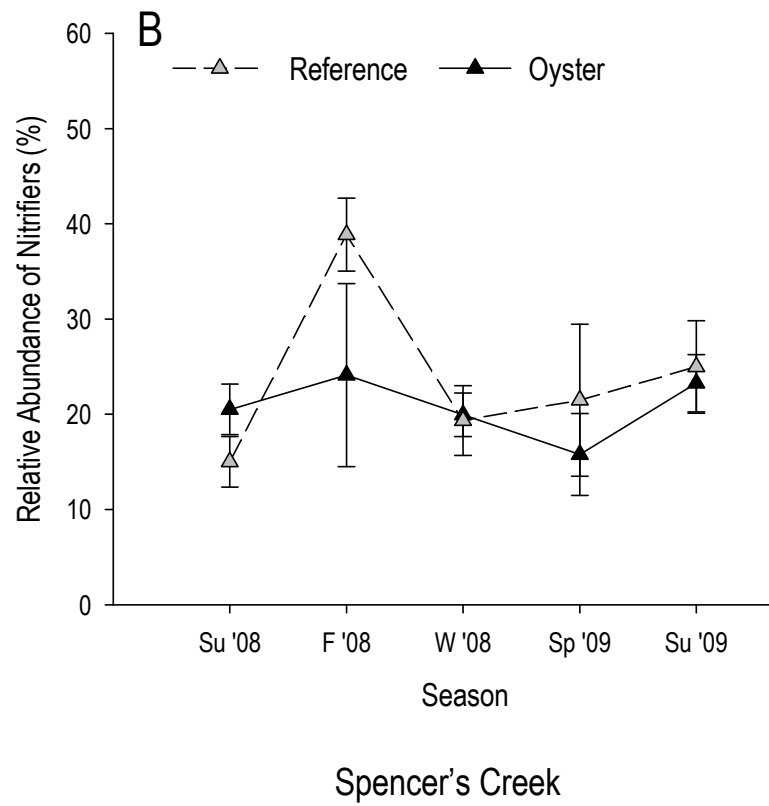
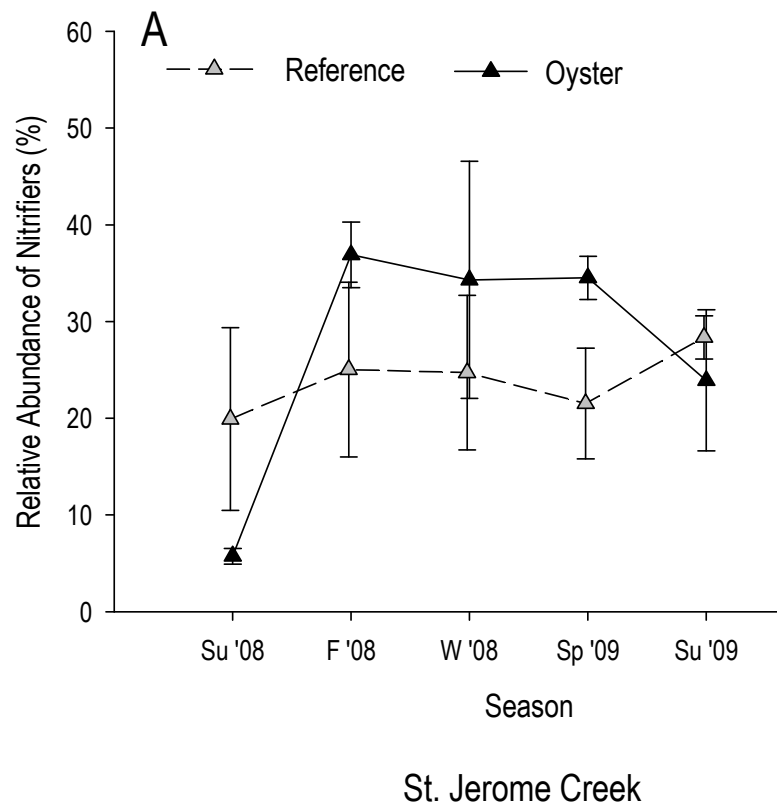
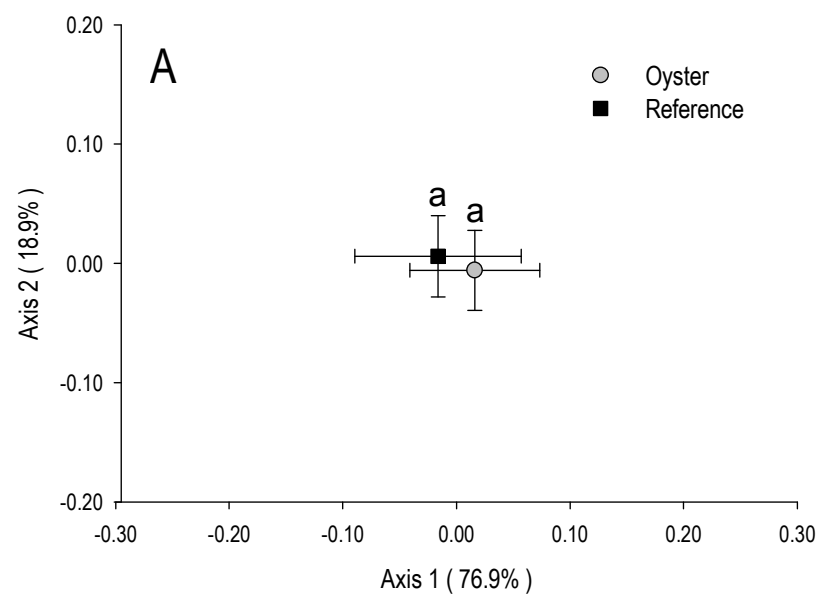
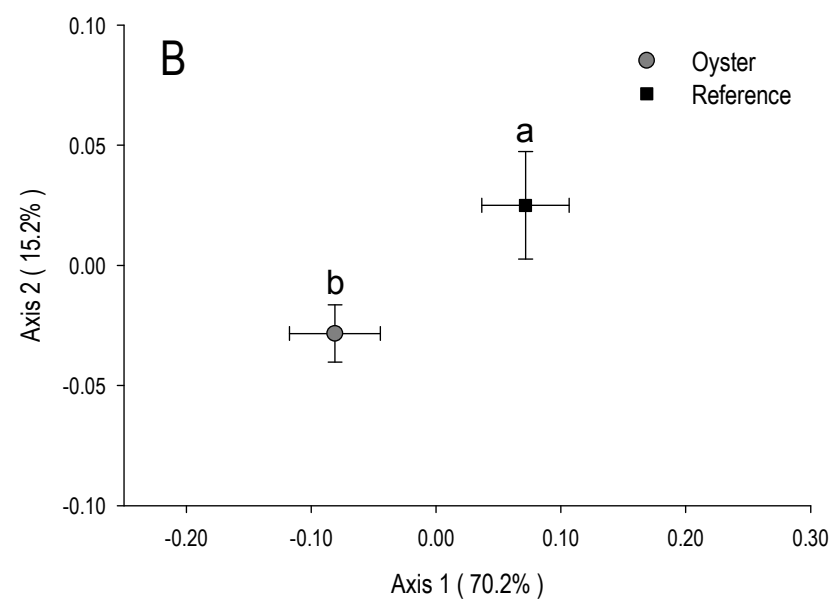


Figure 4.6.



St. Jerome Creek



Spencer's Creek

Figure 4.7.

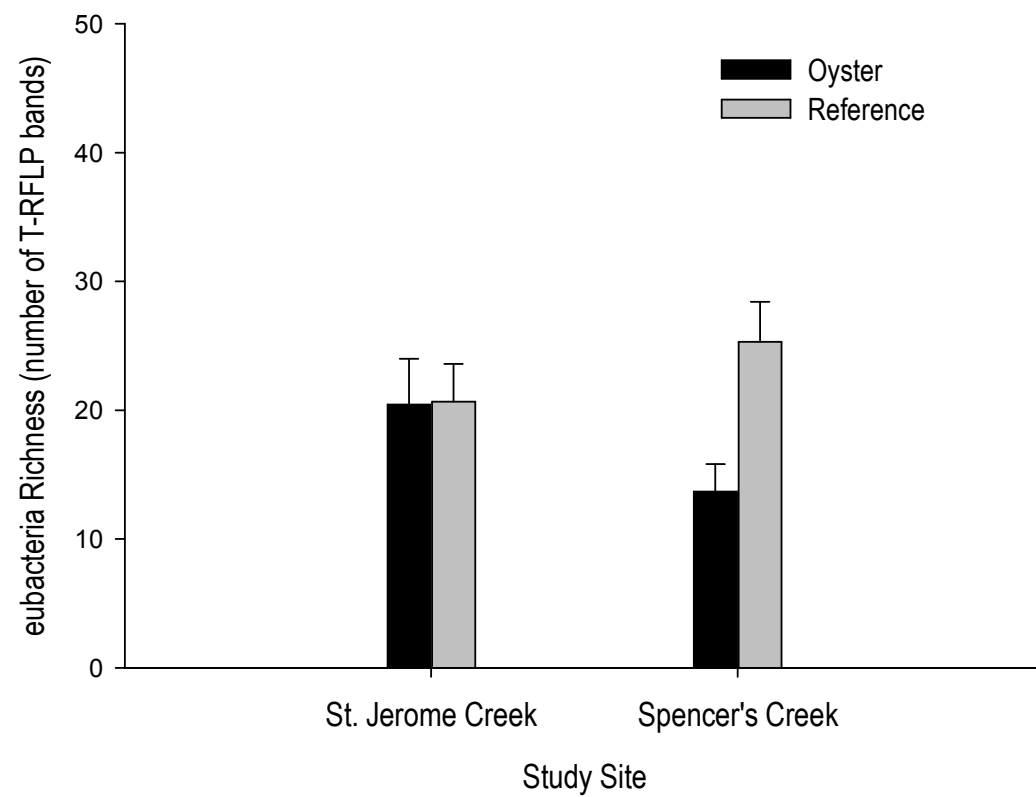
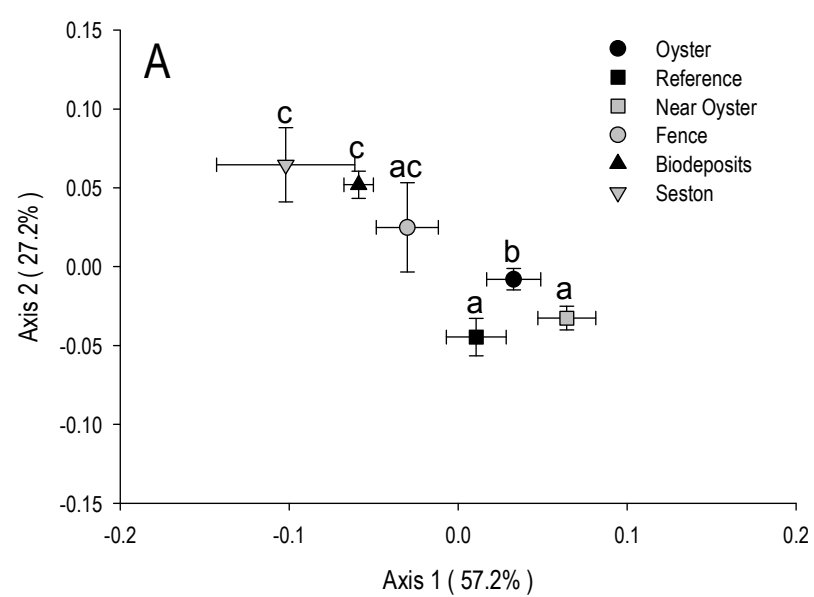
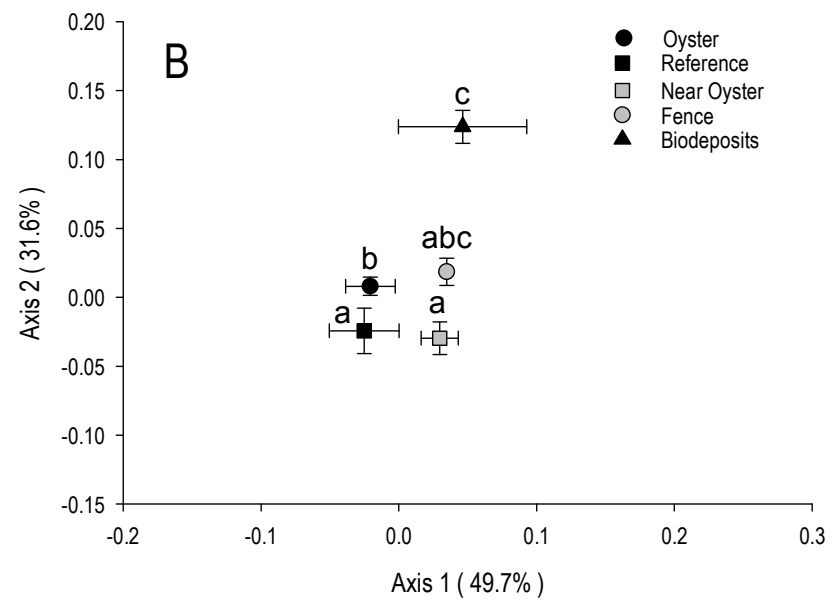


Figure 4.8.

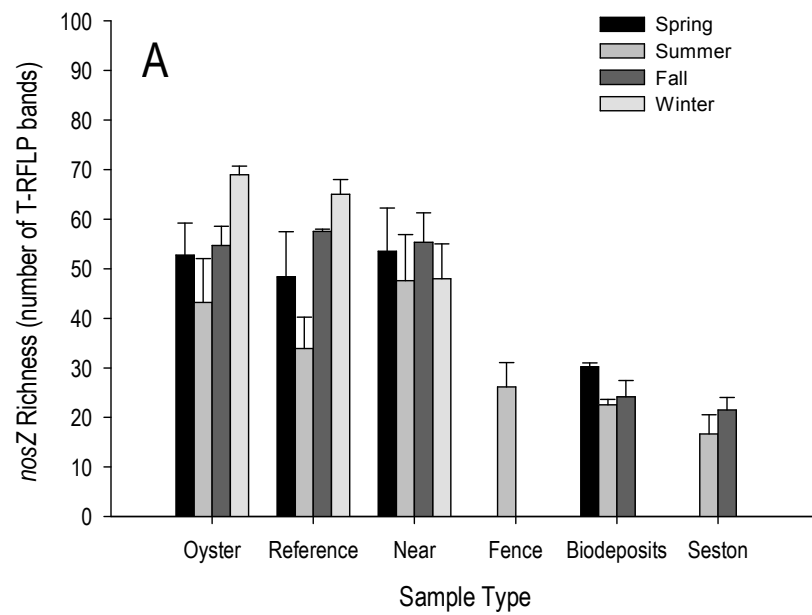


St. Jerome Creek

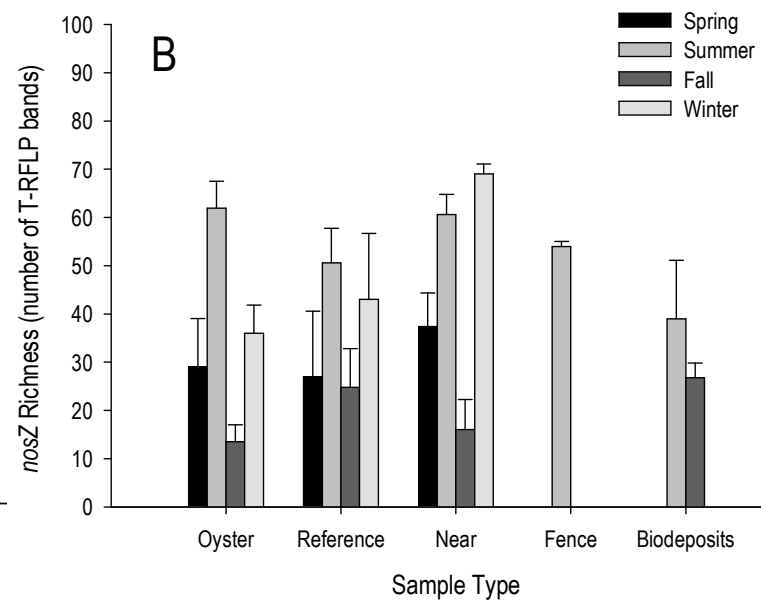


Spencer's Creek

Figure 4.9.

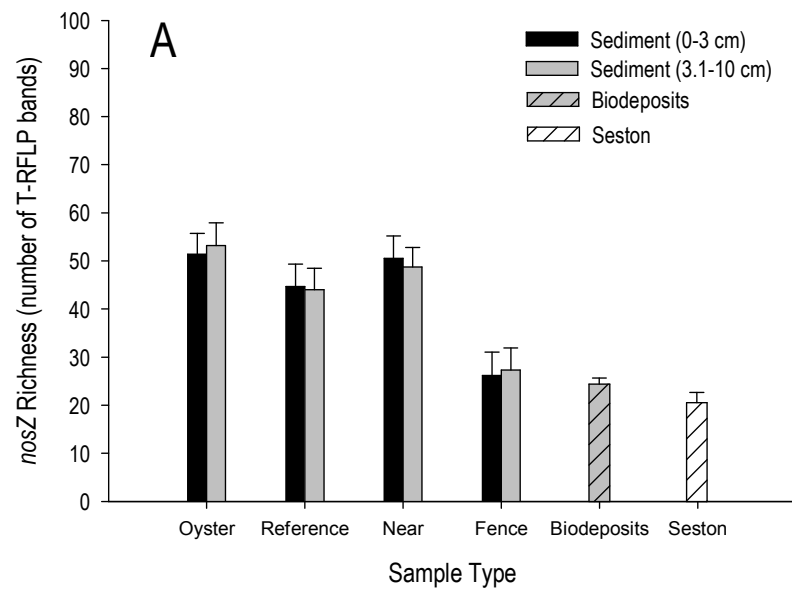


St. Jerome Creek

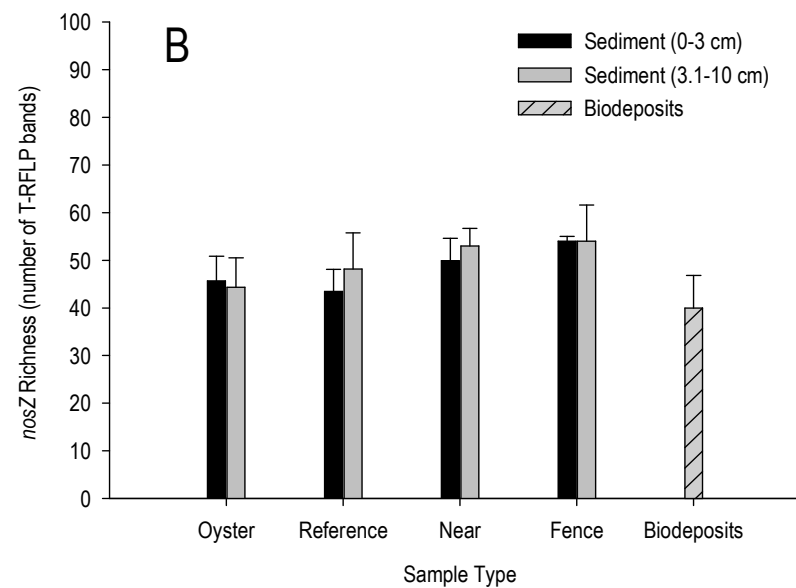


Spencer's Creek

Figure 4.10.



St. Jerome Creek



Spencer's Creek

Figure 4.11.

Chapter 5

Conclusions

Quantifying an ecosystem service is a challenging task because some factors lend themselves to being quantified, whereas others do not. Ecosystems provide benefits fundamental to everyday life, yet the ubiquitous and complex nature of these benefits make it difficult to determine the value of ecological services. Often the services that organisms and ecosystems provide are not well understood, are taken for granted, or we can not perceive their true value until they are gone. The process of ecological quantification in terms that are translatable into economic valuation requires a biologist to approach a research question from a different vantage point. How much is an oyster worth? What value does an oyster add by virtue of its addition to an ecosystem?

Eutrophication of estuaries and coastal marine ecosystems is a problem associated with human population growth worldwide. Oysters and other suspension-feeding bivalves have long been promoted as a means to improve water quality by virtue of their filtration capacity. Loss of over 99% of the wild oyster population in Chesapeake Bay may have confounded the nutrient pollution effect. There has been much speculation surrounding the native oyster's ability to help rid the Bay of excess nutrients, but there has been no clear quantification of such an effect to date. In this study, I investigated the nutrient removal potential of native oyster aquaculture, *Crassostrea*

virginica, in Chesapeake Bay. I evaluated two pathways of permanent nutrient removal, biomass assimilation and oyster biodeposit-stimulated sediment N₂ production, at two commercial-scale oyster floating-raft aquaculture sites.

Oysters are a nutrient sink, as are all organisms, by virtue of the fact that they assimilate nutrients into biomass. What makes oysters (and other suspension-feeding bivalves) unique is their ability to filter large quantities of ambient water and capture nutrients. Oyster cultivation does not require an input of nutrients from outside the system and nutrients assimilated into biomass represent a human endeavor to capture and remove nutrients permanently from the system. Nutrient removal through harvest of oyster biomass is a measure that lends itself to quantification. In this particular case, assimilation services can be quantified and measured with high levels of statistical confidence. I developed models for nitrogen (N), phosphorous (P), and carbon (C) removal using the common market measurement of oyster total shell length. Results were uniform across a number of native oyster strains grown at two sites such that a simple, yet accurate, method is now available for estimating the amount of nutrients removed when harvesting aquacultured oysters. Cultivated oysters had substantially less nutrients than previously thought because they are raised to maximize growth rates and thereby have thinner shells than wild oysters. Although the percent of N and P in oyster shells is low, because so much of the biomass of an oyster is shell, cultivated oysters with thinner shells assimilate fewer nutrients per unit length than wild oysters.

An alternative pathway of N removal, sediment N₂ production, has attracted much interest because of the widespread implications at the ecosystem scale. If oyster biodeposition were to stimulate increased N₂ production, then oysters have the potential

to induce an ecosystem-wide loss of N on a large scale by expanding the sediment N sink. Through oyster cultivation or restoration, oysters could truly have an impact on mitigating eutrophication effects. This pathway involves an interaction of oyster biodeposition and sediment microbial nutrient processing. No evidence was found to support the hypothesis that oyster biodeposition significantly stimulates increased direct denitrification, coupled nitrification-denitrification, or anammox rates at either site. These results were consistent across the two sites which had very different sediment and hydrological conditions. In spring, early summer, and fall, oysters appear to have either no effect or inhibit sediment N_2 production. Only in mid-summer do sediments associated with oyster aquaculture maintain higher N_2 production rates while rates at the reference sites decrease by ~88%. In these few months, oysters may have the potential to maintain N removal rates at $\sim 1 \text{ mmol N m}^{-2} \text{ d}^{-1}$ above baseline rates. In a scenario where oysters covered 25% of Bay sediments and assuming that rates were not inhibited in other seasons, the N removal potential due to oyster biodeposition could be as high as 3% of annual N loads to Chesapeake Bay. However, a more likely scenario that is more in line with these findings is that the overall impact of oyster aquaculture on net sediment N_2 production is no different or slightly less than baseline N removal rates.

Based on direct measures of N_2 production and molecular analysis of the community of denitrifying bacteria, there is considerable evidence that the area of sediment impacted by oyster biodeposition is limited to within 5 m of an oyster array. This is important because sediment N removal rates are based on sediment area, therefore, even if rates were substantially higher in oyster impacted sediments, N removal would be limited by the relatively small sediment footprint of oyster aquaculture.

The previously published massive estimated potential of oyster enhanced N_2 production was based on a hypothesized relationship between oyster biodeposition rates and N_2 production. However, these two variables were found in the present study to be only weakly correlated and therefore the high filtration capacity of oysters does not appear to be related to N removal.

Investigation into the sediment microbial consortia of denitrifying and nitrifying bacterial provided important insight into the dynamics of oyster biodeposit nutrient processing that is not reflected by direct measures of N_2 production. It was evident that oyster biodeposition significantly alters the sediment community of denitrifying bacteria. In seasons when significant differences in N_2 production were observed, these changes in function were paralleled by changes within the sediment denitrifying community. Although the *nosZ* T-RFLP analysis did not provide significant correlation results that allow for prediction of N_2 production, this molecular approach proved useful in revealing changes in N removal. Although production of N_2 gas from oyster biodeposit treated sediments may be no higher than reference sediments (no oyster biodeposits), denitrification may be incomplete in oyster-impacted sediments resulting in production of NO and/or N_2O . No apparent effects of oyster biodeposition were evident in nitrifier abundance patterns at either site, indicating that oyster biodeposition does not enhance coupled nitrification-denitrification by increasing the abundance of nitrifiers in sediments.

Because it is possible to measure the effects of some ecosystem services, we may inadvertently apply higher standards of quantification and verification to such services than to deleterious actions that cause environmental damage. However, to quantify an ecosystem service as a policy initiative, it is important that services can be

measured and/or modeled with a reasonable degree of confidence. If environmental variability of a service is high, the prescriptive measurement may be so costly or technologically difficult that verification is virtually impossible. Oyster nutrient bioassimilation is quantifiable and can be modeled accurately, but N removal *via* sediment N₂ production is so costly and requires such specialized expertise to quantify that prescriptive measures are impractical at this time. Even if oyster biodeposition had proven to remove N in meaningful quantities, because N₂ production is highly variable, initiating a program of verification would likely be impractical due to the complicated and costly nature of taking such measurements seasonally at multiple sites. Molecular approaches are far more accessible and allow for high throughput analyses, but ultimately they are a poor proxy for direct measures to quantify N removal.

Oysters capture large amounts of matter and energy, the fate of which is not completely understood. In this investigation, two removal pathways of oyster-processed nutrients were quantified. Oyster aquaculture bioassimilation provides non-trivial nutrient removal services and may be an important component of nutrient management strategies in the Bay. Conversely, oyster biodeposition did not increase N removal rates *via* sediment N₂ production and the inclination to intertwine oyster policy initiatives with oyster nutrient removal through this pathway may have the unintended consequence of devaluing the perceived benefits of native oysters in Chesapeake Bay. Although the findings of this study did not support large-scale ecosystem benefits of oyster aquaculture in terms of eutrophication mitigation as previously estimated, there are numerous other documented ecosystem services of oysters and other suspension-feeding bivalves.

Vita

Colleen Beth Higgins was born in Schenectady, NY, on March 14, 1971. She graduated from Plano Senior High School, Plano, TX in 1989. She received her Bachelor of Arts in Middle East Area Studies from The George Washington University, Washington, D.C. in 1994. She received a Bachelor of Science in Biology from Virginia Commonwealth University, Richmond, Virginia in 2000. She received a Master of Science in Biology, from Virginia Commonwealth University in 2006.