FEATURE ARTICLE

Denitrification and nutrient assimilation on a restored oyster reef

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ABSTRACT: At a restored reef site and a control site in the Choptank River, Maryland, USA, we partially quantified the effect of oyster reef restoration on the removal of nutrients from the water column by determining seasonal fluxes of oxygen (O_2), ammonium (NH_4^+), combined nitrate and nitrite (NO_{2+3}), di-nitrogen (N_2) and soluble reactive phosphorus (SRP) and by assessing the assimilation of nutrients by macrofauna. Fluxes of O_2, NH_4^+, NO_{2+3} and SRP at the restored site were enhanced by at least one order of magnitude during all seasons. Seasonal denitrification rates at the restored site, measured as flux of N_2-N, ranged from 0.3 to 1.6 mmol N_2-N m^{-2} h^{-1}, with August rates among the highest ever recorded for an aquatic system. In addition to oysters (131 oysters m^{-2}; average shell height = 114 mm; age = 2 to 7 yr), the restored reef provided habitat for 24 585 other macrobenthic organisms per square meter compared to 2265 organisms m^{-2} at the control site. Restoration enhanced the average standing stock of assimilated nutrients by 95 g N m^{-2} and 15 g P m^{-2}. Nitrogen and phosphorus in shells of live oysters and mussels accounted for 47 and 48% of total nitrogen and phosphorus standing stocks, respectively. Our results demonstrate that oyster reef restoration can significantly increase denitrification rates and enhance nutrient sequestration via assimilation into bivalve shells.

KEY WORDS: Restoration · *Crassostrea virginica* · Biogeochemistry · Denitrification · Ecosystem services · Nitrogen · Phosphorus · Water quality
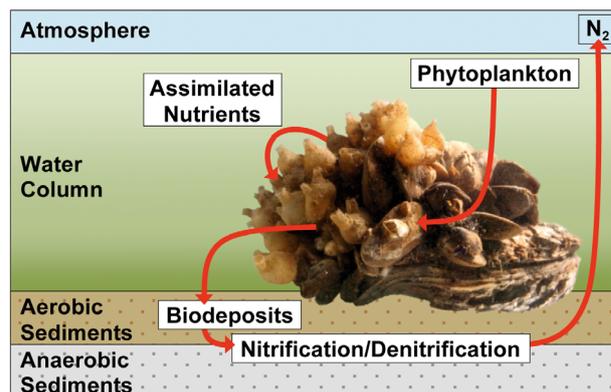
Oyster reefs reduce eutrophication by enhancing denitrification rates and assimilating nutrients into macrofauna.

Image: M. L. Kellogg

INTRODUCTION

Global loss of 85% of oyster reef ecosystems over the past 130 yr (Beck et al. 2011) has led to both growing recognition of the services once provided by healthy oyster reef ecosystems (Grabowski et al. 2012) and increasing interest in restoring these ecosystems. In Chesapeake Bay, USA, populations of the native oyster *Crassostrea virginica* have been reduced to <1% of historic levels through a combination of overharvesting, poor management practices and the impacts of 2 oyster diseases (Wilberg et al. 2011 and references therein). Between 1990 and 2007, oyster reef restoration activities were under-

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taken at 594 locations within Chesapeake Bay and the Maryland and Virginia Coastal Bays (Kennedy et al. 2011). Although many restoration efforts focused on increasing substrate available for settlement of oyster larvae, a substantial number also targeted areas where natural larval supply was a limiting factor. These efforts relied on producing oyster larvae in a hatchery, setting them on adult oyster shell and transplanting those juveniles to the restoration site. Since 2002, this has been the primary method used for oyster reef restoration in the Maryland portion of Chesapeake Bay (Kennedy et al. 2011) and is being used with increasing frequency in other parts of the United States (Brumbaugh & Coen 2009).

The expense of oyster reef restoration, especially when hatchery-produced juvenile oysters are used, raises the question of whether the benefits of oyster reef restoration are worth the investment. Quantifying the benefits of any particular oyster reef restoration effort is complicated because they are diverse, often site-dependent and, in some cases, difficult to measure. Although significant progress has been made in quantifying the benefits of oyster reefs in terms of enhanced (1) filtration and clarity of the water column (Grizzle et al. 2008), (2) abundance and diversity of benthic invertebrates (Rodney & Paynter 2006), (3) abundance and diversity of mobile crustaceans and fishes (Stunz et al. 2010 and references therein) and (4) fishery production (Peterson et al. 2003), less progress has been made in quantifying

the impacts of oyster reefs on nutrient cycling (but see: Newell et al. 2005, Piehler & Smyth 2011, Smyth et al. 2013).

Two poorly quantified benefits of oyster reef restoration are removal of nitrogen via enhanced denitrification and assimilation of nutrients into the tissues and shells of reef organisms. Oysters, along with other reef-associated filter-feeding organisms, modify biogeochemical cycles by filtering large quantities of organic matter from the water column (Fig. 1), most of which is either used directly for growth and maintenance or is deposited on the sediment surface as feces and/or pseudofeces (i.e. biodeposits). In many subtidal environments, a common setting for oyster reef restoration, these biodeposits concentrate organic material in aerobic environments with low light levels. These conditions are conducive to efficient removal of nitrogen by microbially mediated denitrification (Newell et al. 2002). Oyster reefs also provide habitat for an abundant and diverse reef-associated macrofaunal community. As these organisms grow, they assimilate nitrogen and phosphorus. While a significant portion of these nutrients is rapidly recycled back into the system, nutrients assimilated into bivalve shells represent a means of long-term sequestration. Nitrogen removal is likely further enhanced by the activities of high densities of deposit-feeding and bioturbating organisms (Rodney & Paynter 2006, Nizzoli et al. 2007). Restoration of oyster reefs adjacent to deep water in eutrophic estuar-
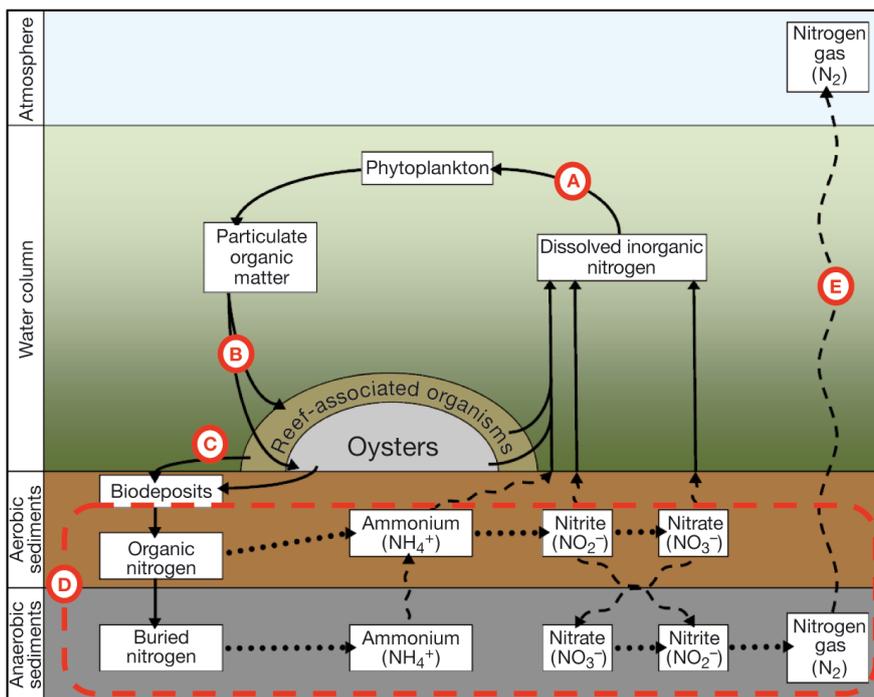

Fig. 1. Primary nitrogen pathways associated with oyster reefs at intermediate depths in Chesapeake Bay, USA: phytoplankton use dissolved inorganic nitrogen for their growth (A), oysters and other reef-associated organisms filter phytoplankton and other particulate organic matter from the water column (B), some of the associated nitrogen is incorporated into organisms and some is deposited on the surface of the sediments (C), and, given the right conditions, a portion of the nitrogen in these biodeposits is transformed into nitrogen gas (D) which diffuses out of the sediments back to the atmosphere (E) where it is no longer available to phytoplankton for growth (diagram adapted from Newell et al. 2005)

ies similar to Chesapeake Bay has the added benefit of removing phytoplankton and other particulates from the water column before they can be deposited in areas with reduced denitrification and enhanced ammonium regeneration due to hypoxic and/or anoxic conditions in summer (Newell et al. 2005).

Prior studies of the effects of oyster communities on nitrogen cycling have included benthic tunnels in marsh creeks (Dame et al. 1989), core incubations to simulate the effects of oyster biodeposits (Newell et al. 2002, Holyoke 2008) and incubations of sediment cores collected adjacent to oyster clumps (Piehler & Smyth 2011, Smyth et al. 2013). All of these studies have either measured fluxes in intertidal or shallow subtidal waters in the photic zone and/or have excluded oysters and other reef-associated organisms from their measurements. Extrapolating from these studies to deeper subtidal reefs is problematic because (1) biogeochemical processes are expected to differ between reefs within and below the euphotic zone (Newell et al. 2005), (2) the physical structure of an oyster reef provides numerous microhabitats that likely facilitate nitrification and denitrification and (3) the burrowing and feeding activities of reef-associated organisms likely alter biogeochemical cycles (Nizzoli et al. 2007 and references therein).

Extrapolating existing data to estimate nutrient assimilation by restored oyster reef organisms is also difficult. Prior studies of oyster assimilation have used selected strains and/or grown oysters under environmental conditions that differ from natural or restored oyster reefs (Higgins et al. 2011, Carmichael et al. 2012). Prior studies documenting the macrofaunal community found on restored subtidal oyster reefs (e.g. Rodney & Paynter 2006) have not measured the nutrient content of each species.

The present study focused on assessing alterations in nutrient dynamics attributable to reef restoration in terms of both the amount of nitrogen removed via denitrification and the amount of nitrogen and phosphorus assimilated into the tissues and shells of oyster reef organisms. While we recognize that biodeposition and subsequent burial of nutrients is also an important nutrient removal pathway (Newell et al. 2005), estimating this loss term was beyond the scope of the present study. We hypothesized that oyster reef restoration in subtidal environments would enhance denitrification rates and increase the standing stock of assimilated nutrients. Using a combination of field sampling and laboratory flux chamber incubations we (1) quantified denitrification rates by comparing fluxes of nitrogen gas in samples collected from a restored oyster reef site (hereafter 'restored site') to those collected from an adjacent site that had not been restored (hereafter 'control site'); (2) assessed seasonal changes in denitrification rates and calculated annual values; and (3) compared the average abundance, biomass and standing stock of nutrients contained in the tissues and shells of organisms at the restored and control sites.

MATERIALS AND METHODS

Study area

Our study focused on a restored oyster reef (38° 34.411′ N, 76° 3.131′ W) in the Choptank River and an adjacent control site (38° 34.308′ N, 76° 3.153′ W) that was suitable for restoration, both located within the Shoal Creek historic oyster bar near Cambridge, Maryland, USA (Fig. 2). Restoration activities in this area included placement of oyster shell in 2003 followed by placement of hatchery-produced juvenile oysters settled on adult oyster shells in 2003, 2006 and 2007 (S. Allen, MD Oyster Recovery Partnership, pers. comm.). Divers identified a 'restored' site (characterized by evidence of restoration activity, i.e. planted shells and oysters) and a 'control' site (characterized by a lack of planted shell and oysters) within this area suitable for our experiments and established a 4 m × 4 m experimental plot at each site (Fig. 3). Because sites were only ~190 m apart and both were at ~4 m depth, physical conditions were expected to be similar.

Both control and restored sites were characterized by firm substratum. At the control site, the substratum surface consisted primarily of sand mixed with fine sediments and shell fragments covering a dense layer of oyster shell, presumably the remains of a past reef. Substratum at the restored site was covered in a layer of exposed oyster shell and clumps of adult oysters from prior restoration efforts. Because oyster density at this site had not yet reached >100 adult oysters m^{-2}, a target density commonly used for oyster reef restoration projects in the Chesapeake Bay, divers collected oyster clumps from the surrounding area and placed them within the experimental plot to increase oyster abundance. All manipulations were completed >12 wk prior to the start of experiments to allow time for the system to equilibrate. Prior to the initial deployment of sampling trays (described below), each 4 m × 4 m experimental plot was subdivided into 4 equal subplots. Within each subplot, 5 potential deployment sites were identified and the sampling sequence was randomly
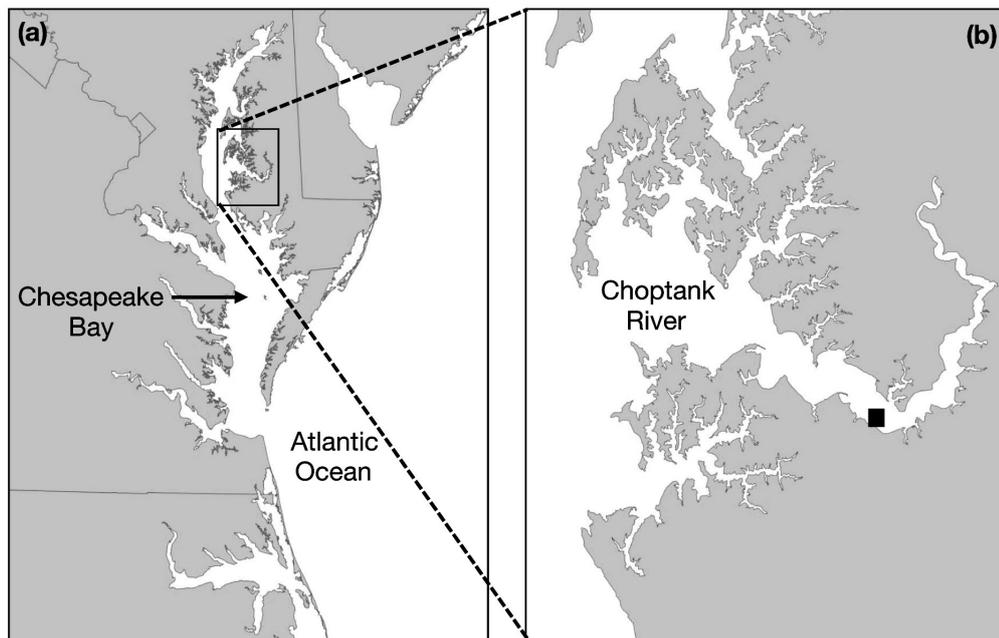
Fig. 2. Map of study area showing (a) the location of Choptank River within Chesapeake Bay and (b) the location of our study sites (■) within the Choptank River. The control site was ~190 m away from the restored site and both fell within the single location indicated on the map

assigned (Fig. 3). One sampling site was used for pilot studies and 4 were used for seasonal sampling.

To characterize environmental conditions, a YSI 6600 was placed at each site to record temperature, sal-

inity and dissolved oxygen every 5 min during each seasonal deployment. We determined the total volume of sediments in sampling trays during each sampling period by measuring the height of the sediments in the tray at 8 locations and multiplying the average height by the area of the tray. To characterize the surface sediments at each site, we collected samples from the top 1 cm of each sampling tray at the end of the August sampling period. Distribution of fine-grained sediment size classes was determined after samples were wet sieved at 64 µm to separate mud from larger sediment particles (Coakley & Syvitski 1991). Total organic content in sediments was determined by combustion at 450°C for 4 h. After removing large pieces of shell, sediments were dried (65°C) and ground and total sediment carbon and nitrogen concentrations were determined using a CHN analyzer. Carbonate carbon was analyzed by gas chromatography after acidification (Stainton 1973), and organic carbon was estimated as the difference between total carbon and carbonate carbon.
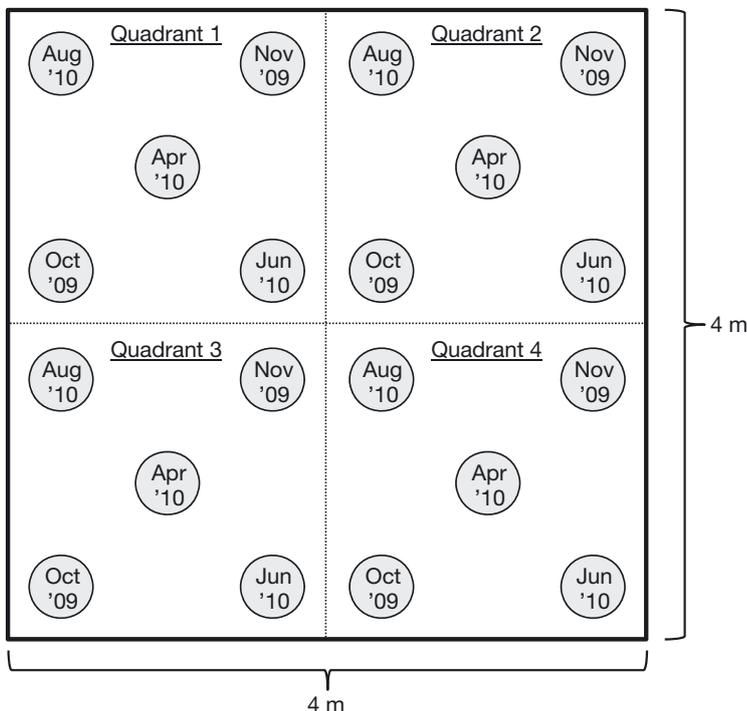

Fig. 3. Diagram of an experimental plot; one plot was located at the restored site, and one at the control site. Each circle designates a sampling location (drawn to scale) and is labeled with the date it was sampled. October samples were collected as part of pilot studies to determine the details of incubation methods. As indicated in the diagram, 4 samples were collected from each site during each sampling period

Biogeochemical flux measurement

Fluxes of oxygen (O_2), ammonium (NH_4^+), combined nitrate and nitrite (NO_{2+3}), dinitrogen (N_2) and soluble reactive phospho-

rus (SRP) were determined in a pilot study (October 2009) and then seasonally (November 2009 and April, June and August 2010) using a combination of *in situ* equilibration with *ex situ* incubation and measurement. Custom-designed incubation chambers consisted of 3 primary components machined from 40.6 cm (16″) outer diameter PVC pipe: a sampling tray (area = 0.1 m^{-2}), a midsection and an upper section (Fig. 4). During each sampling period, divers deployed 8 sampling trays, one in each subplot at each site. Each tray was filled with material from the site (restored site = sediments, oyster shell and oyster clumps; control site = sediments) and embedded in the substratum flush with surrounding sediments. Trays remained in the field for approximately 2 wk to allow the system to equilibrate.
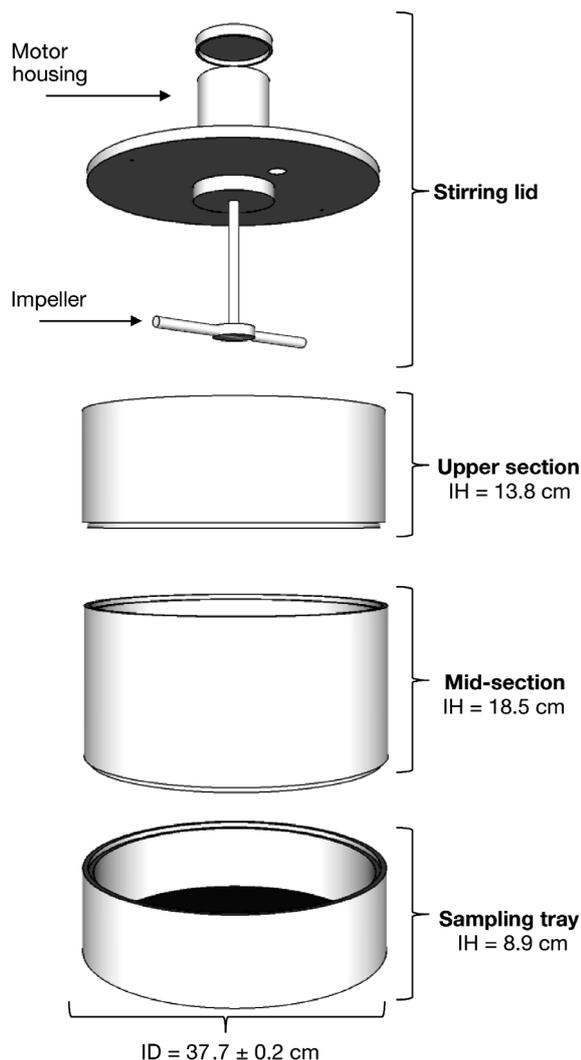

Fig. 4. Diagram of incubation chamber drawn to scale. All major components of the chambers were constructed from PVC. IH: inner height; ID: inner diameter

At the end of each field equilibration period, divers capped the sampling trays underwater by adding the chamber midsection fitted with a lid, allowing collection of reef materials, associated organisms, sediments and a portion of the overlying water column. Samples were transported to the surface and delivered to the University of Maryland Center for Environmental Science's Horn Point Laboratory (HPL) in Cambridge, Maryland, within 2.5 h of collection, where they were placed in a water bath (unfiltered Choptank River water with temperature adjusted to field conditions) inside an environmental chamber and aerated for ≥1 h to bring dissolved oxygen levels to saturation. During aeration, a 500 μm mesh lid on each chamber prevented escape of mobile macrofauna. An additional chamber in the water bath containing only unfiltered Choptank River water served as a seawater control (hereafter 'blank'), bringing the total number of chambers sampled during each set of experiments to 9.

Seasonal flux incubations were carried out in the dark and began ≤5 h after collection of the first sample in the field. Prior to starting incubations, mesh lids and air stones were replaced with stirring lids that sealed the sample from the surrounding water bath and mixed the water column using a motor-driven impeller (Fig. 4). Each stirring lid was fitted with a sampling tube, a water replacement tube that drew water from the water bath and a dissolved oxygen probe (NexSens Model WQ-DO). During incubations, we sampled solutes and dissolved gases a minimum of 4 times from each chamber. For the pilot study, the first incubation began 24 h after sample trays were collected from the field, and incubation of the trays from the restored site was repeated at 28, 48 and 73 h after collection. Otherwise, all incubation methods were identical between the pilot study and seasonal flux measurements. Timing of sampling events was based on real time data from oxygen probes. Total incubation periods varied seasonally from 0.8 to 2.3 h for samples from the restored site and from 17.0 to 24.5 h for samples from the control site. Because incubation periods were much shorter for samples from the restored site than for those from the control site, we were able to incubate these trays twice in succession. Between incubation periods, stirring lids were replaced with mesh lids with air stones and aerated for an hour to return oxygen levels to saturation. Final oxygen concentrations for the restored trays ranged from a low of 0.08 ± 0.02 mmol l^{-1} (mean ± SD, here and throughout) in June to a high of 0.19 ± 0.01 mmol l^{-1} in November.

During each sampling event, water samples were collected for analyses of dissolved gases (O_2, N_2, Ar) and solutes (NH_4^+, NO_{2+3}, SRP). Dissolved gas samples were preserved with 10 μl of 50% saturated $HgCl_2$, sealed, submerged in water and stored at temperatures equal to or below incubation temperatures until analysis. Solute samples were filtered (pore size = 0.45 μm) and frozen for later analysis. Dissolved gas samples were analyzed using membrane-inlet mass spectrometry (Kana et al. 1994). NH_4^+ concentrations were determined using phenol/hypochlorite colorimetry (Parsons et al. 1984). NO_{2+3} concentrations were determined by using automated colorimetric analysis with a detection limit of <0.03 mg l^{-1} (Parsons et al. 1984). SRP analyses followed Parsons et al. (1984).

Fluxes for all analytes were determined using linear regressions fitted to plots of concentration (n ≥ 4 for each chamber) versus time. To remove the influence of water column processes, slopes of regression lines were adjusted using data from the blank chamber when those data indicated significant flux of an analyte. Fluxes were considered significant when the regression line had an R^2 ≥0.80 and the difference between data in a time course was greater than the precision of the analytical method. To estimate average hourly fluxes for the year, we applied the average of the 4 measured rates to the 8 mo period from April through November, assigned zero values to the winter months and divided by 12. This method was intentionally chosen to generate conservative estimates. To estimate the total amount of nitrogen removed via denitrification annually, we multiplied the average hourly flux by the number of hours in a year.

We estimated total remineralized nitrogen fluxes (ΣN) as the sum of NO_{2+3}, NH_4^+ and N_2-N fluxes. This method works even when NO_{2+3} fluxes are directed into the sediment because negative fluxes result in either denitrification or dissimilatory nitrate reduction to ammonium, both of which are captured by this calculation. Nitrification and denitrification efficiencies describe the proportion of total nitrogen remineralization resulting from nitrification and denitrification, respectively. We did not measure nitrification directly, but calculated it from the sum of the NO_{2+3} and N_2-N fluxes to the water column. Thus, nitrification efficiency can be expressed as:

$$\text{Nitrification Efficiency (\%)} = \frac{(N_2\text{-N}) + (NO_{2+3})}{\Sigma N} \times 100 \quad (1)$$

where N_2-N is di-nitrogen flux, NO_{2+3} is combined nitrate and nitrite flux and ΣN is total inorganic nitrogen flux.

Similarly, denitrification efficiency can be expressed as:

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

To examine nutrient fluxes relative to organic matter oxidation, we calculated the stoichiometric ratios of C:N:P for each set of samples collected and compared them to the expected Redfield ratio of 106C:16N:1P for marine algae. Although we were unable to measure carbon remineralization during each sampling period, we did measure O_2 flux on each occasion. Assuming the primary drivers of oxygen demand in our system were metazoan respiration, aerobic microbial respiration and the reoxidation of reduced species resulting from alternate terminal electron acceptors [i.e. Fe(II), H_2S], we used an O_2:CO_2 flux ratio of 1:1 to estimate total carbon mineralization. Because SRP is the product of organic phosphorus remineralization, fluxes of SRP were used directly to estimate phosphorus remineralization. Ratios of C:N:P were estimated using regression lines fitted to measured seasonal fluxes of SRP versus ΣN, ΣN versus O_2 and SRP versus O_2.

Macrofaunal abundance, biomass and nutrient content

We compared nutrient assimilation into macrobenthic organisms between restored and control sites by estimating the average standing stock of assimilated nutrients at each site using the samples collected for seasonal flux incubations. At the end of each incubation, material in each sampling tray was rinsed through a sieve series (mesh sizes: 12.5, 4.0 and 1.0 mm) and all organisms >1.0 mm were retained. Whenever possible, we retained a minimum of 25 individuals from each major faunal group from each tray for nutrient analyses. To collect organisms attached to or living within large shells and shell fragments, all materials retained on the 12.5 mm sieve were further processed by (1) removing all macrofauna visible to the naked eye, (2) gently scrubbing all surfaces with a soft brush, (3) re-examining materials under 1.75× magnification, (4) soaking in successive freshwater baths until no additional organisms were recovered from the bathwater (~2 to 3 h) and (5) carefully destroying materials to remove any organisms retained within them. In November, the shells of all live oysters were broken apart to remove organisms. With the exception of one tray in April which contained only 3 live oysters,

Table 1. Dates of deployment for each sampling period and environmental conditions during deployment (mean ± SD). Data were collected at 5 min intervals throughout the deployment period. Data for salinity and dissolved oxygen (DO) are not reported for the control site during the November sampling period due to sonde malfunction

Sampling period	Deployment period	Average temperature (°C) Control	Restored	Average salinity (ppt) Control	Restored	Average DO (mg l^{-1}) Control	Restored
Nov	29 Oct–10 Nov 2009	14.5 ± 1.0	14.5 ± 1.0		11.6 ± 0.9		7.8 ± 0.6
Apr	6–20 Apr 2010	15.2 ± 0.9	15.1 ± 1.0	6.8 ± 0.7	7.0 ± 0.7	7.0 ± 1.4	6.9 ± 1.2
Jun	7–21 Jun 2010	26.0 ± 0.7	25.7 ± 0.6	7.6 ± 0.3	8.2 ± 0.4	6.1 ± 0.7	5.4 ± 0.7
Aug	10–26 Aug 2010	27.8 ± 0.8	27.8 ± 0.7	9.5 ± 0.3	10.1 ± 0.4	5.9 ± 0.7	5.2 ± 0.8

5 live oysters were randomly selected from each tray in April, June and August for analyses of barnacle abundance. All oysters and mussels collected from the ≥12.5 mm size fraction were frozen for later analyses. All other organisms retained on sieves ≥1.0 mm were preserved in 10% buffered formalin for ≥48 h followed by rinsing and transfer to 70% ethanol.

All organisms collected from sampling trays were counted (using subsampling as needed), identified to the lowest practical taxonomic level, dried to constant weight at 60°C and weighed to the nearest 0.1 mg. Samples from each major faunal group were ground to a fine powder using a mortar and pestle prior to nutrient analyses. Nitrogen was analyzed with an automated CHN analyzer, and phosphorus was analyzed by extraction of P from combusted samples using 1 N HCl followed by colorimetric analyses (Aspila et al. 1976). When sufficient material existed, 3 replicates were analyzed from each faunal group. The total amount of nitrogen and phosphorus assimilated was determined by multiplying the total dry weight of each group by its percentage of nitrogen and phosphorus. For mussels and oysters >10 mm in shell height, shells and soft tissue were analyzed separately. For some groups, replicate analyses were made for different components of the sample (e.g. gravid versus non-gravid xanthid crabs), and total nutrient content was calculated using a weighted mean percentage based on the abundance and biomass of each component. Because estimating secondary production rates was beyond the scope of this project, seasonal standing stock assimilation values were averaged to estimate annual standing stocks of assimilated nitrogen (TN_{ss}) and phosphorus (TP_{ss}) at each site and do not represent an annual rate of assimilation. During the course of sampling and sample processing, we observed nothing to suggest that the presence of trays significantly impacted macrofaunal abundance, biomass, or behavior.

RESULTS

Differences in environmental conditions between the restored and control sites were minor and well below values we consider biologically significant (Table 1). In contrast, sites differed significantly in the total sediment volume in sampling trays (restored site = 6.4 ± 1.5 l, control site = 4.4 ± 1.1 l) and sediment characteristics (Table 2). Sediments at the restored site had lower median grain size and higher organic content. Percentages of total carbon, organic carbon, total nitrogen and total phosphorus per gram dry weight (DW) of sediments were an order of magnitude higher at the restored site than at the control site.

Biogeochemical fluxes

The pilot study was instructive for examining the trajectory of incubations over a short period of time. Over the 2 d period we measured nitrogen fluxes, ammonium fluxes steadily decreased, with net ammonium uptake exhibited on the third day (Fig. 5). During the same time period, NO_{2+3} fluxes and, to a much lesser extent, N_2-N fluxes increased.

Table 2. Sediment characteristics at the control and restored sites in August. Data are reported as means ± SD

Sediment characteristics	Site Control (n = 4)	Restored (n = 3)
Median grain size (µm)	457.2 ± 30.4	25.5 ± 22.6
Sand (%)	97.3 ± 1.9	37.0 ± 12.4
Total organics (%)	0.7 ± 0.3	10.8 ± 3.1
Total carbon (%)	0.418 ± 0.074	6.533 ± 0.676
Organic carbon (%)	0.360 ± 0.053	4.196 ± 0.561
Total nitrogen (%)	0.028 ± 0.011	0.333 ± 0.040
Total phosphorus (%)	0.009 ± 0.002	0.082 ± 0.005

Respiration rates for the restored site were high (Fig. 6), with oxygen demand ranging from 12.87 ± 1.61 mmol O_2 m^{-2} h^{-1} in November to 38.82 ± 4.35 mmol O_2 m^{-2} h^{-1} in June. Oxygen demand for the control site was much lower, ranging from 1.14 ± 0.32 mmol O_2 m^{-2} h^{-1} in August to 1.54 ± 0.17 mmol O_2 m^{-2} h^{-1} in April. Average hourly oxygen demand was 20 times higher for the restored site (17.80 mmol O_2 m^{-2} h^{-1}) than for the control site (0.91 mmol O_2 m^{-2} h^{-1}). Oxygen demand was positively correlated with bottom water temperature ($R^2 = 0.99$) for the restored site, but not for the control site ($R^2 = 0.34$).

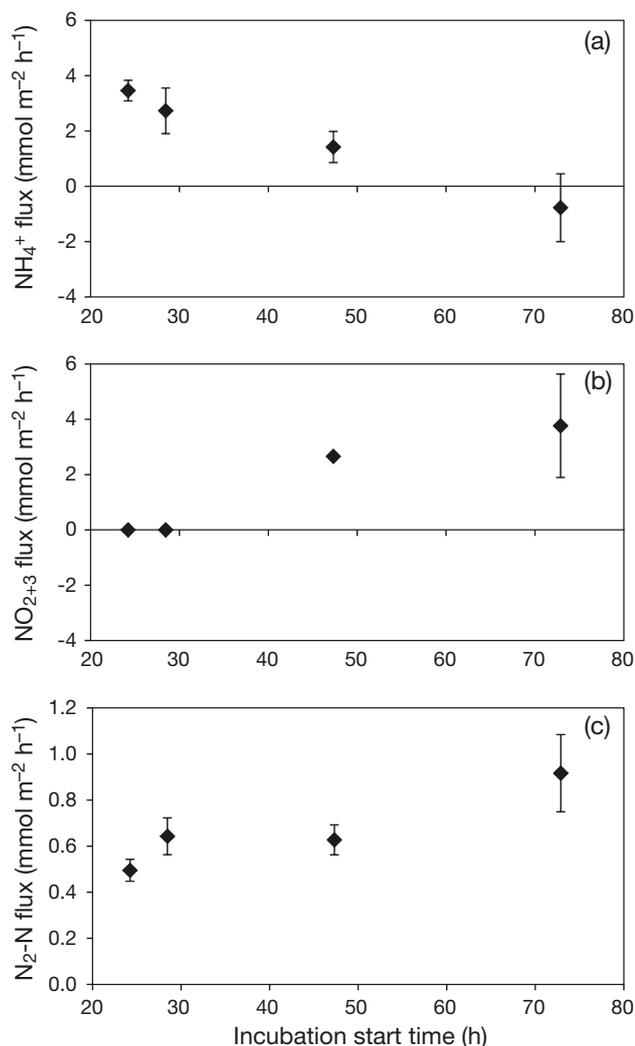Similar seasonality was observed for the release of ammonium from the sediments into the water column at the restored site (Fig. 7a). Ammonium fluxes for the restored site ranged from 0.62 ± 0.10 mmol NH_4^+ m^{-2} h^{-1} in November to 3.51 ± 0.45 mmol NH_4^+ m^{-2} h^{-1} in August, while those at the control site were much lower, ranging from 0.05 ± 0.07 mmol NH_4^+ m^{-2} h^{-1} in June to 0.12 ± 0.05 mmol NH_4^+ m^{-2} h^{-1} in August. Average hourly ammonium flux for the restored site (1.24 mmol NH_4^+ m^{-2} h^{-1}) was 23 times greater than that for the control site (0.05 mmol NH_4^+ m^{-2} h^{-1}).

Fluxes of combined nitrate and nitrite for the restored site also peaked in late summer and were much higher than values for the control site (Fig. 7b). Fluxes for the restored site ranged from 0.46 ± 0.07 mmol NO_{2+3} m^{-2} h^{-1} in November to 1.83 ± 0.11 mmol NO_{2+3} m^{-2} h^{-1} in August compared to control site fluxes ranging from 0.01 ± 0.02 mmol NO_{2+3} m^{-2} h^{-1} in April to 0.07 ± 0.01 mmol NO_{2+3} m^{-2} h^{-1} in August. Average hourly NO_{2+3} flux for the restored site was 0.72 mmol NO_{2+3} m^{-2} h^{-1} compared to 0.02 mmol NO_{2+3} m^{-2} h^{-1} for the control site, reflecting high rates of nitrification in the reef environment.

Denitrification rates (estimated from fluxes of N_2-N) for the restored site were highest in August (1.59 ± 0.22 mmol N_2-N m^{-2} h^{-1}) and lowest in November (0.25 ± 0.06 mmol N_2-N m^{-2} h^{-1}; Fig. 7c). Again, rates for the control site were much lower, ranging from 0.04 ± 0.01 mmol N_2-N m^{-2} h^{-1} in April to 0.11 ± 0.02 mmol N_2-N m^{-2} h^{-1} in August. Average hourly N_2-N flux for the restored site (0.50 mmol N_2-N m^{-2} h^{-1}) was 12 times greater than for the control site

Fig. 5. Changes ($\pm$SD) in (a) NH_4^+ flux, (b) NO_{2+3} and (c) N_2-N flux over time with repeated incubations in October (n = 4 except for the incubation at 24 h where n = 3). Negative values represent fluxes from the water column to the benthos
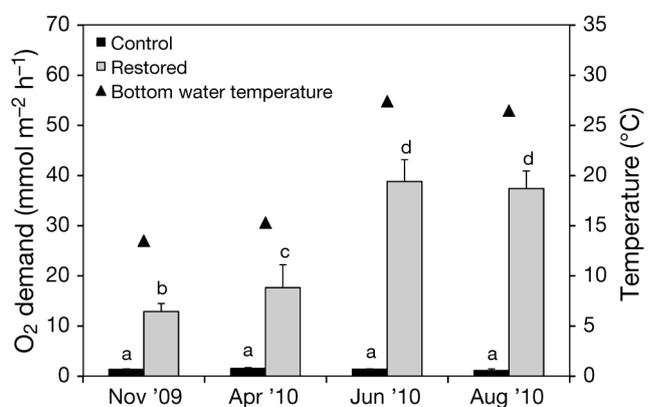

Fig. 6. Seasonal fluxes of oxygen (= sediment oxygen demand; left axis) and bottom water temperature (right axis). Error bars represent 1 standard deviation (n = 4 except for the restored site in August where n = 3). Two-way ANOVA indicated a significant interaction between site and season (p < 0.001). Letters are used to indicate significant differences between levels within each main effect based upon 1-way ANOVA followed by Tukey's post hoc comparison ($\alpha = 0.05$). Bars that share a letter are not significantly different
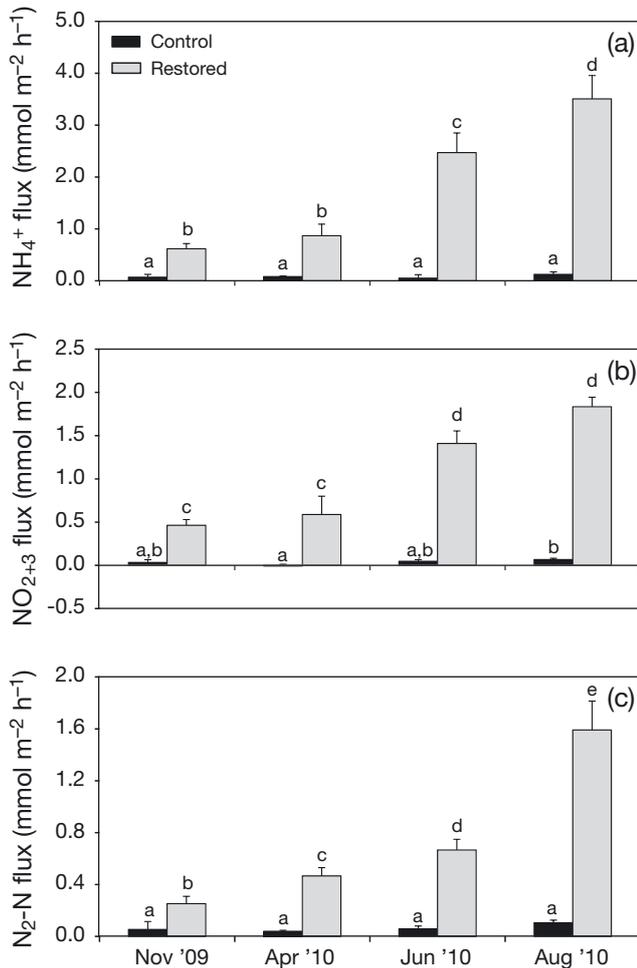
Fig. 7. Seasonal fluxes of (a) NH_4^+, (b) NO_{2+3} and (c) N_2-N. Error bars represent 1 standard deviation (n = 4 except for the restored site in August where n = 3). Negative values represent fluxes from the water column to the benthos. Two-way ANOVA indicated a significant interaction between site and season for all 3 analytes (p < 0.001). Letters are used to indicate significant differences between levels within each main effect based upon 1-way ANOVA followed by Tukey's post hoc comparison (α = 0.05). Bars that share a letter are not significantly different
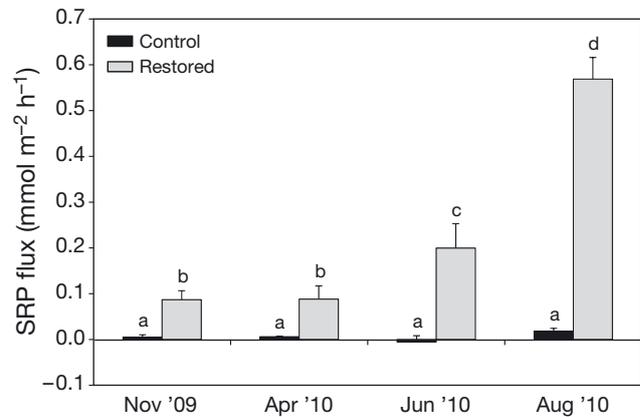

Fig. 8. Seasonal fluxes of soluble reactive phosphorus (SRP). Error bars represent 1 standard deviation (n = 4 except for the restored site in August where n = 3). Negative values represent fluxes from the water column to the benthos. Two-way ANOVA indicated a significant interaction between site and season (p < 0.001). Letters are used to indicate significant differences between levels within each main effect based upon 1-way ANOVA followed by Tukey's post hoc comparison (α = 0.05). Bars that share a letter are not significantly different

(0.04 μmol N_2-N m^{-2} h^{-1}). Estimated annual removal for the restored site was 61 g N m^{-2} yr^{-1} compared to 5 g N m^{-2} yr^{-1} for the control site.

Patterns in SRP flux were similar to those observed for ammonium (Fig. 8), increasing at the restored site from 0.09 to 0.20 mmol P m^{-2} h^{-1} during the first 3 sampling periods to 0.57 mmol m^{-2} h^{-1} in August. SRP fluxes were significantly lower for the control site than for the restored site in all seasons, ranging from −0.01 to 0.02 mmol m^{-2} h^{-1}. Average hourly SRP flux at the restored site was 0.16 mmol m^{-2} h^{-1} compared to 0.00 mmol m^{-2} h^{-1} at the control site.

For the restored site, total nitrogen fluxes ranged from 1.33 ± 0.11 to 6.93 ± 0.27 mmol N m^{-2} h^{-1} compared to only 0.11 ± 0.02 to 0.30 ± 0.07 mmol N m^{-2} h^{-1} for the control site (Fig. 9a). Nitrogen flux from the restored site was dominated by ammonium, corresponding to high rates of respiration and consequent nitrogen remineralization.

Nitrification efficiencies for the restored site ranged from 46 ± 4% in June to 55 ± 5% in April and those for the control site ranged from 33 ± 4% in April to 76 ± 20% in June (Fig. 9b). Nitrification efficiencies were significantly higher for the restored site than for the control site samples in April, but this pattern had reversed by June. Season did not significantly influence efficiencies at the restored site, but did have an impact at the control site.

Denitrification efficiencies for the restored site ranged from 15 ± 2% in June to 25 ± 7% in April and those for the control site ranged from 17 ± 17% in November to 42 ± 15% in June (Fig. 9c). June was the only sampling period in which there was a significant difference between sites. Although season had no effect at the restored site, denitrification efficiencies were significantly higher in June than in November or April at the control site.

Using data from all of our samples (n = 31), regression lines were fitted to fluxes of SRP versus ΣN, ΣN versus O_2, and SRP versus O_2, resulting in ratios of 15P:1N, 7O_2:1N and 116O_2:1P, respectively (Fig. 10). Using a 1:1 ratio of O_2:CO_2 resulted in a C:N:P ratio of 116:15:1.
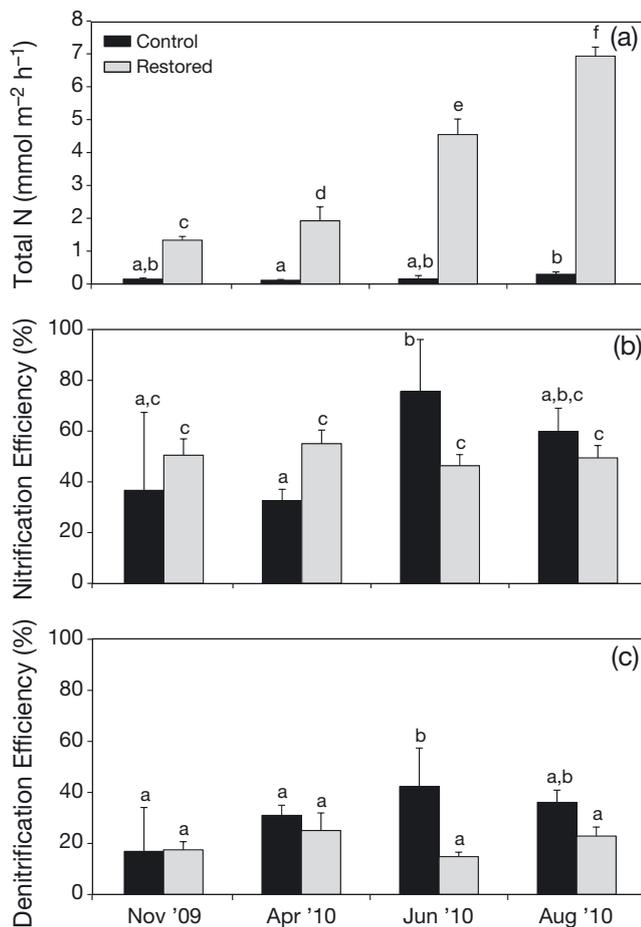
Fig. 9. (a) Total inorganic nitrogen fluxes, and (b) efficiency of nitrification and (c) denitrification for control and restored sites. Error bars represent 1 standard deviation (for total nitrogen, n = 4 except for the restored site in August where n = 3; for efficiencies, n = 4 except for the restored site in August and the control site in November and April where n = 3). In all cases, 2-way ANOVAs indicated a significant interaction between site and season (total nitrogen: p < 0.001; denitrification: p = 0.030; nitrification: p = 0.005). Letters are used to indicate significant differences between levels within each main effect based upon 1-way ANOVA followed by Tukey's post hoc comparison (α = 0.05). Bars that share a letter are not significantly different

Fig. 10. Stoichiometric plots using all individual core fluxes of O_2, ΣN (sum of NH_4^+, NO_{2+3} and N_2-N) and SRP. We assume that the stoichiometric ratio of $CO_2:O_2$ during decomposition was 1:1; this implies that the reduced products of anaerobic decomposition were reoxidized. (a) $\Sigma N:SRP$ ratio was 15, (b) $CO_2:\Sigma N$ was 7 and (c) $CO_2:SRP$ was 116, yielding a C:N:P of 116:15:1, similar to the Redfield elemental ratio for marine algae (106:16:1)

Macrofaunal abundance, biomass and nutrient content

Prior to completion of macrofaunal abundance and biomass analyses, a laboratory fire resulted in the loss of some samples from the restored site. As a result, reported abundance and biomass values for barnacles and biomass values for crabs underestimate actual values.

Macrofaunal abundances were higher in samples from the restored site than from the control site for all organisms except clams (Table 3). The 3-fold greater
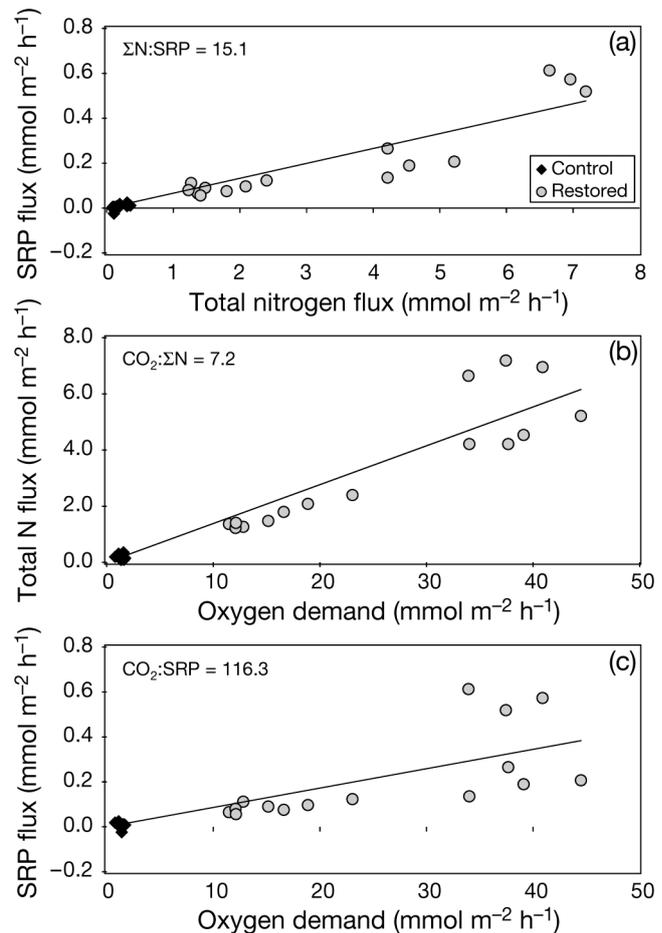
average abundance of clams at the control site was driven by high abundances of small clams (<10 mm), especially in the April and June sampling periods (3317 ± 875 and 1638 ± 584 ind. m^{-2}, respectively). Among the other major faunal groups, sessile species were either completely absent or 2 orders of magnitude less abundant at the control site than at the restored site. Greatest differences among mobile species were found for polychaete worms and xanthid crabs, which were also 2 orders of magnitude less abundant at the control site.

Macrofaunal biomass (inclusive of shell) was greater at the restored site than at the control site for all faunal groups (Table 3). Although 86% of this difference in biomass between sites can be attributed to oysters, the restored site also had 2564 ± 634 g m^{-2}

Table 3. Average abundance and biomass (inclusive of shell) of major faunal groups from restored and control sites. Numerical and/or biomass dominant species listed in parentheses under each faunal group. DW: dry weight

Faunal group	Abundance (ind. m^{-2}, ±SD)		Biomass (g DW m^{-2}, ±SD)	
	Control	Restored	Control	Restored
Amphipods	340.6 ± 558.4	7067.3 ± 1847.0	0.3 ± 0.5	3.5 ± 3.7
(*Melita nitida*, *Apocorophium lacustre*, *Apocorophium simile*)				
Anemones	0 ± 0	893.8 ± 1279.3	0 ± 0	5 ± 8.8
(*Diadumene leucolena*)				
Barnacles	0 ± 0	3769.6 ± 3552.8	0 ± 0	110.7 ± 91.4
(*Balanus* spp.)[a]				
Clams	1456.4 ± 1372.9	481.4 ± 726.2	10.8 ± 12.3	57.8 ± 56.9
(*Mya arenaria*, *Macoma balthica*, *Mulinia lateralis*)				
Crabs	6.2 ± 6.5	217.7 ± 46.1	0.2 ± 0.3	87.5 ± 79.2
(*Eurypanopeus depressus*)[a]				
Fish	10.7 ± 14.9	141.4 ± 85.8	0.3 ± 0.3	18.6 ± 12.8
(*Gobiosoma bosc*, *Opsanus tau*)				
Mussels	13 ± 10.8	1563.3 ± 573	0.2 ± 0.2	2279.2 ± 598.3
(*Ischadium recurvum*)				
Polychaete worms	435.1 ± 149.9	10415.3 ± 3001.1	0.4 ± 0.1	12.1 ± 3.8
(*Alitta succinea*)				
Shrimp	2.8 ± 3.3	35.1 ± 47.1	0 ± 0.1	1.5 ± 1.6
(*Palaemonetes pugio*)				
Non-oyster macrofauna	**2264.9 ± 1952.4**	**24584.9 ± 5921.6**	**12.1 ± 12.6**	**2575.8 ± 634.3**
Oysters	0 ± 0	130.7 ± 15	0 ± 0	15422.2 ± 1505.8
(*Crassostrea virginica*)				
Total macrofauna	**2264.9 ± 1952.4**	**2415.6 ± 5936.1**	**12.1 ± 12.6**	**17998.0 ± 2021.0**

[a]Based on partial dataset due to losses resulting from laboratory fire

more non-oyster biomass than the control site. Much of this difference was driven by sessile organisms attached to oyster shells, but the biomasses of most mobile faunal groups were also 2 orders of magnitude greater for the restored site. Clam biomass was >5 times higher for the restored site than the control site, a difference primarily attributable to the larger average size of *Mya arenaria* for the restored site.

Percentages of nitrogen and phosphorus in major faunal groups and their components (e.g. oyster shell and oyster tissue) generally followed expected patterns, with highest values found in organisms without significant calcium carbonate structures (Table 4). The restored site average standing stock of nutrients was greater than that at the control site by 95.09 ± 14.55 g N m^{-2} and 15.17 ± 2.46 g P m^{-2}, primarily due to assimilation by bivalves. Oysters (tissue + shell) accounted for 65 and 67% of TN$_{ss}$ and TP$_{ss}$, respectively (Table 5). Mussels, the second largest contributor, accounted for 25% of TN$_{ss}$ and 17% of TP$_{ss}$. When combined, the shells of live oysters and mussels >10 mm accounted for 47% of TN$_{ss}$ and 48% of TP$_{ss}$. Of the other organisms found at the restored site, only fish and crabs accounted for >1% of TN$_{ss}$ and TP$_{ss}$. Xanthid crabs contained 4% TN$_{ss}$ and 8% of TP$_{ss}$. Fish contributed 2 and 4%, of TN$_{ss}$ and phos-

phorus, respectively. Together, non-oyster macrofauna at the restored site contained 35% of TN$_{ss}$ and 33% of TP$_{ss}$.

Table 4. Percentages of nitrogen (N) and phosphorus (P) in tissues and shell of major faunal groups. Sample size (n) is the number of samples analyzed. Because it was necessary to pool individual organisms for many analyses, the total number of individuals per sample ranged from 1 to 90

Faunal group	n	N (%, ±SD)	P (%, ±SD)
Amphipods	4	4.53 ± 0.58	1.99 ± 0.07
Anemones	3	9.17 ± 0.13	1.33 ± 0.07
Barnacles	4	0.99 ± 0.16	0.14 ± 0.03
Clams—mixed spp.	6	1.42 ± 0.38	0.10 ± 0.03
Clams—*Mya* spp.	6	2.38 ± 0.29	0.28 ± 0.06
Crabs—gravid	3	4.15 ± 0.71	1.40 ± 0.12
Crabs—not gravid	6	3.98 ± 0.65	1.37 ± 0.33
Fish—blennies	2	10.86	3.84
Fish—gobies	6	10.60 ± 0.11	3.61 ± 0.30
Fish—skilletfish	1	9.37	4.59
Fish—toad fish	1	11.04	3.66
Mussels—shell	4	0.47 ± 0.04	0.04 ± 0.01
Mussels—tissue	4	10.93 ± 0.67	1.35 ± 0.32
Oyster—shell	3	0.21 ± 0.08	0.04 ± 0.01
Oyster—tissue	3	9.27 ± 0.60	1.26 ± 0.18
Polychaete worms	6	6.84 ± 1.12	1.07 ± 0.14
Shrimp—gravid	2	8.95	2.44
Shrimp—not gravid	4	9.35 ± 0.66	2.59 ± 0.37

Table 5. Assimilated nitrogen and phosphorus in average standing stock biomass (inclusive of shell) of major faunal groups at restored and control sites

Faunal group	Nitrogen (g m^{-2}, ±SD)		Phosphorus (g m^{-2}, ±SD)	
	Control	Restored	Control	Restored
Amphipods	0.012 ± 0.021	0.160 ± 0.167	0.0051 ± 0.0094	0.0703 ± 0.0733
Anemones	0.000 ± 0.000	0.455 ± 0.807	0.0000 ± 0.0000	0.0660 ± 0.1171
Barnacles	0.000 ± 0.000	1.096 ± 0.905	0.0000 ± 0.0000	0.1550 ± 0.1280
Clams	0.173 ± 0.196	1.344 ± 1.333	0.0146 ± 0.0164	0.1560 ± 0.1552
Crabs	0.010 ± 0.011	3.515 ± 3.185	0.0034 ± 0.0038	1.2068 ± 1.0935
Fish	0.030 ± 0.036	1.996 ± 1.391	0.0101 ± 0.0122	0.6779 ± 0.4643
Mussels	0.002 ± 0.002	24.055 ± 6.890	0.0002 ± 0.0003	2.5825 ± 0.7551
Oysters	0.000 ± 0.000	61.759 ± 6.082	0.0000 ± 0.0000	10.1241 ± 0.9351
Polychaete worms	0.025 ± 0.009	0.827 ± 0.260	0.0039 ± 0.0013	0.1294 ± 0.0406
Shrimp	0.003 ± 0.005	0.137 ± 0.151	0.0008 ± 0.0013	0.0365 ± 0.0402
Non-oyster macrofauna	**0.254 ± 0.218**	**33.587 ± 8.856**	**0.0381 ± 0.0251**	**5.0804 ± 1.6152**
Oysters	0.000 ± 0.000	61.759 ± 6.082	0.0000 ± 0.0000	10.1241 ± 0.9351
Total macrofauna	**0.254 ± 0.218**	**95.346 ± 14.571**	**0.0381 ± 0.0251**	**15.2054 ± 2.4460**

DISCUSSION AND CONCLUSIONS

Biogeochemical fluxes

Measurement approach

Measurement of the sediment–water exchange of substances generally requires sealing a part of the sediment community into a chamber, either *in situ* (e.g. benthic landers) or *ex situ* (e.g. cores), and measuring the change of the solute or gas concentration over time (e.g. Newell et al. 2002). Alternative approaches include measuring differences in inflow and outflow concentrations in flow-through incubations (e.g. Piehler & Smyth 2011) or *in situ* measurements of oxygen fluxes using eddy correlation (e.g. Berg et al. 2003). In designing a system to measure net benthic fluxes, there are a number of key considerations: (1) inclusion of sufficient benthic habitat to provide good representation of benthic biota and physical structure; (2) minimizing disturbance of surface sediments where the most reactive material and abundant microbial communities are generally found; (3) maintenance of *in situ* temperatures which affect both microbial processes and the accuracy of the N_2:Ar approach to measuring denitrification (Kana et al. 1994); (4) avoiding excessive oxygen depletion or accumulation/depletion of other chemical species (e.g. ammonium, hydrogen sulfide) that can affect coupled nitrification/denitrification, sediment–water exchange of SRP and benthic organism activity; and (5) incorporation of reasonable boundary-layer physical characteristics.

Our approach takes each of these issues into consideration. By excavating discrete segments of reef (area = 0.1 m^{-2}) and placing them in trays that were re-imbedded in the substratum, a reasonable subset of the whole community was sampled. Disturbance associated with moving the whole community into trays was addressed by allowing ~2 wk of equilibration between manipulation and sampling, a duration that exceeds measured turnover rates for carbohydrates under aerobic conditions in Chesapeake Bay (Harvey et al. 1995) and thus allowed sufficient time for resupply of labile organic matter to compensate for any losses during excavation. Close proximity between the field sites and the laboratory limited negative effects due to alterations in temperature or oxygen saturation. In the laboratory, sampling trays were immediately placed in an aerated water bath with temperature and salinity matched to field conditions. An impeller in each chamber provided flow within the range of flow speeds observed in the field and sufficient to ensure thorough mixing of the water column without resuspension of sediments.

Multiday incubation of samples collected in October demonstrated that nitrogen fluxes change significantly as samples are held in the laboratory. As NH_4^+ fluxes decrease, NO_{2+3} fluxes increase (Fig. 5), likely as a result of increased rates of nitrification. By the end of the incubation, there was also a slight increase in denitrification rates. Thus, the longer samples are held prior to incubation, the more likely it is that field nitrification and denitrification rates will be overestimated due to laboratory artifacts.

Seasonal fluxes

Fluxes of O_2, NH_4^+, NO_{2+3}, N_2-N and SRP were all at least an order of magnitude greater for the

restored site than at the control site. Because we did not observe significant macrofaunal mortality during incubations, we are confident that these fluxes represent ongoing biological and biogeochemical processes and cannot be attributed to mortality of organisms within the chambers. The greatest differences in fluxes between the 2 sites were observed in NH_4^+ and NO_{2+3} for which rates were as much as 2 and 3 orders of magnitude higher, respectively, at the restored site than at the control site. Although high compared to the control site, ammonium fluxes fell within the range of ammonium fluxes previously measured for oyster reefs. Dame et al. (1989) report an average summer ammonium flux of ~5 mmol m^{-2} h^{-1} from an oyster reef in South Carolina. In contrast, our fluxes of N_2-N were much higher than those previously reported for oyster reef environments. Previous studies of denitrification associated with oyster reefs (Piehler & Smyth 2011, Smyth et al. 2013), oyster biodeposition (Newell et al. 2002) and oyster aquaculture (Holyoke 2008) have relied on incubations of sediment cores and have not included living oysters within these cores. These studies report denitrification rates ranging from 0.0 to 0.33 mmol N_2-N m^{-2} h^{-1}, rates substantially lower than our observed rates of 0.25 to 1.59 mmol m^{-2} h^{-1}.

Compared to fluxes of oxygen, nitrogen and phosphorus previously measured in soft sediment environments in Chesapeake Bay and its tributaries, the rates we measured for the restored site are far higher. A comprehensive review of oxygen and nutrient exchange measurements from Chesapeake Bay reported maximum values for oxygen demand, ammonium flux and nitrate flux of 9 mmol O_2 m^{-2} h^{-1}, 2.17 mmol NH_4-N m^{-2} h^{-1} and 0.28 mmol NO_{2+3}-N m^{-2} h^{-1}, respectively (Boynton & Bailey 2008), compared to our maximum values of 39 mmol O_2 m^{-2} h^{-1}, 3.51 mmol NH_4-N m^{-2} h^{-1} and 1.83 mmol NO_{2+3}-N m^{-2} h^{-1}. Our maximum N_2-N flux (1.59 mmol N_2-N m^{-2} h^{-1}) was also higher than any previously published values for soft sediments in Chesapeake Bay, where denitrification rates seldom exceed 0.18 mmol N_2-N m^{-2} h^{-1} (Kemp et al. 2005, J. C. Cornwell & M. S. Owens unpubl. data). Our August SRP flux (0.57 mmol m^{-2} h^{-1}) was higher than any previously reported values for Chesapeake Bay sediments (Cowan & Boynton 1996), including SRP releases observed with seasonal hypoxia (~0.15 mmol m^{-2} h^{-1}; Boynton 2000) or elevated pH (~0.11 mmol m^{-2} h^{-1}; Seitzinger 1991).

The denitrification and nitrification rates we observed for the restored site are among the highest values ever reported for an aquatic environment. Calculated nitrification rates for the restored site

ranged from 0.72 to 3.43 mmol N m^{-2} h^{-1}. We are unaware of comparable rates in any ecosystem. A comprehensive review of coastal denitrification rates found 2 rates (1.58 and 1.26 mmol N_2-N m^{-2} h^{-1}) similar to our highest rate, but no others that were even half that value (Joye & Anderson 2008). A cross-system analysis of 107 datasets examined denitrification rates in aquatic systems during the warmest month and found only 3 studies, all riverine, with higher denitrification rates than those found at our restored site in August (Piña-Ochoa & Álvarez-Cobelas 2006).

Because our nitrification and denitrification estimates are based on accumulation of NO_{2+3} and N_2-N in the overlying water, our estimates represent 'net' fluxes and cannot be used to tease apart the contributions of all possible nitrogen pathways. Dissimilatory reduction of nitrate to ammonium (DNRA) can be an important sink for NO_{2+3} in some environments (An & Gardner 2002). If DNRA was significant in our system, then our calculated nitrification rates underestimate actual nitrification rates. Although anammox has not been shown to be an important process in Chesapeake Bay sediments (Rich et al. 2008), the observed co-occurrence of oxidized NO_{2+3} and NH_4^+ prevents us from eliminating the possibility that anammox could have occurred in our incubations. While we are not able to distinguish between the various pathways that lead to N_2-N production, by measuring fluxes of N_2-N in the water column, we did accurately measure the net effects of these pathways.

Although rates of nitrification and denitrification were consistently higher for the restored site than at the control site, efficiencies were not. Nitrification efficiency is a measure of the likelihood that ammonium molecules will be transformed into nitrite and nitrate via nitrification. Similarly, denitrification efficiency is a measure of the likelihood that nitrate and nitrite molecules will be transformed into nitrogen gas via denitrification. Both efficiencies are useful for comparisons between sites or systems. At the 2 sites we studied, patterns in nitrification and denitrification efficiencies varied, but the highest single values for each were found at the control site. However, it is important to consider these efficiencies in the context of total nitrogen fluxes which were far higher at the restored site. In Chesapeake Bay, the efficiency of coupled nitrification–denitrification is spatially and seasonally variable, with low efficiencies in sub-pycnocline low oxygen mainstem environments (Kemp et al. 2005). In fine-grained Choptank River sediments, the efficiency of denitrification can range from <10% in the summer to >50% in spring and fall

(Owens 2009). In contrast to our results and other data from Chesapeake Bay, data from intertidal oyster reef sediments in North Carolina suggest denitrification efficiencies of approaching 100 % (Piehler & Smyth 2011, Smyth et al. 2013). In eutrophic coastal environments, inefficient coupled nitrification–denitrification is generally attributed to low rates of nitrification because of minimal oxygen penetration into sediments (Kemp et al. 1990). On restored oyster reefs, direct excretion of ammonium to the water column is also a likely contributing factor.

We are confident that the high denitrification rates we measured are representative of actual rates rather than an artifact of our measurement approach for several reasons. First, fluxes measured for the restored site were consistently high between replicate runs of the same sample, across samples within each sampling period and across seasons. Second, our overall composition of remineralization was 116C:15N:1P. This ratio is similar enough to the expected Redfield ratio for marine algae of 106C: 16N:1P to confirm that our fluxes accounted for the majority of nitrogen and phosphorus transformations. Third, by the nature of their structure and faunal composition, oyster reefs provide ample microhabitats conducive to the establishment of communities of nitrifying microbes. Nitrification requires contact between remineralized nitrogen and the aerobic nitrifying community. In soft sediments, oxygen penetration is limited and nitrifiers often inhabit only the top few millimeters of sediment. In contrast, oyster reefs have as much as 50 m^2 of surface area per square meter of reef (Bahr 1974), providing ample habitat for nitrifiers on the exterior of live and dead oysters. Nitrification activity has also been found directly associated with the surfaces of living organisms, including polychaetes, amphipods and the surfaces of bivalve soft tissues (Welsh & Castadelli 2004). Finally, denitrification rates on oyster reefs are expected to be highest for reefs in oxic waters below the euphotic zone where microbes need not compete with benthic algae for nitrogen compounds and where reduced oxygen levels do not inhibit coupled nitrification–denitrification. The restored site was located at a depth of ~4 m, where it received very little or no light. During our study, dissolved oxygen levels never dropped to anoxic or hypoxic levels in the vicinity of the restored site. These factors combined with an abundant supply of organic matter likely led to optimal conditions for both nitrification and denitrification.

The role that restored oyster reefs play in controlling levels of SRP in the water column is less straight-forward. Phosphorus fluxes in estuarine sediments vary widely and are highly dependent upon local factors including sediment characteristics, degree of eutrophication, pH levels and bioturbation (Howarth et al. 2011). The large SRP effluxes observed at the restored site are unusual in aerobic Chesapeake sediments (Boynton & Kemp 1985) and suggest poor binding of P to sediments, direct excretion of SRP from the macrofaunal community to the water column, or both. Regardless of the mechanism, the highly efficient recycling of SRP to the water column indicates that biogeochemical processes at the restored site were not a significant sink for phosphorus and that the primary mechanism for potential phosphorus retention is assimilation into the tissues and shells of reef organisms.

Macrofaunal abundance, biomass and nutrient content

Macrofaunal abundances at the restored site were greater than those previously reported for restored oyster reefs in the Maryland portion of Chesapeake Bay (Rodney & Paynter 2006), but within the range reported for natural reefs in Chesapeake Bay (e.g. Larsen 1985). Average standing stock biomass, assimilated nitrogen and assimilated phosphorus at the restored site were 2 to 3 orders of magnitude higher than at the control site. The percentage of nitrogen in oyster soft tissues in the present study were 8 to 32 % higher than those previously reported (Newell 2004, Higgins et al. 2011, Carmichael et al. 2012), while the percentages of phosphorus were 52 to 58 % higher (Newell 2004, Higgins et al. 2011). Percentages of nitrogen and phosphorus in oyster shell in the present study were 30 and 60 % lower, respectively, than previously reported for wild oysters (Newell 2004), but fell within the range of values reported for oyster aquaculture (Higgins et al. 2011). We are unaware of any previous work that has attempted to estimate the nutrient assimilation (soft tissues and shell) by all macrofauna from a natural or restored oyster reef.

Variation in the percentage of nitrogen and phosphorus contained in oyster shell and tissue is relatively small compared to reported variation in the estimated total nutrient content per oyster. At the restored site, an average individual oyster contained 0.49 ± 0.08 g N and 0.08 ± 0.14 g P, with 0.24 ± 0.05 g N and 0.03 ± 0.01 g P bound in tissue and 0.26 ± 0.06 g N and 0.05 ± 0.01 g P bound in shell. Despite similar shell heights and lower tissue biomass, our

oysters contained 25% more nitrogen and 62% more phosphorus than the largest size class of oysters examined by Higgins et al. (2011). Previous work on wild oysters provides estimates of total nitrogen and phosphorus content that are 5 and 97% greater, respectively, than the values in the present study despite smaller shell heights (Newell 2004). Estimates of nitrogen in the soft tissue of oysters with a shell height of 76.2 mm given by Carmichael et al. (2012) range from 16% lower than our values to 60% higher. Some of the variation in estimates of nutrient content per oyster in these studies stems from differences in the percent nitrogen and phosphorus estimates used in calculations, but most results from the differences in dry weights of oyster tissue and/or shell between studies. These differences highlight both the need for additional studies to document the range in % N and % P in oyster tissue and shell in relation to varying environmental conditions and the necessity of using site-specific relationships between oyster shell height, tissue dry weight and shell dry weight when calculating total nutrients per oyster.

Although we did not directly address the rates of nutrient assimilation or the fate of those nutrients once assimilated, our work demonstrates that significant amounts of nutrients are sequestered in the shells of *Crassostrea virginica* and the mussel *Ischadium recurvum* (Table 4). Including only the shells of oysters and mussels >10 mm, we estimate that 31.71 ± 3.15 g N m^{-2} and 6.04 ± 0.60 g P m^{-2} are contained in oyster shell and an additional 10.02 ± 2.60 g N m^{-2} and 0.85 ± 0.22 g P m^{-2} are contained in mussel shell. Combining these values, we estimate that 47% of TN$_{ss}$ and 48% of TP$_{ss}$ are assimilated into the shells of oysters and mussels. Because the shells of these organisms are expected to persist long after the organisms die, a significant portion of the standing stock of nutrients may be sequestered for years, decades, or even centuries if shells become buried below the taphonomically active zone (Waldbusser et al. 2011).

It is important to reiterate that we have not measured assimilation rates, but rather the standing stock of nitrogen and phosphorus assimilated into soft tissues and shells. While a substantial portion of the nutrients in shells are likely sequestered for considerable time, those in soft tissues will cycle through the system. Regardless of how long nutrients are retained within reef organisms and the structures they create, the δ^{15}N signatures of benthic macrofauna commonly increase with increasing anthropogenic nitrogen load, confirming that they assimilate anthropogenically derived nitrogen from their food sources (e.g. Martinetto et al. 2006, Carmichael

et al. 2012). To the extent that this nitrogen flows through benthic and pelagic food webs without being remineralized, it will reduce the pool of nitrogen available for phytoplankton growth.

In addition to assimilating nutrients, oyster reef macrofauna play a significant role in altering biogeochemical cycling. Filter-feeding organisms were the most abundant functional group at the restored site and are the most likely explanation for the higher organic content of sediments at this site. Deposit-feeding organisms including polychaetes and amphipods were also extremely abundant. Numerous studies have demonstrated that reworking of sediments by both infaunal filter feeders and deposit feeders alters organic matter turnover and nutrient recycling (reviewed by Welsh 2003) and can enhance denitrification rates (e.g. Nizzoli et al. 2007). Bacterial nitrification activity is also directly associated with the surfaces of many macrofaunal species including *Neanthes succinea*, the most abundant species of polychaete in our samples, and *Corophium insidiosum*, an amphipod species closely related to 2 of our most abundant species (Welsh & Castadelli 2004).

Water quality implications

To investigate the potential for restored reefs to impact regional-scale water quality, we used annual denitrification rates from the present study in combination with estimates of restorable substratum area to calculate (1) the percentage of annual external nitrogen inputs to the Choptank River that could be removed by restoring oyster reefs to all suitable areas in this tributary and (2) the percentage of available bottom that would need to be restored to reduce nitrogen loads by an amount equivalent to recently mandated nitrogen reductions for the Choptank River. We chose to focus on nitrogen removal via denitrification because our data were not suited to estimating rates of assimilation, we did not collect data on nitrogen burial, and published estimates of oyster reef nitrogen burial rates (e.g. Newell et al. 2005) are based on data from soft sediment environments rather than oyster reefs. To estimate the amount of substratum in the Choptank River that is suitable for restoration, we used a combination of data from 3 separate surveys. According to the Maryland Bay Bottom Survey conducted between 1974 and 1983, the Choptank River had 6439 ha of oyster shell and hard bottom at that time (Smith et al. 2001). More recent side-scan sonar survey data that overlap many of these areas (2007 to 2012; NOAA Chesa-

peake Bay Office and Maryland Geological Survey, unpubl. data) show that only 61% of the area originally identified as shell or hard bottom is currently suitable for oyster reef restoration, resulting in a total of 3914 ha. Ground truth data, collected by a stratified random patent tong survey in Harris Creek, a tributary of the Choptank River, indicate that only 44% of this area contains shell as the primary substratum (Paynter et al. 2012). Assuming these data are representative of the small-scale distribution of shell within areas identified as restorable bottom based on sonar surveys, we estimate that there are 1722 ha of restorable bottom in the Choptank River.

Subtracting the annual denitrification rate at our control site from that at our restored site yields an estimate of 556 kg N ha^{-1} yr^{-1} removed as a result of reef restoration. Published estimates of external nitrogen inputs to the Choptank River range from 1.5×10^6 kg N yr^{-1} (USEPA 2010) to 2.5×10^6 kg N yr^{-1} (Lee et al. 2001). Using 2.0×10^6 kg N yr^{-1} as the annual nitrogen load to the Choptank, we estimate that restoring oyster reefs to all suitable areas in the Choptank River would remove ~48% of the total external nitrogen inputs annually. Along with other tributaries throughout the Chesapeake Bay, the reductions in nitrogen loads required to meet newly mandated restrictions have been published for all segments of the Choptank River. Adding all of the required reductions and entering zeros for any segments which already meet these requirements, we estimate that a total of 216 219 kg N need to be removed from the system to meet current restrictions. According to our calculations, this amount of nitrogen could be removed by restoring oyster reefs to 23% of the available bottom.

There are important caveats to this conclusion. Scaling our values up to larger spatial scales assumes that (1) average oyster densities comparable to those at the restored site can be achieved on much larger scales; (2) denitrification rates scale linearly with oyster density, a requirement given that a large reef with the same average density will be spatially heterogeneous; and (3) differences in environmental conditions between restoration sites within the Choptank River do not significantly alter denitrification rates. It is important to recognize that oyster reef restoration is not a substitute for reduction in land-based inputs to the system, but rather a potential safety net to reduce additional downstream impacts. For example, all of the Choptank River segments that exceed allowable nitrogen loads are upstream of areas where salinity levels make oyster reef restoration feasible. Thus, nitrogen inputs will still have deleteri-

ous impacts within the Choptank River system regardless of the level of oyster reef restoration. However, downstream oyster reefs have the potential to remove nutrients before they reach the main stem of Chesapeake Bay and contribute to environmental degradation there, an especially important function in late summer when denitrification rates on the reefs are high and those in deeper water often decline dramatically (Newell et al. 2005).

Although nitrogen loading to the Choptank River falls within the range of nitrogen loading rates considered by Carmichael et al. (2012), our estimates indicate that restored oyster reefs could have greater impacts on estuarine water quality than previously thought. The difference between our estimates and those calculated and reviewed by Carmichael et al. (2012) stem from several sources. First, our goal was to estimate the potential nitrogen removal by successfully restored mature oyster reefs to all currently viable areas within the Choptank River. The restored site in our study had 326 ± 106 g DW oyster tissue m^{-2}, a value that falls well within the range of oyster soft tissue biomass reported for natural reefs (e.g. Bahr 1976), but is far greater than that considered by Newell et al. (2005) when modeling the potential for oysters to remove nitrogen from the Choptank River. Newell et al. (2005) modeled nitrogen removal at biomass densities of 1 to 10 g DW m^{-2} and estimated that oysters could remove 0.6 to 6% of nitrogen inputs to the Choptank River via denitrification and burial. Because their model scales directly to oyster soft tissue biomass, applying their calculations to the biomass density in our study results in estimated nitrogen removal rates even greater than our own. In addition, ~8.5% of the Choptank River is currently suitable for restoration, a percentage greater than that considered by Carmichael et al. (2012) in their calculations. Although our estimates are high relative to previous ones, they systematically underestimate the potential rates of nitrogen removal by restored oyster reefs because they do not include estimates of either long-term assimilation of nitrogen into shell or burial of nitrogen in biodeposits in sediments.

The concept of using bivalve populations to ameliorate anthropogenic impacts on water quality is not a new one. Early studies focused on the ability of bivalves to filter phytoplankton from the water column (e.g. Officer et al. 1982). Although the filtration of phytoplankton and other particulate organic material from the water column is the first step in pathways that lead to nutrient removal by bivalves, the fate of that phytoplankton varies widely between species and environmental settings. Many studies

have focused on the potential impacts of aquacultured bivalve populations on water quality (e.g. Lindahl et al. 2005, Higgins et al. 2011). While it is evident that harvesting cultured bivalves would remove a significant amount of nutrients, few studies have measured comparable positive impacts resulting from enhanced denitrification in the underlying sediments, with some reporting the potential for negative impacts (e.g. Nizzoli et al. 2006, Holyoke 2008, Minjeaud et al. 2009, Higgins et al. 2013). Most studies that have estimated the water quality benefits of restored or natural bivalve populations have relied heavily on indirect estimates of their impacts. Newell et al. (2005) based denitrification rates in their model on laboratory studies of enhanced denitrification rates in soft sediments after addition of simulated biodeposits (Newell et al. 2002), and their burial estimates were based upon burial rates of nitrogen and phosphorus for soft sediments (Boynton et al. 1995). These values have been used by subsequent modeling efforts (e.g. Carmichael et al. 2012). To the best of our knowledge, Piehler & Smyth (2011) and Smyth et al. (2013) report the only previously published values for denitrification based on measured rates for materials collected from a restored or natural *Crassostrea virginica* reef. The contrast in rates between those studies and our own highlights the need to clarify the influence of environmental factors (e.g. tidal regime, light regime, water depth, salinity and primary production) and reef structural characteristics (e.g. oyster abundance and biomass, macrofaunal assemblage and structural complexity) on denitrification and nutrient sequestration rates, and thereby refine our understanding of the role restored oyster reefs play in nutrient removal.

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