POTENTIAL OF RIBBED MUSSELS (*GEUKENSIA DEMISSA*) TO ENHANCE GROWTH AND NITROGEN-REMOVAL SERVICES IN RESTORED SALT MARSHES

A Final Report of the Tibor T. Polgar Fellowship Program

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Zhu, J., C. Zarnoch, J.S. Gosnell, M. Alldred, and T. Hoellein. 2020. Potential of Ribbed Mussels (*Geukensia demissa*) to Enhance Growth and Nitrogen-removal Services in Restored Salt Marshes. Section III: 1-42 pp. *In* D.J. Yozzo, S.H. Fernald, and H. Andreyko (eds.), Final Report of the Tibor T. Fellowship Program, 2017. Hudson River Foundation.

ABSTRACT

Salt marshes are decreasing worldwide. Restoration projects address marsh loss, yet it remains unclear how well restored marshes grow, expand, and function in eutrophic waters. In natural marshes, mutualistic interactions among plants, animals, and microbes can sustain marsh growth, yet reduced diversity of restored marsh communities may inhibit their recovery. Management plans that incorporate species interactions may be needed to improve restoration outcomes. This study explored how a facultative mutualism between Atlantic ribbed mussels, *Geukensia demissa*, and cordgrass, *Spartina alterniflora*, may enhance marsh growth and nitrogen cycling in a eutrophic setting. Experimental plots containing live mussels, mussel shells, or no mussels (control) were created in Jamaica Bay, NY. After nine weeks, sediment and plant characteristics and sediment cores were collected for use in continuous-flow through incubations with ambient conditions and treatments enriched with stable isotope-labeled nitrate ($^{15}NO_3^-$). Denitrification in marsh plots with live mussels was significantly higher than the other treatments. Live mussels likely enhanced denitrification as biodeposits increased sediment organic carbon and anaerobic conditions. Mussel treatments did not impact cordgrass growth, possibly due to the eutrophic conditions at the study site or the short duration of the field trials. Ribbed mussels may be a valuable addition for salt marsh restoration projects in eutrophic estuaries since they increase the ecosystem service of nitrogen removal. Future work should focus on long-term effects of ribbed mussels on nitrogen removal and cordgrass biomass in restored marshes to determine how the mutualism impacts restoration success as sites age.

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INTRODUCTION

Salt marshes provide many functions to human and ecological communities. Marshes protect coastlines from erosion, provide shelter and food for diverse groups of fauna and flora, and contribute to nutrient storage and cycling (Deegan et al. 2012; Zedler and Kercher 2005; Costanza et al. 1997). Salt marshes have decreased 50-80% worldwide from historical levels due to anthropogenic influences (Grabowski et al. 2012), including the synergistic impacts of nitrogen (N) pollution, sea-level rise, dredging, reduced sediment input, and erosion (Hartig et al. 2002; NYCDEP 2007). In salt marshes in the Northeastern Atlantic region of the United States, for example, large inputs of anthropogenic N have reduced organic matter storage and sediment stability, which worsens erosion (Deegan et al. 2012; Wigand et al. 2014). Rising sea levels threaten salt marshes when they are unable to grow vertically at the same rate as water levels rise, and vertical growth could be limited by high nitrogen levels (Watson et al. 2014).

Given the global decrease of marsh coverage and the resulting loss in ecosystem services they provide, marsh protection and restoration is a goal of many coastal conservation efforts. Salt marsh restoration is designed to prevent or remediate environmental damage by optimizing the delivery of critical ecosystem services (Gedan et al. 2009) such as intercepting polluted runoff (Shutes 2001) or mitigating effects of sea level rise (Hazelden and Boorman 2001). Recovery of marshes after anthropogenic disturbance is slow under natural conditions but can potentially be accelerated through management practices that promote marsh growth or moderate negative impacts of environmental stressors (Broome et al. 1988). Various restoration methods have been

developed and employed in marsh restoration projects, such as altering hydrology, increasing elevation, and plantings (Broome and Craft 2009). Research is needed to evaluate the success of restoration efforts (Ruiz-Jaen and Aide 2005; Staszak and Armitage 2013).

Species interactions can have major consequences for salt marsh growth and ecosystem processes (Angelini et al. 2016; Silliman and Zieman 2001; Silliman et al. 2004; Silliman et al. 2005); however, common marsh restoration practices rarely account for community interactions. For example, Atlantic ribbed mussels (*Geukensia demissa*) engage in a facultative mutualism with cordgrass (*Spartina alterniflora*), which is the dominant plant of the low marsh along the eastern coast of North America (Bertness 1984). A facultative mutualism is a positive relationship in which interacting species can benefit from the association without depending on the relationship to survive and reproduce (Bertness 1984). Ribbed mussels attach themselves to the stems and roots of cordgrass with proteinaceous byssal threads which increases the structural stability of the marsh surface. When mussels suspension-feed, they pump water over marsh substrate, oxygenating the sediments and alleviating plant stress from anoxia (Bertness 1984). The production of feces and pseudofeces, collectively known as biodeposits, can enhance cordgrass growth by increasing sediment nutrients. In return, cordgrass provides the mussels with refuge from predators and shelter from heat and desiccation stress (Bertness and Grosholz 1985). Previous studies have shown that the ribbed mussels-cordgrass mutualism in salt marshes can enable the ecosystem to recover from or tolerate disturbances (Bertness et al. 2015) and enhance recovery from drought by increasing soil water storage and reducing soil salinity stress (Angelini et al. 2016).

The presence of ribbed mussels may also increase N removal and recycling in restored salt marshes (Bilkovic et al. 2017). Denitrification is the microbial respiratory process of using organic carbon as an energy source and reducing nitrate (NO_3^-) or nitrite (NO_2^-) to nitrogen gas (N_2). Therefore, denitrification provides the important ecosystem service of permanent N removal from ecosystems (Seitzinger 1988). Denitrifying bacteria are heterotrophs and are limited by availability of organic carbon (C), NO_3^-, and anaerobic conditions (Davis et al. 2004). High denitrification rates have been reported in areas where organic content of the sediment is enhanced by biodeposition (Piehler and Smyth 2011). Ribbed mussels and other bivalves may affect denitrification via biodeposition and diffusion of water column NO_3^- to sediment through the shell burrow (Hoellein et al. 2015; Welsh et al. 2015; Turek and Hoellein 2015; Humphries et al. 2016; Bilkovic et al. 2017). Nutrients from C and N rich biodeposits can diffuse into the sediment and be made available to marsh plants and sediment microbes (Kautsky and Evans 1987; Giles et al. 2006; Nizzoli et al. 2006). The accumulation of biodeposits increases sediment organic content, microbial activity, and sediment oxygen demand (Rodhouse and Roden 1987; Wotton and Malmqvist 2001; Nizzoli et al. 2006; Hargrave et al. 2008; Cranford et al. 2009). High oxygen demand may lead to the formation of anoxic microsites (Fenchel 1992; Hylleberg 1975), which can also enhance denitrification. Highly eutrophic systems may lead to enhanced organic C deposition since high seston loads result in the greater production of bivalve pseudofeces (Galimany et al. 2013). Denitrification may also be promoted by bivalve burrows which increase surface area for the diffusion of water column NO_3^- or alter oxygen levels in sediment (Turek and Hoellein 2015). The combined act of active suspension feeding and burrow

ventilation allows for oxygen-rich water to penetrate deeper into the sediment (Norkko and Shumway 2011) which may promote coupled nitrification-denitrification. For example, the combination of ribbed mussels and cordgrass resulted in higher denitrification rates in marsh sediments as compared to when either were alone (Bilkovic et al. 2017).

The impacts of mussels on denitrification may be especially important for marsh restoration projects. Young salt marshes generally have low denitrification rates due to C and NO_3^- limitation (Tyler et al. 2003; Broome and Craft 2009). N fixation often occurs at greater rates than denitrification in young mashes (Piehler et al. 1998); however, as marshes age, N fixation decreases, and denitrification increases (Tyler et al. 2003). Denitrification rates in natural marshes can be up to 44 times greater than restored salt marshes due to oxygen inhibition at low tide and porewater nutrients being flushed out at high tide in restored marshes (Thompson et al. 1995). Most ecological functions in restored marshes require 5-15 years to reach the original levels of natural marshes, which is about the time needed to accumulate 1000 g C/m^2 and 100 g N/m^2 in the soil (Craft et al. 2003). This suggests that either recovery is slow, or that the post-disturbance act of restoration has altered ecological functions to a state different from reference conditions. The reciprocal positive interaction between ribbed mussels and cordgrass could therefore be important in promoting the growth of restored salt marshes and increasing N removal.

Ribbed mussel introductions are rarely included during salt marsh restorations, and it is not clear how C and N-rich conditions typical of eutrophic waters could impact mutualistic interactions. Eutrophic conditions could alleviate N and C limitations for both cordgrass and denitrifying bacteria, thus reducing the role of mussel biodeposits and

burrows in sustaining denitrification. High N loadings are expected to decrease cordgrass belowground biomass as nutrients are acquired from the water column rather than the marsh sediment. Cordgrass will favor aboveground growth over belowground growth (Deegan et al. 2012; Alldred et al. 2017) which results in less C stored in marsh sediment. A reduction in belowground biomass will likely affect marsh N cycling since the rhizosphere mediates coupled nitrification-denitrification (McGlathery et al. 2007; Aoki and McGlathery 2018).

To consider the impacts of species interactions on cordgrass growth, sediment characteristics, and ecosystem functioning in eutrophic systems, a field study was conducted in 2017 at a young restored marsh in eutrophic Jamaica Bay, NY. Marshes in Jamaica Bay have been deteriorating rapidly with an average loss of 13 hectares per year and are also the target of multiple restoration projects (Rafferty et al. 2011; Wigand et al. 2014; Campbell et al. 2017). Plots were created in the field and assigned one of three treatments: live mussels, empty mussel shells, and no mussels. The inclusion of live mussels in plots was expected to increase aboveground and belowground biomass of marsh plants as mussels increased nutrient availability through biodeposition and promoted N diffusion through their burrows. A mussel shell treatment was included to compare the physical effect of mussel presence (shell and burrow) with the effect of filtration and biodeposition of live mussels. In addition to monitoring field plots, a sediment core incubation study was performed using cores collected from the field plots to examine how mussels impact denitrification under ambient and nitrate-enriched conditions in this young restored marsh. The combined effects of mussel presence and

biodeposition was hypothesized to increase denitrification compared to the control and mussel shell treatments.

METHODS

Study site

Yellow Bar Hassock (Figure 1) is a large salt marsh island in Jamaica Bay (NY, USA) that has declined in area since the 1950s (Hartig et al. 2002). In 2012, approximately 286,700 m^3 of dredged sand from Ambrose Channel and Rockaway Inlet (NY Harbor) was transferred to the northern half (17 hectares) of the island and graded to the desired elevation (Elias et al. 2012). Cordgrass was then seeded in middle-elevation areas while high elevation areas were planted with *Spartina patens* and *Distichlis spicata* plugs (NPS 2015). Subsequent monitoring has shown that restoration at Yellow Bar successfully increased elevation and coverage of cordgrass (Campbell et al. 2017).

Experimental field plots

Fifteen 0.25 m^2 experimental plots (Figure 2) were established in the southwest corner of the restored portion of Yellow Bar (40.61 °N, 73.83°W) in the summer of 2017. The plots were located near the marsh edge in two transects parallel to the edge of the waterline (Figure 2). Plots were spaced approximately ~1-1.5 m from each other and all were at a similar elevation. Plots were established so that they contained 18- 23 naturally-occurring cordgrass stems (mean (se): 79.47(1.70) stems m^{-2}).

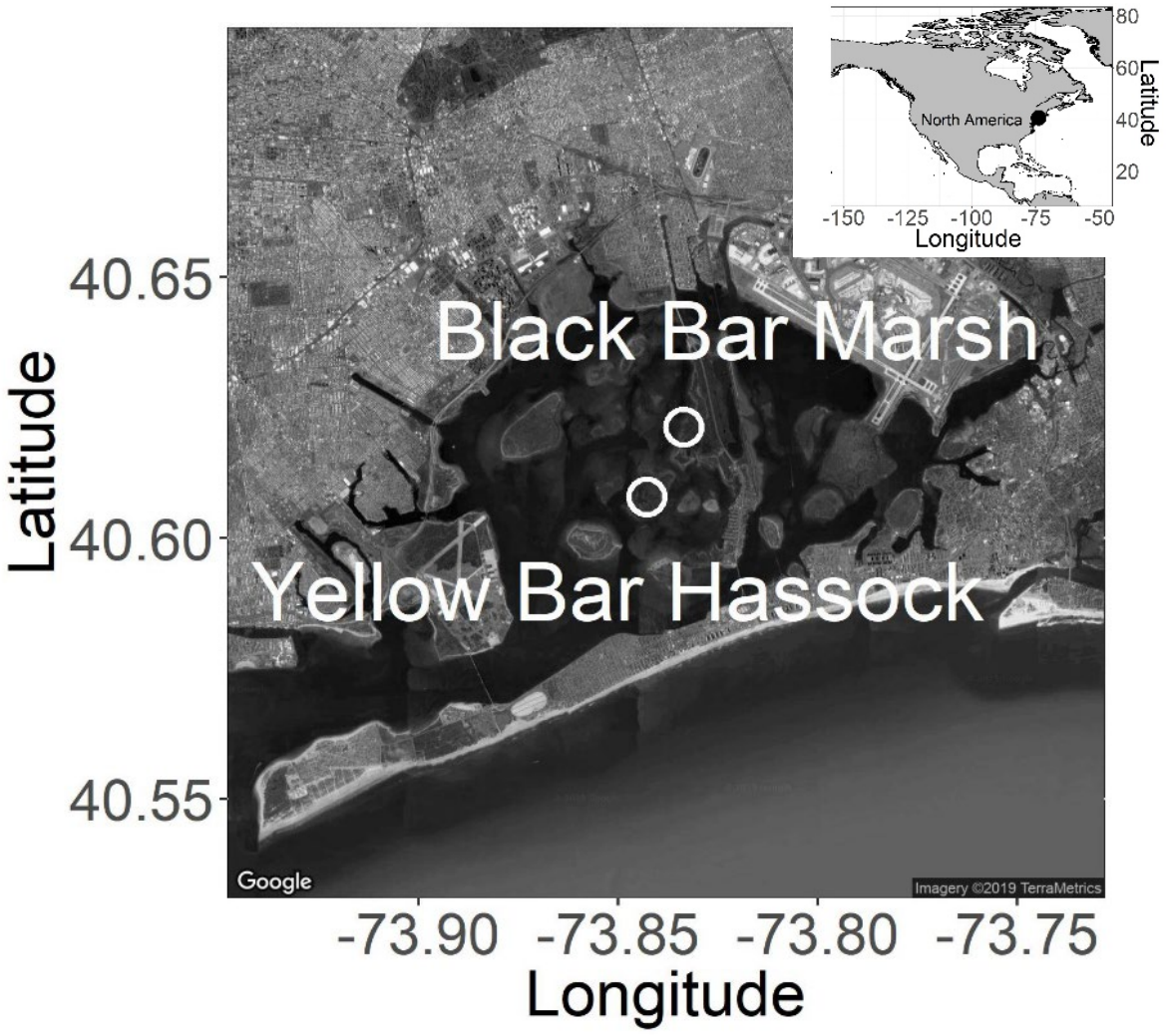

Figure 1. **Map of Yellow Bar Hassock and study site. Inset shows location in North America.**

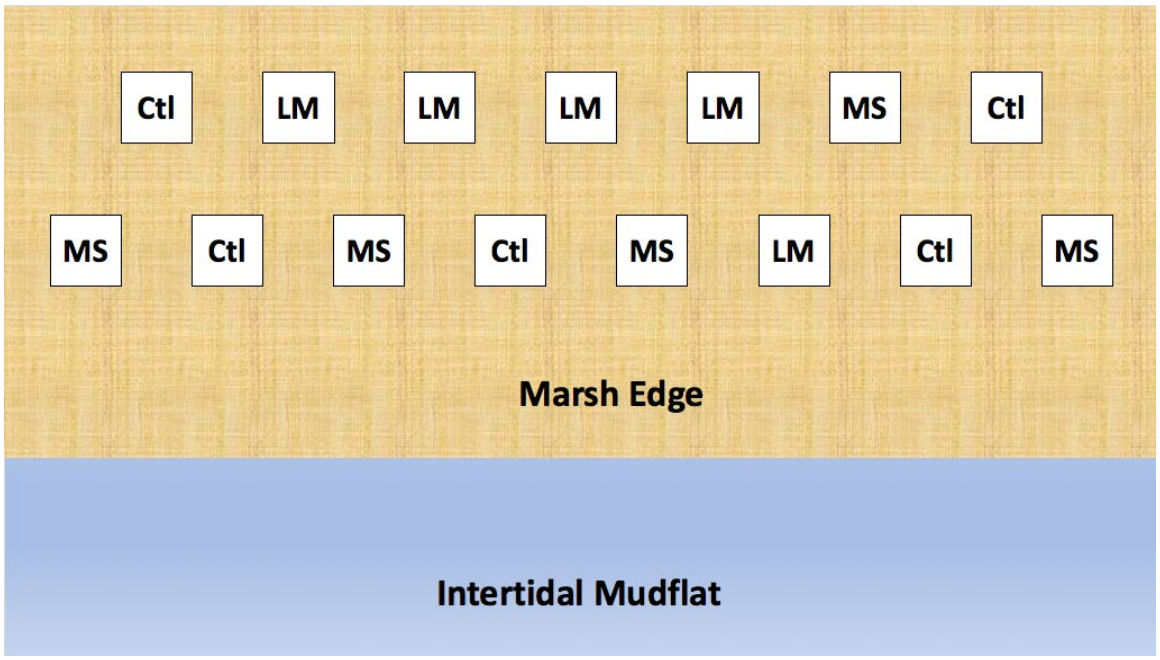

Figure 2. **Diagram of plot location relative to marsh shore. Treatment for each plot was distributed randomly. Ctl = control plots, MS = mussel shells plots, LM = live mussels plots.**

The plots consisted of the following treatments: 1) cordgrass alone serving as a control, 2) cordgrass with live ribbed mussels embedded in the sediment (LM plots hereafter), and 3) cordgrass with empty ribbed mussel shells embedded in the sediment (MS plot hereafter; Figure 3). Empty ribbed mussel shells used in the MS treatment were sealed together using marine epoxy. The MS plot treatment was applied to account for the physical presence of mussels and its effects on sediment characteristics and processes (i.e., change in friction velocity and organic matter accumulation; Sanford and Chang 1997). Each treatment had five replicate plots for a total of 15 plots, and the location of treatments were randomly assigned throughout the study area. The mussel shells and live mussels were collected from Black Bank marsh which is ~1 km north of Yellow Bar. Black Bank is a degraded marsh that has become fragmented due to loss of elevation and

vegetation (Wigand et al. 2014; Campbell et al. 2017). Ribbed mussels used in the plots had a mean shell length of 70.85 mm (SE = 0.11 mm; n = 70). The live mussels and shells were pushed into the sediment within the plots so that ~50% of the shell was buried (Jost and Helmuth 2007; Bertness et al. 2015). Each of the LM and MS plots had a density of 400 mussels (~1,600 mussels m^{-2}). Salt marshes in the northeastern US commonly have ribbed mussel populations of 2,000-3,000 m^{-2} (Bertness and Grosholz 1985) but densities of 10,000 m^{-2} have been observed in the eutrophic Jamaica Bay, likely due to its high primary production (Franz 2001). Monitoring of naturally-recruiting ribbed mussel populations at restored salt marshes in Jamaica Bay, however, has shown densities < 1,000 m^{-2} (Freynk 2018). Therefore, the experimental density used in this study, while similar to densities observed in other ecosystems, likely represents both the lower limits of natural population sizes and an achievable restoration goal.

Initial cordgrass and sediment characteristics were measured on 22 June 2017 to ensure all plots were similar. The treatments were then established by adding live mussels and mussel shells on 19-21 July 2017. Established plots were monitored for ~9 weeks, with sediment data collected again on 14 August 2017 and 29 September 2017. Cordgrass biomass samples were collected only during the final sampling date of 29 September. Due to the short growth period between plot establishment ~20 July and sampling on 14 August, the focus for the included methods and analysis was on data collected in September. Intact sediment cores were collected on 18 September 2017 and incubated for ~48 hours at Baruch College for measuring nutrient fluxes, oxygen demand, and denitrification rates.

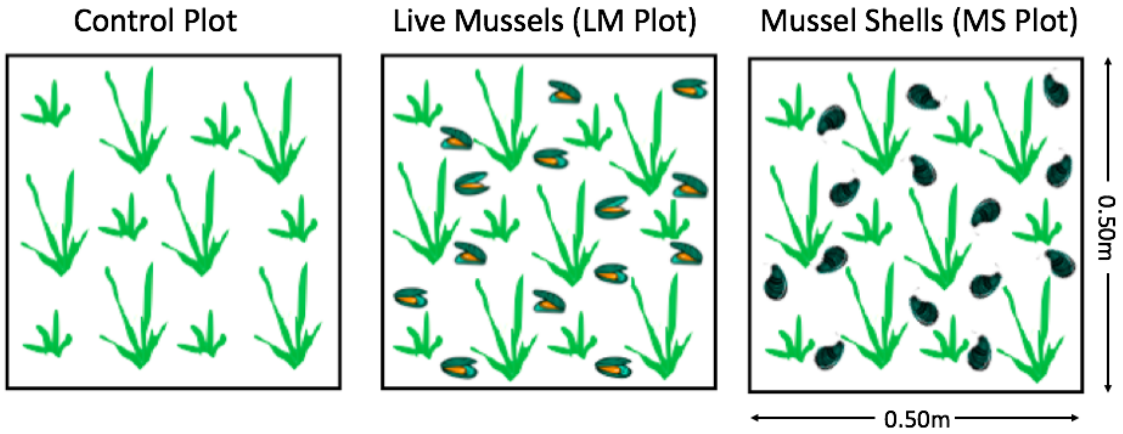

Figure 3. **Schematic of treatment plots. Control plots (N = 5) had cordgrass only, LM plots (N = 5) had live mussels partially buried in the sediment with cordgrass, and MS plots (N = 5) had closed empty mussel shells partially buried in sediment with cordgrass.**

Cordgrass and sediment sample collection and processing

Initial cordgrass and sediment measurements were taken on 22 June 2017. The number of stems in each plot were recorded and the height of five randomly-selected stems (distance from the sediment surface to the tip of the cordgrass stem) were measured. A sediment sample was taken from each plot with a modified 25 mm-diameter syringe to a depth of 3 cm and brought back to Baruch College for analysis. Each sediment sample was homogenized and then subsamples were taken to measure sediment characteristics. Subsamples were dried at 60°C until a constant weight, and then re-weighed to determine bulk density, percent moisture, and porosity. Sediment organic matter (OM) was determined following loss on ignition at 500°C (Benfield 2006). Sediment total organic carbon and total nitrogen was determined by treating with 25% HCl and redrying at 60°C (Nieuwenhuize et al. 1994). C:N content of homogenized

samples was then measured on a Series II 2400 CHN Analyzer (Perkin Elmer Life and Analytical Sciences, Shelton, CT) with acetanilide as a standard.

At the end of the experiment (29 September 2017), stem density and heights were recorded for all cordgrass stems from each plot. All stems were cut at the sediment surface, and aboveground biomass was recorded after drying cordgrass at 60°C for at least 48 hours. Two stems from each plot were reserved to measure C:N ratio. Stems were cut to 5 cm long sections, dried at 60°C until a constant weight, ground into homogenous samples using a mortar and pestle, and analyzed for C:N content as described above. Belowground biomass (i.e. roots and rhizomes) was sampled by inserting 30 cm long x 7.6 cm diameter acrylic sediment cores into marsh soil to a depth of 15 cm. Three replicate cores were taken from each plot (n = 45). The belowground material was wet-sieved through a 1.0 mm mesh sieve to remove sediment, placed in paper bags, dried at 60°C until a constant weight, and weighed to determine biomass. Bulk density, moisture, porosity, OM, and C:N of belowground material were measured following the procedures noted above (N=3 replicates per plot).

Benthic nutrient and gas fluxes from continuous-flow core incubations

The impacts of treatments on nutrient and gas fluxes were measured by using intact sediment cores from each plot in continuous-flow incubation studies (Hoellein et al. 2015; Zarnoch et al. 2017; Bilkovic et al. 2017). The intact sediment cores (30 cm long x 7.6 cm diameter) were collected from each of the plots on 19 September 2017 (2 months after plot establishment) using a PVC coring device (Gardner et al. 2006) and brought back to the laboratory. Each core contained ~15 cm of sediment along with 3

mussels (applicable to only MS and LM treatment cores; equivalent to 661.3 mussels/m^2) and cordgrass. Stems in the cores were cut and plugged with silicone gel to reduce oxygen and organic C leakage (Caffrey et al. 2007). All cores were placed in a water bath adjusted to the in-situ water temperature (22°C). Two continuous-flow core incubation experiments were conducted. In the first set of measurements, ambient site water (NO_x^- = 8 μmol L^{-1}) flowed through the cores (ambient incubation hereafter). In the second set of measurements, site water was enriched with $^{15}NO_3^-$ to determine total denitrification and N fixation (NO_x^- concentration = 24 μmol L^{-1}). The experiments were performed sequentially with each lasting 24 hours. The flow rate through the cores was 1.1 ml min^{-1}, making the total turnover time 3.5 hours in each core. After the core incubations, 10 ml of sediment from each core were collected for sediment analysis and analyzed for OM and C:N content as previously described.

Concentrations of soluble reactive phosphorus (SRP), NH_4^+, and NO_x^- were analyzed in water samples collected from inflow carboys and from outflow samples of each core (Figure 4). Water was collected and then filtered through a 0.2 micron nylon filter (Thermo Scientific, Rockwood, TN, USA) into 3, 20 ml scintillation vials and frozen until analysis. Samples were analyzed following established protocols (SRP: antimonyl tartrate method following Murphy and Riley 1962; NH_4^+: phenol, hypochlorite method following Solorzano 1969; NO_x^-: cadmium reduction method following APHA 1985), with a Seal AQ2+ discrete nutrient analyzer (Seal Analytical Inc., Mequon, WI).

Samples for dissolved gases ($^{28}N_2$, $^{29}N_2$, $^{30}N_2$, $^{32}O_2$, and ^{40}Ar) were collected directly from carboys for inflow measurements and outflows were dripped directly into triplicate 12 ml Labco Exetainer ® vials (Lampeter, Wales, UK) so that vials overflowed

for several volumes. Samples were poisoned with 200 µl of 50% zinc chloride, then capped and stored underwater at 4°C. Samples were analyzed using membrane inlet mass spectrometry (MIMS; Bay Instruments, Easton, MD, USA; Kana et al. 1994) at Loyola University Chicago, IL. The MIMS had a peristaltic pump that pulled the sample out of the vials to extract the dissolved gases across a membrane under vacuum. The standard used for the MIMS was artificial seawater held at 22°C and a salinity of 27.2 ppt (Circulating Bath, VWR International, Radnor, PA, USA), stirred at a low speed to equilibrate to atmospheric gases (Lab Egg RW11 Basic, IKA Works, INC., Wilmington, NC, USA). O_2 and N_2 concentrations were determined by using the ratio with Ar following standard protocol (Hoellein et al. 2015; Kana et al. 1994). All MIMS samples were corrected for instrument drift using standard at the beginning and throughout the run.

Nutrient (SRP, NO_x^-, NH_4^+) and gas (O_2, N_2) fluxes were calculated by subtracting the concentration in the outflow from the concentration in the inflow, multiplying by the pump flow rate, and dividing by the surface area of the core (flux units = µmol element m^{-2} h^{-1}). The $^{28}N_2$ data were used to determine the net N_2 flux of the control incubation, whereas the sum of dissolved gases of $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ were used to calculate total denitrification in the $^{15}NO_3^-$ enriched incubation. The production of $^{29}N_2$ and $^{30}N_2$ in the enriched incubation were considered as an index of direct denitrification of the $^{15}NO_3^-$. Nitrification, percentage of coupled nitrification-denitrification, and denitrification efficiency were calculated for each core, focusing on $^{28}N_2$ flux for ambient cores and the sum of $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ fluxes for enriched cores. Nitrification was calculated by summing the NO_x^- and N_2 fluxes (Kellogg et al. 2013). The percentage of

coupled nitrification-denitrification was calculated by dividing the calculated nitrification by the N_2 fluxes and multiplying by 100%. If NO_x^- fluxes were positive all denitrification was assumed to be coupled (Gonzalez et al. 2013). Lastly, denitrification efficiency was calculated by dividing N_2 efflux by the sum of the N_2, NH_4^+, and NO_x^- effluxes (only positive values used) and multiplying by 100 (Eyre and Ferguson 2009).

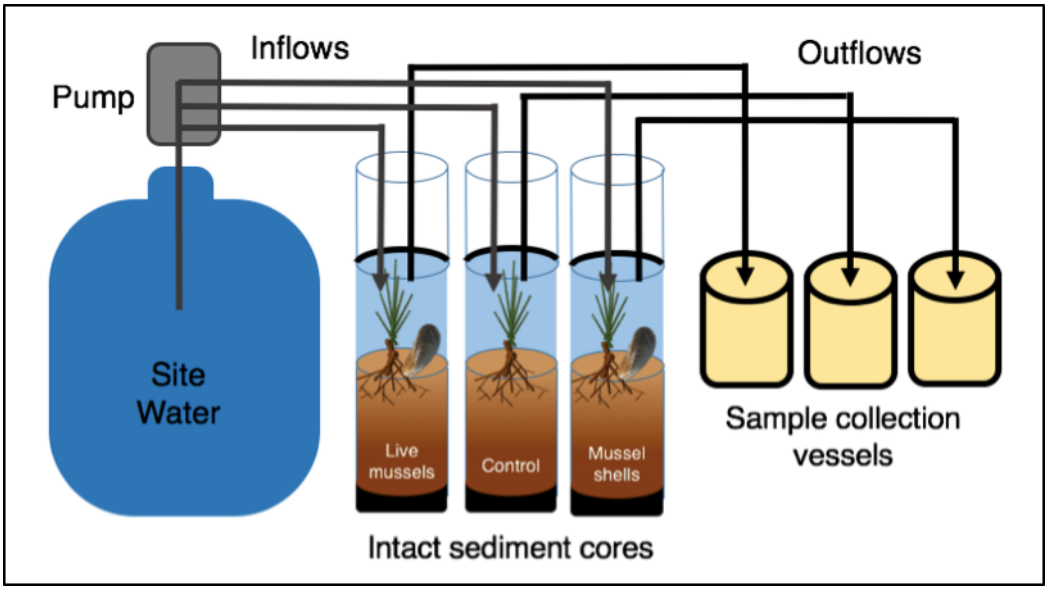

Figure 4. Diagram of lab incubation set-up. Intact sediment cores were collected from Yellow Bar field manipulation plots at the end of the study.

Statistical analysis

Data were analyzed using appropriate statistical approaches in R (R Core Team 2016; Zuur et al. 2009). One-way ANOVAs with treatment as a factor were used to access data collected once per plot or core (plot stem density, June and July sediment data, core sediment data) ('aov' function from stats package; R Core Team 2016). Mixed-effect models with treatment as a fixed factor and sampling unit as a random

effect were used to assess data collected multiple times from a plot or core (stem heights and widths, belowground biomass and plant C:N, September sediment data, core incubation flux data) to account for within sampling unit variation and pseudoreplication ('lme4' package, Bates et al. 2015; 'car package', Fox and Weisberg 2011). P-values were found by analyzing Type III sums of squares for models using the ANOVA function ('car package'). When significant differences were found among treatments, post-hoc tests were carried using the 'TukeyHSD' function ('stats' package; R Core Team 2016) for ANOVAs and the 'lsmeans' package for linear mixed-effects models ('lsmeans' package; Lenth 2016) to compare treatment means. Relationships among OM, sediment oxygen demand, and denitrification in ambient cores were also analyzed using regression.

Graphs and figures were produced using the 'ggplot2'and 'ggmap' packages, and the 'plyr' and 'reshape' package were used for data manipulation (Kahle and Wickham 2013; Wickham 2007, 2009, 2011). Map data was provided by Google Earth and Natural Earth.

RESULTS

Initial plot measurements

Plant and sediment characteristics in study plots were compared prior to setting up the experiment. No significant differences were observed in stem density ($F_{2,12} = 0.659$, $p = 0.535$) or stem height ($X^2_2 = 0.459$, $p = 0.795$) among plots. Sediment conditions also showed no significant differences among plots (bulk density: $F_{2,12} = 0.860$, $p = 0.448$; % moisture: $F_{2,12} = 0.389$, $p = 0.686$; porosity: $F_{2,12} = 0.425$, $p = 0.663$; OM: $F_{2,12} = 0.036$, $p = 0.964$; C:N: $F_{2,12} = 0.885$, $p = 0.438$).

Impact of treatments on cordgrass and sediment characteristics

Plant characteristics were measured and compared among study plots at the end of the study period in September which was ~9 weeks after treatments were established. Results showed no differences in aboveground plant characteristics including stem density ($F_{2,12} = 0.276$, $p = 0.763$), stem height ($X^2_2 = 1.719$, $p = 0.423$), AG biomass ($F_{2,12} = 0.808$, $p = 0.708$), and C:N molar ratio ($F_{2,12} = 3.222$, $p = 0.076$) among treatments (Table 1). In contrast, changes in plant density and height were observed in all plots from the start to the end of the experimental period. Stem density increased from 79.4 stems m^{-2} to 319.2 stems m^{-2} and mean stem height increased from 23.7 cm to 39.0 cm from the start to the end of the experiment. Results also showed no significant differences among treatments for belowground biomass ($X^2_2 = 0.820$, $p = 0.663$) and C:N molar ratio ($X^2_2 = 4.683$, $p = 0.096$) using linear mixed-effects models (Supplemental Table 1). C:N ratios were lowest in the live mussel treatment for both above- and belowground biomass but were not statistically different from the other treatments.

Table 1. Mean (standard error) of aboveground (AG) and belowground (BG) cordgrass traits measured at the end of the study. The carbon:nitrogen (C:N) data are expressed as molar ratios. Treatments include the control plots (C), empty mussel shell plots (MS), and live mussel plots (LM).

Cordgrass trait	C	MS	LM
AG biomass (g m^{-2})	252.25 (43.94)	243.66 (64.53)	206.08 (47.58)
Stem density (# m^{-2})	300 (26.32)	319.19 (43.03)	338.4 (38.17)
Stem height (cm)	40.05 (1.46)	36.88 (1.40)	35.69 (1.05)
AG C:N	126.18 (15.14)	72.09 (9.61)	87.18 (15.14)
BG biomass (g m^{-2})	698.88 (533.8)	601.02 (296.56)	230.45 (67.38)
BG C:N	76.41 (6.70)	72.09 (8.56)	50.66 (4.55)

The effect of the live mussel and shell treatments on sediment varied among the characteristics measured. There was no effect of the treatments on bulk density (X^2_2 value = 1.005, p = 0.605); moisture (X^2_2 value = 1.316, p = 0.518), porosity (X^2_2 value = 1.281, p = 0.527); and C:N (X^2_2 value = 1.652, p = 0.438). In contrast, OM differed significantly among treatments (X^2_2 value = 15.842, p < 0.001; Figure 5). OM was significantly higher in plots compared to control (t = -3.133, p = 0.022) and mussel shell plots (t = -3.693, p = 0.008).

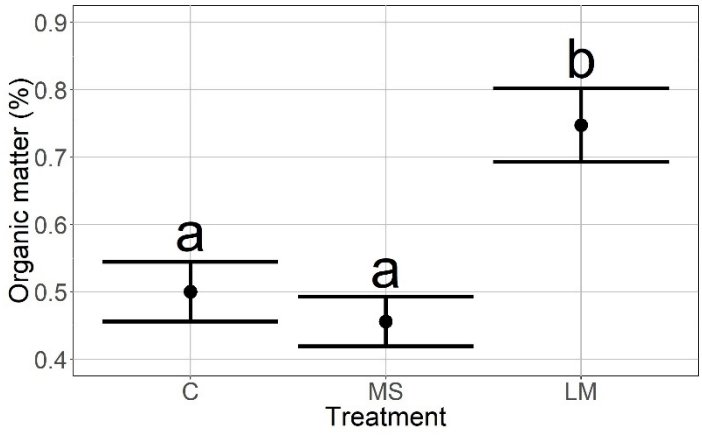

Figure 5. Percent sediment organic matter in at the end of the experiment. Sediment organic matter was higher in live mussel (LM) plots than control (C) or mussel shell (MS) treatments ~9 weeks following mussel introductions. Dots represent the mean and error bars represent standard error.

Continuous-flow core incubations

Live mussels had a significant effect on two of the three solutes measured. Significant differences existed among treatments for fluxes of SRP (X^2_2 value = 18.287, p < 0.001; Figure 6A) and NO_x^- (X^2_2 value = 17.596, p < 0.001; Figure 6B) in the ambient core incubations. Post-hoc analysis indicated that LM plots had significantly greater SRP

efflux and NO_x^- uptake than control and MS plots (post-hoc tests, all $p < 0.01$). In contrast, NH_4^+ fluxes did not differ statistically among plot treatments (X^2_2 value = 3.391, p = 0.184; Figure 6C).

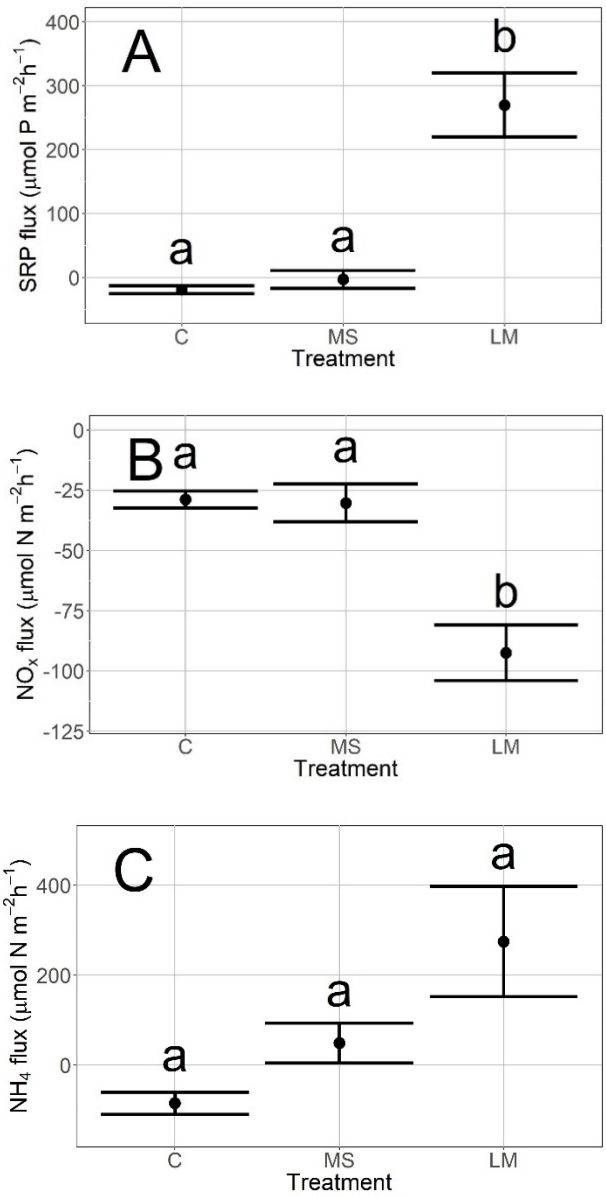

Figure 6. **A) Mean (±SE) flux of soluble reactive phosphorus (SRP), B) NO_x^-, and C) NH_4^+ flux from continuous-flow core incubations under ambient conditions. Dots represent mean and error bars represent standard error.**

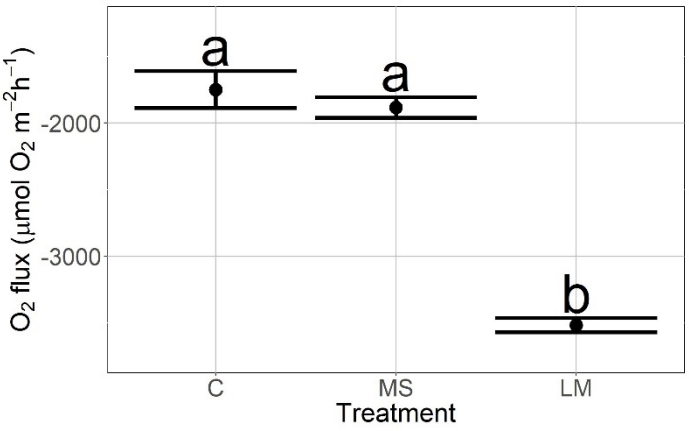

Figure 7. **Oxygen flux from the ambient trial of the continuous-flow core incubations. O_2 flux LM plots was significantly greater than the other treatments incubations ($p < 0.001$). C = control plots, MS = mussel shells plots, LM = live mussels plots. Dots represent the mean and error bars represent standard error.**

Gas fluxes also showed significant effects of the mussel treatments. Oxygen fluxes were significantly different among treatments (X^2_2 value = 19.249, $p < 0.001$; Figure 7) with LM plots showing greater O_2 demand than the control and MS treatments (all $p < 0.009$). For N_2 flux, there was a significant interaction between core (ambient vs enriched) and plot (Control, MS, LM) treatment on (X^2_2 value = 36.038, $p < 0.001$; Figure 8) that was driven by differences in N_2 flux from LM and MS plots between core treatments. Examining the ambient and enriched results separately showed LM plots always leading to highest rates of denitrification in both core trials (ambient: X^2_2 value = 9.711, $p = 0.008$; enriched: X^2_2 value = 47.582, $p < 0.001$). In the ambient core incubation, LM plots had significantly higher $^{28}N_2$ efflux than the MS treatment (t-ratio value = -2.832, $p = 0.037$) and marginally higher flux than the control treatment (t-ratio value = -2.543, $p = 0.062$; Figure 8A). In the enriched core incubation, LM plots had significantly greater total N_2 efflux than both the control (t-ratio value = -6.148, $p <$

0.001) and MS plots (t-ratio value = -5.783, $p < 0.001$; Figure 8B). Nitrogen fixation (11µmol N m^{-2} h^{-1}) was observed in only one core from the control treatment during the enriched trial. In both ambient and enriched trials, the LM plots had the highest nitrification rate, lowest DN efficiency, and lower coupled nitrification-denitrification than control plots (Table 2).

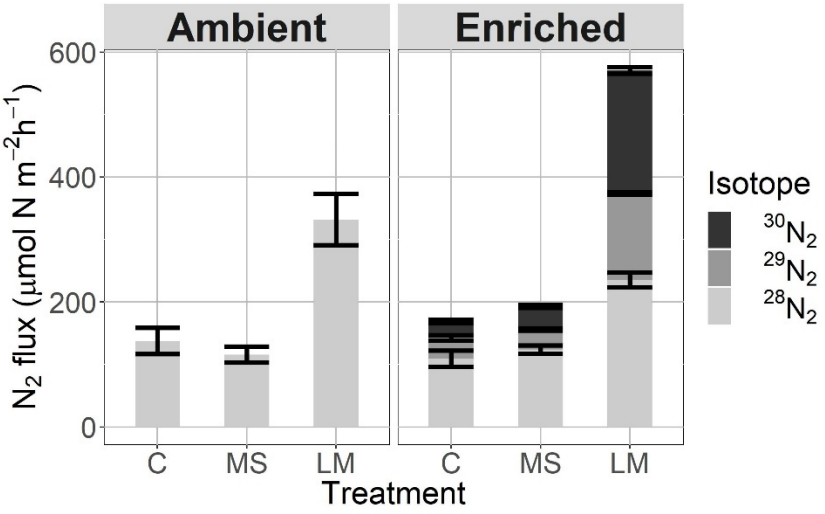

Figure 8. **N_2 flux including contributions of $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ fluxes from ambient and enriched trials. Mean and standard error are represented as horizontal bars.**

Table 2. **Mean (standard error) of calculated nitrification rate, percent of denitrification coupled to nitrification, and denitrification efficiency from ambient trial. Treatments include the control plots (C), empty mussel shell plots (MS), and live mussel plots (LM).**

	Treatment		
	C	MS	LM
Calculated nitrification rate (µmol N m^{-2} h^{-1})	109.09 (43.49)	85.59 (22.85)	239.65 (80.64)
Denitrification coupled to nitrification (%)	84.35 (5.32)	62.52 (15.27)	65.09 (8.34)
Denitrification efficiency (%)	83.31 (16.69)	72.37 (13.75)	0.96 (16.84)

Linear regression result showed a significant relationship between sediment oxygen demand and core OM under ambient conditions (slope = 1160.9, $F_{1,13} = 6.993$, $R^2 = 0.350$, $p = 0.020$; Figure 9A). Regression results also showed a significant positive relationship between N_2 flux and sediment oxygen demand from the ambient trial (slope = 0.102, $F_{1,13} = 13.695$, $R^2 = 0.513$, $p = 0.003$; Figure 9B).

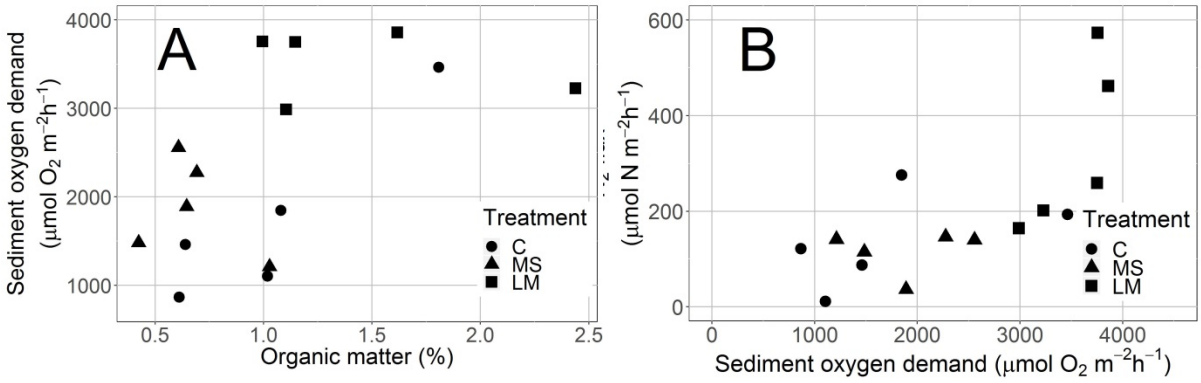

Figure 9. **A) Linear regression of sediment oxygen demand and core %OM from ambient incubation. B) Linear regression of net N_2 flux and sediment oxygen demand from the ambient incubation.**

DISCUSSION

The study results demonstrate the importance of the mussel-cordgrass mutualism in enhancing the ecosystem service of N removal via denitrification at a recently restored salt marsh in eutrophic Jamaica Bay, NY. Bilkovic et al. (2017) also found this mutualism important in enhancing denitrification when examining conditions in a natural marsh. Rates from both studies were similar (~411 μmol N m^{-2} h^{-1} in Bilkovic et al. 2017 and ~350 μmol N m^{-2} h^{-1} in the current study), which was unexpected as this study was

performed at a young restored marsh (five years) while the other measurements were from natural marshes. Studies at restored marshes have typically found that N fixation is predominant over denitrification during early development (Currin and Paerl 1998) and it could take >15 years before denitrification in restored marshes reaches equivalency of natural reference marshes (Broome and Craft 2009). The high rates of denitrification observed in this study is likely due to the highly eutrophic conditions of Jamaica Bay. With an abundance of N and C substrates, denitrifying microbes in both control and mussel treatments were conducting denitrification at high rates.

Enhanced cordgrass biomass in the live mussel treatment was not observed, possibly due to the eutrophic conditions in Jamaica Bay, soil conditions in the restored marsh, and the relatively short duration of the experiment. Eutrophic conditions at the study sites mean that the plants may not be N limited and therefore not benefit from the live mussels adding organic matter and increasing N uptake in the sediment. However, the soils in the restored marsh were relatively sandy, so it is possible that over time the mussel-derived nutrients in the marsh soil could enhance marsh growth. Measuring these effects will likely take longer than the nine-week study conducted here, and justify study of long term analysis of the mutualistic relationships that could benefit marsh growth. Overall, the high N loads into Jamaica Bay may decouple the mutualism between mussels and cordgrass just as other forms of disturbances have impacted mutualistic interactions (de Fouw et al. 2018; Hoek et al. 2017; Palmer et al. 2008). Therefore, the addition of mussels to marsh restoration programs in eutrophic ecosystems may enhance N removal via denitrification but may not enhance cordgrass biomass in the short term.

Mussel impact on denitrification

The increase in denitrification in plots containing live mussels was likely enhanced by mussel biodeposition increasing organic C availability and creating a more anaerobic environment. Both OM and denitrification rates were ~two times higher in LM plots (Figs. 5 and 8, respectively) compared to control and mussel shell plots. Denitrification may have been limited by organic carbon at the study site since it was a young restored marsh with much lower organic matter than what would be found at a natural marsh (Craft et al. 2003). Decomposition of biodeposits likely altered sediment redox conditions to promote denitrification (Poulin et al. 2007). The positive relationship between organic matter and sediment oxygen as well as the positive relationship between sediment oxygen demand and net N_2 flux support this argument. Sediment oxygen demand and SRP efflux was highest in the live mussel plots which suggests that these plots had the most reduced sediment conditions. The SRP fluxes observed in the control and mussel shell treatments were consistent with most measurements taken within coastal sediments (-25 to 100 μmol P m^{-2} h^{-1}; Boynton et al. 2018). The higher rates of SRP efflux (270 μmol P m^{-2} h^{-1}) from the live mussel plots suggest that anoxic conditions promoted SRP desorption from iron oxides and/or transformation to iron sulfate (Kemp et al. 2005).

The $^{15}NO_3^-$ enrichment provided additional insight into mechanisms for enhanced denitrification. The total N_2 flux in the control treatment was found to change -4% due to enrichment, 55% in the mussel shell treatment, and 63% in the live mussel treatment (Figure 8). The lack of response in the control treatment indicates that this denitrifying community was not NO_3^- limited. Calculations show that 84% of the measured

denitrification in the control plots was coupled to nitrification so NO_3^- demand from denitrifying bacteria was largely met by nitrification. In addition, water column NO_x^- was 8 μmol L^{-1} during the control incubations which could support direct denitrification (Seitzinger et al. 2006). The increase in $^{29}N_2$ and $^{30}N_2$ flux in the mussel shell and live mussel treatments (Figure 8) may have been due the physical presence of the mussels altering anaerobic conditions and increased NO_3^- diffusion. The presence of mussels on the marsh surface likely reduced horizontal flux of O_2 into the sediment due to lower flow velocity. This would then increase anaerobic microsites and promote denitrification potential. Similarly, altered friction velocity over oyster reefs likely creates microsites that support high rates of denitrification (Kellogg et al. 2013; Humphries et al. 2016; Sanford and Cheng 1997). Other studies have found that bivalve burrows or the presence of shell hash could enhance direct denitrification as it increases the sediment surface area (Turek and Hoellein 2015; Hoellein et al. 2015).

The increase in $^{29}N_2$ and $^{30}N_2$ fluxes in the mussel shell and live mussel treatments may have also been caused by NO_3^- diffusion through mussel burrows; however, the N_2 and NO_x^- flux were similar between the control and mussel shell treatments in the ambient trial. This suggests that NO_3^- diffusion through mussel burrows alone could not enhance denitrification and that enriched conditions coupled with anaerobic sediment would more likely lead to the increase in $^{30}N_2$ production in the mussel shell treatment during the enriched trial. The mussel shell plots also had only 63% of denitrification coupled to nitrification as compared to 84% in the control treatment. This difference is likely due to nitrification being inhibited by O_2 availability in the mussel shell treatment. Other organisms (e.g., fiddler crabs, *Uca pugnax,* Bertness 1985; Laverock et al. 2011)

that are more active bioturbators can impact the diffusion of oxygenated water into the sediment and increase the exchange of solutes from the water column to sediment. Active burrowers may also allow for deeper penetration of biodeposits to areas below the surface where denitrifying bacteria do not have to compete for C with other sediment microbes (Norkko and Shumway 2011). Calculated nitrification was highest in the live mussel plots likely due to NH_4^+ excretion and ammonification. Bruesewitz et al. (2008) found the NH_4^+ rich waste of zebra mussels increased sediment nitrification which increased NO_3^- availability for denitrification. Collectively, these results suggest that mussel additions to young restored marshes may enhance denitrification by alleviating the limitations of C availability and anaerobic conditions. The highly eutrophic system may have also reduced competition for N between denitrifying bacteria and cordgrass which allowed for high rates of denitrification (Hamersley and Howes 2005).

No effect of mussels on cordgrass biomass

The marsh site was expected to be N limited due to its young age, low sediment organic matter, and coarse sediment. This would then result in the mussel addition positively affecting cordgrass biomass; however, results indicate that the addition of live ribbed mussels and mussel shells did not enhance cordgrass biomass. Positive feedback interactions between ribbed mussels and cordgrass have been documented by previous studies and shown to enhance salt marsh resilience and marsh recovery (Angelini et al. 2016; Bertness et al. 1984; Crotty and Bertness 2015; Derksen-Hooijberg et al. 2017). For example, mussels reduced soil salinity stress, increased cordgrass aboveground growth, and promoted survival during drought (Angelini et al. 2016). In addition,

Derksen-Hooijberg et al. (2017) found cordgrass growth and clonal expansion increased by 50% due to mussel presence because co-transplanted mussels increased nutrients in the porewater and reduced sulfide stress. However, that study was conducted over a period of over 16 months while this study was performed over three months. The absence of an increase in cordgrass biomass in response to the mussel addition is possibly due to the short time frame of the experiment. However, other studies have shown responses to positive interactions between mussels and cordgrass over a period of two months (Crotty and Bertness 2015) and four months (Bertness et al. 1984). The impacts of mussels on cordgrass biomass may be context-dependent and the eutrophic conditions of this study system may have reduced positive feedbacks between mussels and cordgrass. Longer analyses will be required to make that determination.

Fertilization experiments simulating high N loadings (Valiela et al. 1976; Turner et al. 2009; Deegan et al. 2012) and field measurements across N loading gradients (Darby and Turner 2008; Alldred et al. 2017) show N addition increases aboveground biomass and reduces belowground biomass in cordgrass. For example, Alldred et al. (2017) found that in marshes which span a land-use gradient in Long Island, NY, sites with high dissolved inorganic N, have 60-70% less belowground biomass. In this study, above- and belowground biomass measurements were low compared to natural marshes and were indicative of a relatively young restored marsh (Craft et al. 1999; 2003). A degrading natural marsh located ~1.3 km from the study site had reduced belowground biomass and a high above: belowground biomass ratio compared to a stable marsh in Jamaica Bay (Wigand et al. 2014), which is consistent with the previous studies. Jamaica Bay is a sediment limited system and marsh elevation is sustained through organic matter

accumulation (Peteet et al. 2018). Future studies should document above and belowground biomass at restored marshes in Jamaica Bay over a longer time period in order to understand the combined effect of N loading and sand substrates.

Sediment characteristics that change across long time scales with marsh maturity may affect the role of mussels in providing plants with nutrients in restored marshes of Jamaica Bay and other eutrophic locations. Analyses showed live mussels did not increase above and belowground biomass nor did they alter C:N ratios, suggesting the cordgrass was not N limited. The cordgrass likely received adequate N through water column N, organic matter deposition, and sediment N recycling. In addition, the low above and belowground biomass in the plots likely reduced competition for available N with sediment microbes, allowing for high rates of denitrification. As the marsh ages and N demand increases, there may be a shift towards greater reliance upon biogenic processes that could alter cordgrass allocations to growth, sediment N recycling, and competition for N with denitrifying bacteria. These changes with marsh maturity may also affect the role of mussels, increasing the possibility that mussels will be important for marsh nutrients.

Implications for management

The study results indicate that mussel additions to a restored salt marsh in Jamaica Bay, NY, will not increase cordgrass biomass at this stage of marsh maturity, however, the mussel-cordgrass interaction may provide other measurable benefits which are critical to evaluate marsh restoration projects. For example, mussel addition to salt marsh plots increased sediment organic matter, which is a critical component of ecosystem structure

and growth in the restored salt marshes in Jamaica Bay. Study plots with live mussels increased organic matter ~200% as compared to control plots. Since Jamaica Bay marshes rely upon organic matter deposition as compared to mineral sediments to sustain themselves (Peteet et al. 2018), mussel biodeposition may be a critical subsidy to maintaining marsh elevation. Mussel C deposition is likely to be important to the development of restored marshes as well (Craft et al. 2003) and contributes to carbon sequestration (i.e., blue carbon) in restored marshes.

In addition to benefits for organic matter deposition, mussel addition increased denitrification, which is a valuable ecosystem service. For example, mussel addition increased denitrification 140% compared to the control plots (ambient NO_3^- = 8 μmol L^{-1}) and denitrification was increased 235% under the enriched conditions (NO_3^- = 24 μmol L^{-1}). Water column NO_3^- varies seasonally in Jamaica Bay and often exceeds enriched conditions used in this study (Hoellein and Zarnoch 2014). Including mussels in marsh restoration programs will significantly increase the ecosystem service on N removal provided by restored marshes even in their early stages of development. This an important measurable outcome in restoration projects whose value can be quantified monetarily and used in cost-benefit analyses (Piehler and Smyth 2011; Zarnoch et al. 2017). This study suggests future analyses of salt marsh restoration will benefit from careful calculations of the monetary value of marsh-mussel denitrification, which can help justify the use of mussels to sustain healthy and valuable ecosystem services.

ACKNOWLEDGEMENTS

This study was funded by the Hudson River Foundation through a Tibor T. Polgar Fellowship awarded to JZ and a Hudson River Fund award (013/15A) that supported TJH, MKA, and CBZ. Additional funding was provided by the Professional Staff Congress of CUNY to JSG and CBZ. We appreciate the support of Gateway National Recreation Area and their advice on sampling design and permitting. We are thankful for field assistance from Shannon Jenkins, Renee Montelbano, and Beth Freynk. We also appreciate laboratory and field assistance of the high school students participating in the CUNY College Now Program and the Rockaway Waterfront Alliance Environmentor Program at Baruch College.

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