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Living oysters and their shells as sites of nitrification and denitrification

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ABSTRACT

Oysters provide a critical habitat, are a food resource for higher trophic levels and support important commercial fisheries throughout the world. Oyster reefs can improve water quality by removing phytoplankton. While sediment denitrification may be enhanced adjacent to oyster reefs, little is known about nitrification and denitrification associated with living oysters and their shells. We measured nitrification and denitrification in living oysters (*Crassostrea virginica* and *Crassostrea gigas*) and empty oyster shells. Nitrification was similar between live oysters and empty oyster shells, however, denitrification was enhanced significantly on living oysters compared to shells. This is the first demonstration of nitrification and denitrification associated with living oysters and their shells. Our data suggest that loss of historic oyster reefs has likely affected the resilience of estuaries to eutrophication. The additional benefit of oyster mediated denitrification should be considered in restoration of oyster reefs as a tool for managing eutrophication.

1. Introduction

Nearshore ecosystems containing oyster reefs are among the most valuable and productive marine habitats on earth, from both ecological and economic perspectives. Over the past century, areal coverage of oyster reefs and oyster biomass have declined 64% and 88%, respectively (Zu Ermgassen et al., 2012). Declines in oysters have been attributed to disease, overharvesting, burial by excessive sedimentation, pollution-induced mortality and other human impacts (Luckenbach et al., 2005, Lotze et al., 2006, Jackson, 2008, Beck et al., 2011). The loss and degradation of oyster habitat represents a substantial threat to the sustainability of coastal marine resources. As a foundation species that produce hard substrate, healthy oysters are essential for maintaining reefs and sustaining optimal ecosystem services including food production, stabilizing shorelines, burial of carbon and improving water quality (Grabowski and Peterson, 2007, Grabowski et al., 2012, zu Ermgassen et al., 2013).

Oyster reefs serve as habitat for over 200 species of fish and invertebrates (Karnauskas et al., 2013). Möbius coined the term "biocoenosis" in 1877 to describe the distinct assemblage of invertebrate and fish species that colonized oyster reefs (Möbius, 1877), since then this concept has been expanded to include microbes (Colwell and Liston, 1960, Nocker et al., 2004, La Valley et al., 2009). Distinctive microbial communities are associated with gills, digestive glands, and gonads (Hernández-Zárate and Olmos-Soto, 2006), as well as stomach and guts of oysters (King et al., 2012, Trabal Fernández et al., 2014, Chauhan et al., 2014). Increasingly, these efforts have focused on understanding the relationship between nitrogen transforming microorganisms and macrofauna (Wahl et al., 2012, Steif, 2013, Mouton et al., 2016).

In addition to harboring diverse prokaryotic and eukaryotic communities, oysters and similar bivalve filter feeders sequester organic matter and nutrients, potentially ameliorating the negative effects of phytoplankton blooms caused by anthropogenic nutrient pollution (Newell et al., 2005). However, ammonium excretion can recycle some of the nitrogen to the water column, where it is available to support additional algal growth (Boucher and Boucher-Rodini, 1988, Mazouni, 2004). Two key nitrogen transformations, nitrification and denitrification, have also been examined in bivalve communities. Nitrification is the sequential oxidation of ammonium to nitrite and then nitrite to nitrate. In estuarine and marine environments, nitrification often limits denitrification, a process during which nitrate is reduced to dinitrogen gas (Jenkins and Kemp, 1984, Seitzinger, 1988). Denitrification is a particularly important process because it removes fixed nitrogen from the ecosystem. Nitrate release from oysters and scallops cultured on ropes is evidence of nitrification associated with bivalves (Mazouni, 2004, Richard et al., 2007). In addition, significant rates of nitrification and denitrification in a variety of bivalve communities have been measured (Welsh and Castadelli, 2004, Svenningsen et al., 2012, Heisterkamp et al., 2013, Kellogg et al., 2013, Smyth et al., 2013,

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Welsh et al., 2015). While there have been many studies focused on examining whether sediments with bivalve biodeposits stimulate denitrification (Newell et al., 2005), results are mixed. Some studies find enhancement adjacent to reefs (Piehler and Smyth, 2011, Smyth et al., 2013, Kellogg et al., 2014), while others do not (Nizzoli et al., 2006, Higgins et al., 2013, Mortazavi et al., 2015). Thus, oysters themselves may have the greatest impact on nitrification and denitrification, rather than sediments adjacent to oyster reefs.

Oysters generate a continuous supply of ammonium through excretion. Therefore, we hypothesized that nitrogen transformations associated with living oysters would be greater than on oyster shells only. To test this hypothesis, we compared the rates of nitrification, denitrification, ammonium excretion and respiration by living oysters and their associated microbial communities with biofilms on shells of recently dead oysters (referred to as shell). We examined nitrification rates and ammonium fluxes in two different species *Crassostrea virginica* and *Crassostrea gigas* and their shells to see if there were species level differences. Denitrification and respiration were only measured on *C. virginica*.

2. Methods

2.1. Experimental design

We collected live oysters, oyster shells and water from Santa Rosa Sound in Pensacola Bay, Florida in October 2011, June 2012, June 2013 and July 2013 and from Tomales Bay, California in September 2011. Subtidal oysters, *C. virginica*, were collected from a pier in Santa Rosa Sound, Florida and are referred to as Pensacola Bay. Hog Island Oyster Company donated aquaculture grown oysters, *C. gigas*, from Tomales Bay. Oysters were transported to the lab in water from their field site and experiments began within 4 h of collection for batch experiments and within 24 h of collection for flow-through experiments. We selected the number of oysters and shells so that each replicate contained approximately the same cross sectional area, thus the numbers ranged from 1 to 6 individuals per replicate (Table 1). All incubations were conducted in the dark to minimize the effect of attached algae on nitrogen transformations.

Whole, unfiltered water from the collection site was used to incubate oysters or shell for all experiments. Two different types of experiments were conducted. Batch experiments measuring nitrification and nutrient fluxes were conducted on all dates except July 2013. For the batch experiments, oysters, shells or water only controls were incubated at 23 °C in 1 L of water amended with NH_4^+ to a final concentration of 10 μM with aeration using aquarium pumps. Water samples were collected from ambient water and from experiments at 0 and 24 h and analyzed for NO_2^-, $NO_3^- + NO_2^-$ and NH_4^+. All treatments were replicated in triplicate, except for Tomales Bay, which had 4 replicates per treatment. We conducted flow-through experiments to measure nitrification, denitrification, nutrient and oxygen fluxes in 2013. Oysters or shell were incubated in gas-tight plexiglass core tubes held in an environmental chamber at *in situ* temperature. Core tube diameter was 9.5 cm and length was 24.5 cm. In June 2013 following batch experiments, live oysters and shell were transferred to tubes and incubated in a flow-through system. A tube with ambient water only was the control. Water was amended with $^{15}NO_3^-$ (98 at.%) to a final concentration of 20 μM. Flow rate was 0.18 L h^{-1}. In July 2013, we incubated live oysters at two nitrate concentrations, either 10 or 40 μM $^{15}NO_3^-$ (98 at.%) in triplicate at a similar flow rate as the June experiment to examine the effect of different NO_3^- concentrations on denitrification rates. A water-only control at each NO_3^- concentration was incubated alongside the oyster treatments. Triplicate samples of inflow and outflow water from each plexiglas tube were collected after a 24 h stabilization period. We used photographs of oysters and shells to determine cross sectional area and shell height. Shell height was measured as the length of the oyster from umbo to the margin of the valve.

2.2. Analytical methods

Ammonium concentration was measured fluorometrically using an *o*-phthaldialdehyde and borate buffer reagent (Holmes et al., 1999). Nitrate + nitrite concentrations were measured using cadmium reduction to nitrite with subsequent addition of sulfanilamide and N-1 naphthyl ethylenediamine dihyrochloride (Jones, 1984). Water samples were collected in glass vials with no headspace for analyses of dissolved oxygen, $^{29}N_2$ and $^{30}N_2$. N_2 concentrations and isotopic ratios in the overlying water were determined with a quadrupole mass spectrometer equipped with a flow-through silicone capillary membrane (Kana et al., 1994), a furnace to remove oxygen (Eyre et al., 2002) and a Channeltron/Faraday secondary electron multiplier. Dissolved oxygen concentrations were measured with Unisense oxygen microelectrodes calibrated according to the manufacturer. The isotopic composition of $^{15}NO_3^- + {}^{15}NO_2^-$ was determined after conversion to N_2O (Sigman et al., 2001) at the UC Davis Stable Isotope Facility. Before analysis, $^{15}NO_3^- + {}^{15}NO_2^-$ samples were diluted with $^{14}NO_3^-$ so that enrichment was <10 at.%.

2.3. Calculations and statistics

Nitrogen transformation rates were calculated per unit of shell cross sectional area. Nitrification rates in the batch experiments were calculated as the production of $NO_3^- + NO_2^-$ during the 24 h incubation period. Because NH_4^+ concentrations were amended to 10 μM, batch experiments are closer to potential nitrification than *in-situ* rates. For all flow-through experiments, oxygen, nutrient and N_2 fluxes were measured as the difference between effluent and influent concentrations multiplied by the flow rate. To determine the contribution of oysters or shell, water only fluxes were subtracted from oyster or shell fluxes. Water only fluxes were <5% of the oyster fluxes for denitrification. These values were divided by the shell cross sectional area (e.g. Eq. 1)

Table 1
Shell cross sectional area, shell height of oysters and shell, and average number of oysters used in each experiment. Mean ± S.E.

Estuary	Treatment	Date	Experiment type	Shell area (cm^2)	Shell height (mm)	Average number of oysters or shell
Tomales Bay	*C. gigas*[a]	Sep 2011	Batch	81.1 ± 12.3	79.2 ± 4.2	1.75
	Shell[a]			74.1 ± 6.2	45.6 ± 3.4	4.5
Pensacola Bay	*C. virginica*[b]	Oct 2011	Batch	37.7 ± 3.6	75.5 ± 8.0	1
	Shell[b]			58.2 ± 8.4	67.8 ± 14.9	2
Pensacola Bay	*C. virginica*[b]	Jun 2012	Batch	63.8 ± 8.9	79.5 ± 15.2	1.3
	Shell[b]			41.4 ± 9.2	82.8 ± 9.8	1
Pensacola Bay	*C. virginica*[b]	Jun 2013	Batch & Flow through	78.9 ± 4.0	82.6 ± 16.3	2.3
	Shell[b]			62.1 ± 3.8	71.5 ± 12.3	2
Pensacola Bay	*C. virginica*[c]	Jul 2013	Flow through	106.1 ± 15.2	95.0 ± 8.6	1.3

[a] n = 4.
[b] n = 3.
[c] n = 6.

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to determine the rate per shell area. Nitrification rate (R) was calculated as the isotopic dilution of $^{15}NO_x^-$ as in Rysgaard et al. (1993)

$$R = \left[\frac{C_{NOx}(e-i)}{(0.366-e)}\right] \cdot \frac{FR}{shell\_area} \quad (1)$$

where C_{NOx} is the $NO_3^- + NO_2^-$ (NO_x^-) concentration in inflowing water, e is the $^{15}NO_x^-$ fraction in effluent, i is the $^{15}NO_x^-$ fraction in influent, 0.366 is the background ^{15}N content, FR is the flow rate, and $shell\_area$ is the cross sectional area of oysters (or shell) in each core tube.

Denitrification rates were calculated according to the isotope pairing technique (Neilsen, 1992)

$$D_{15} = (^{14}N^{15}N) + 2(^{15}N^{15}N) \quad (2)$$

$$D_{14} = \left[\frac{^{14}N^{15}N}{2(^{15}N^{15}N)}\right] D_{15} \quad (3)$$

where D_{15} is the denitrification based on $^{15}NO_3^-$, $^{14}N^{15}N$ is the flux of $^{29}N_2$, $^{15}N^{15}N$ is the flux of $^{30}N_2$, and D_{14} is the denitrification based on $^{14}NO_3^-$. Total denitrification is $D_{15} + D_{14}$.

We used *t*-tests and ANOVA to test for differences in nitrification rates between oyster and shell treatments with R (Table 2) using rank order nitrification data with R (R core Team, 2015). *t*-Tests were used for Tomales Bay batch and the June flow-through experiments. We used an ANOVA for Pensacola Bay batch experiments (October 2011, June 2012, June 2013) since these experiments were conducted on the same species and using the same technique to look for significance between treatments, date and their interaction. Significant factors were tested using a Tukey HSD. Linear regression analyses of denitrification rate as a function of nitrate concentration were conducted for denitrification normalized to the shell cross sectional area.

3. Results

Overall, nitrification rates were similar among our experimental treatments (living oysters versus shell only) and among different species (*Crassostrea virginica* and *Crassostrea gigas*), ranging from near zero to 112 nmol $cm^{-2}_{(shell\ area)}\ d^{-1}$ (Fig. 1). Nitrification rates did not significantly differ between living oysters and oyster shell ($p > 0.07$) (Fig. 1, Table 2). However, date was significant with rates in October 2011 being significantly lower than in 2013 (Tukey HSD, $p = 0.02$). Rates were also similar among batch experiments where nitrification was measured via the accumulation of $NO_3^- + NO_2^-$ and flow-through experiments where nitrification was measured as the isotope dilution of $^{15}NO_3^-$ (Fig. 1).

In contrast to essentially equivalent nitrification rates, denitrification rates associated with living oysters were over three times greater than those of empty oyster shells, 269 ± 37 nmol $cm^{-2}_{(shell\ area)}\ d^{-1}$ and 74 ± 17 nmol $cm^{-2}_{(shell\ area)}\ d^{-1}$, respectively (Fig. 2, Table 3). Additionally, denitrification rates were consistently greater than nitrification rates. On average, denitrification was 3.6 times greater than nitrification with living oysters, and 3.3 times greater than with empty oyster shells.

The fluxes of nitrite and ammonium were an order of magnitude higher on oysters than in shell in both flow-through (Table 3) and batch experiments (Table 4). Ammonium fluxes were similar between *C. gigas* (1121 ± 100 nmol $cm^{-2}_{(shell\ area)}\ d^{-1}$) and *C. virginica* measured in batch (1415 ± 230 nmol $cm^{-2}_{(shell\ area)}\ d^{-1}$) or flow-through (1232 ± 385 nmol $cm^{-2}_{(shell\ area)}\ d^{-1}$) experiments. Oxygen consumption in oyster treatments was about double that of shell treatment (Table 3).

Table 2
Statistical analyses comparing nitrification rate in oyster versus shell (Treatment) and date for *C. gigas* and *C. virginica* from batch and flow through experiments.

Species	Type of experiment	Test	Contrast	p Value
C. gigas	Batch	*t*-test	Treatment	0.43
C. virginica	Batch	ANOVA	Treatment	0.07
			Date	0.03
			Treatment × Date	0.07
C. virginica	Flow through	*t*-test	Treatment	0.57

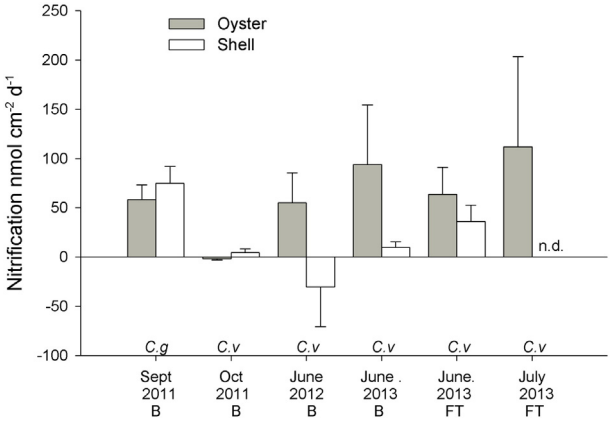

Fig. 1. Nitrification rate of living oysters or oyster shell from Tomales Bay (C.g. = *Crassostrea gigas*) or Pensacola Bay (C.v. = *Crassostrea virginica*). Rates (nmol N $cm^{-2}_{(shell\ area)}\ d^{-1}$) based on accumulation of $NO_3^- + NO_2^-$ in batch experiments (B) or isotopic dilution of ^{15}N-NO_3^- (FT) in flow-through experiments. Mean + S.E., n = 4 (Tomales Bay), n = 3 (Pensacola Bay). n.d. no data.

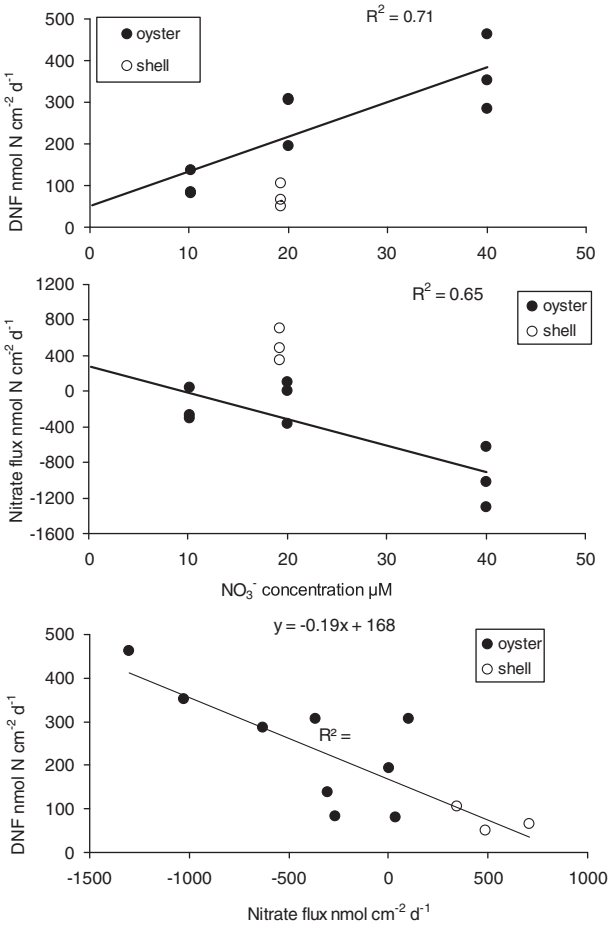

Fig. 2. Denitrification rates (DNF) (top panel) and nitrate flux (middle panel) in live *Crassostrea virginica* and oyster shell (nmol N $cm^{-2}_{(shell\ area)}\ d^{-1}$) at varying NO_3^- concentrations (µM). Denitrification versus nitrate flux (bottom panel).

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Table 3
Fluxes of nitrite, nitrate + nitrite, ammonium, and oxygen and denitrification rates (nmol $cm^{-2}_{(shell\ area)}\ d^{-1}$) at varying nitrate concentrations (μM) in flow-through experiments.

Measurement	*C. virginica*	Shell	*C. virginica*	*C. virginica*
Date	Jun 2013	Jun 2013	Jul 2013	Jul 2013
NO_3^- concentration μM	20	20	10	40
NO_2^- flux	71.1 ± 22.9	4.4 ± 2.7	4.9 ± 6.0	92.8 ± 17.1
$NO_3 + NO_2$ flux	−88 ± 143	515 ± 106	−179 ± 108	−985 ± 194
NH_4^+ flux	1391 ± 133	180 ± 164	500 ± 86	1806 ± 623
O_2 flux	−6884 ± 214	−3570 ± 276	−6591 ± 475	−5629 ± 560
Denitrification	269 ± 37	74 ± 17	100 ± 19	367 ± 52

Mean ± S.E., n = 3.

4. Discussion

Our nitrification results are consistent with studies of other benthic bivalves that measured significant nitrification in their tissues or on their shells (Welsh and Castadelli, 2004, Svenningsen et al., 2012, Heisterkamp et al., 2013, Welsh et al., 2015). While it is difficult to make a direct comparison among these studies, Welsh and Castadelli (2004) found that potential nitrification on interior and exterior surfaces combined were 37.2 and 42.3 nmol $cm^{-2}_{(surface\ area)}\ d^{-1}$ for *Tapes philippanarum* and *Mytilus galloprovicialis*, respectively. Despite the fact that they normalized to surface area while we normalized to cross sectional area, their values are within the range of our values of 75 and 15 nmol $cm^{-2}_{(shell\ area)}\ d^{-1}$ for shells of *C. gigas* and *C. virginica*, respectively (Fig. 1). They found that specific activity associated with the shells represented about 50% of the total activity associated with these bivalves.

The three-fold increase in denitrification relative to nitrification in our experiments was similar to those from netted dog welks, *Hinia reticulate*, but different than the mollusks *Mytilus edulis* and *Littorina littorea* (Heisterkamp et al., 2013). In *Mytilus* and *Littorina,* nitrification and denitrification were equally important in N_2O production with no consistent difference between live animals and empty shells (Heisterkamp et al., 2013). There are also species specific differences in coupled nitrification-denitrification (D_n), with D_n representing 95% of total denitrification in sediments with *Macoma baltica* (Bonaglia et al., 2014), but only 30% of total denitrification on rope culture of *M. galloprovincialis* (Nizzoli et al., 2006). While we did not observe consistent differences in nitrification between *C. virginica* and *C. gigas*, species or habitat specific differences may be important.

In the flow-through experiments with nitrate additions, denitrification and nitrate uptake were proportional to nitrate concentrations (Fig. 2). Denitrification represented about 20% of the total nitrate uptake. This is similar to observations from marine sediments (Dong et al., 2009, Hou et al., 2012), but somewhat lower than measurements from rope culture of *M. galloprovincialis* or sediments with *T. philippinarum* where denitrification was about 25 to 33% of nitrate uptake (Nizzoli et al., 2006).

4.1. Implications for system level N removal

Oyster abundances are variable with generally high abundance historically in the mid-Atlantic and Southeastern US (Dame, 1979, Luckenbach et al., 2005, Zu Ermgassen et al., 2012) and low abundance in newly restored sites (Kellogg et al., 2013) or in the northern Gulf of Mexico (Zu Ermgassen et al., 2012, Brown et al., 2014). Sediment and bivalve-associated denitrification are rarely measured at the same time and place. In a mesocosm study, oysters and oysters plus sediments were a source of N_2, suggesting that net denitrification occurred when oysters were present, while nitrogen fixation occurred in the bare sediments (Smyth et al., 2013). Denitrification from Manila clams (*Ruditapes philippinarum*) alone was 70% of incubations with sediments plus clams (Welsh et al., 2015). In contrast, denitrification rates were similar between sediments with *Macoma baltica* and those without while rates of dissimilatory nitrate reduction to ammonium were higher in sediments with *M. baltica* (Bonaglia et al., 2014). The most comprehensive study examining denitrification was conducted at an oyster restoration site using an ex-situ approach (Kellogg et al., 2013). At the site, which included a mixture of oysters, other invertebrate species and sediments, denitrification rates were 12 times greater than in bare sediment (Kellogg et al., 2013). If we calculate our rates per individual oyster and then scale to an average oyster abundance of 130 oysters m^{-2} (Kellogg et al., 2013), denitrification would be 40.6 μmol $m^{-2}\ h^{-1}$, 1, oxygen consumption would be 2320 μmol $m^{-2}\ h^{-1}$, while ammonium flux would be 444 μmol $m^{-2}\ h^{-1}$. These rates are similar to those measured on oyster reefs (Dame et al., 1989, Dame et al., 1992) or in experimental manipulations that included oysters (Kellogg et al., 2013, Smyth et al., 2013).

Our results support the theory that oyster reefs are hot spots for denitrification within estuaries. Enhanced nitrification and denitrification on healthy oyster reefs may provide a key pathway to the ultimate removal of fixed nitrogen from estuarine environments. Thus, restoration of oyster reefs has the potential to provide ecosystem services beyond those inherent in the physical structure they provide or the food web they support or impact (zu Ermgassen et al., 2013, Kellogg et al., 2014). This benefit might not be limited to oysters, but could include other epibenthic (because oxygen is required for nitrification) filter feeders, for example mussels or other epibenthic bivalves. Although our understanding of the impacts to ecosystem services provided by oyster reefs and our ability to predict their responses to stressors and natural or anthropogenic disturbances is incomplete, this study reveals the key role that nitrifiers and denitrifiers directly associated with living oysters can play in the removal of fixed nitrogen from estuaries. Understanding the environmental conditions that favor nitrogen removal and accounting for all ecosystem components contributing to nitrogen removal from coastal ecosystems is critical for modeling these processes and for improving future nutrient management strategies.

Table 4
Fluxes of nitrite, nitrate + nitrite, ammonium (nmol $cm^{-2}_{(shell\ area)}\ d^{-1}$) from batch experiments. Mean ± S.E., n = 4 (Tomales Bay) n = 3 (Pensacola Bay).

Estuary	Treatment	Date	NO_2^-	$NO_3^- + NO_2^-$	NH_4^+
Tomales Bay	*C. gigas*	Sep 2011	22.2 ± 3.8	58.4 ± 14.8	1121 ± 100
	Shell		16.7 ± 3.5	75.0 ± 17.1	−20 ± 22
Pensacola Bay	*C. virginica*	Oct 2011	4.0 ± 0.9	−1.7 ± 1.2	1570 ± 261
	Shell		−0.4 ± 0.4	4.4 ± 4.0	−6 ± 5
Pensacola Bay	*C. virginica*	Jun 2012	40.7 ± 22.6	55.3 ± 30.1	961 ± 63
	Shell		9.9 ± 4.6	−30.2 ± 40.5	434 ± 116
Pensacola Bay	*C. virginica*	Jun 2013	28.3 ± 11.0	94.0 ± 60.4	1713 ± 339
	Shell		1.4 ± 0.5	9.8 ± 5.5	11 ± 167

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