

Benthic nutrient fluxes and limited denitrification in a sub-tropical groundwater-influenced coastal lagoon

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ABSTRACT: Benthic processes in estuarine systems can alleviate nutrient over-enrichment by removing nitrogen (N) via denitrification, or further intensify eutrophication by supplying N back to the water column. The main objectives of this study were to (1) examine the magnitude and seasonality of benthic fluxes and denitrification, and (2) determine the relative importance of benthic versus submarine groundwater discharge (SGD) N fluxes in Little Lagoon, a shallow, sub-tropical, groundwater-influenced coastal lagoon in the North Central Gulf of Mexico. In the summer, when low water column nitrate (NO_3^-) concentration ($<0.2 \mu\text{M}$) occurred in tandem with high hydrogen sulfide concentration ($>3000 \mu\text{M}$), rates of net N_2 fixation ($61.7 \pm 4.5 \text{ SE } \mu\text{mol N m}^{-2} \text{ h}^{-1}$) exceeded rates of net N_2 efflux ($51.6 \pm 4.9 \text{ SE } \mu\text{mol N m}^{-2} \text{ h}^{-1}$). Unlike many estuarine systems where denitrification is coupled to nitrification, sulfidic sediments caused denitrification to primarily be supported by water column NO_3^- in Little Lagoon. Gene copy numbers indicated that markers for N_2 fixation, sulfate reduction, and dissimilatory nitrate reduction to ammonium were more abundant than markers for denitrification and nitrification. SGD N fluxes were up to 42 times larger than the benthic rates, and both the benthos and SGD provided dissolved organic nitrogen to the water column. This study presents evidence of limited benthic N fluxes in supplying N to the water column compared to input associated with SGD.

KEY WORDS: Nitrogen cycle · Denitrification · Benthic flux · Sediment · Hydrogen sulfide · Submarine groundwater discharge

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INTRODUCTION

Eutrophication is of particular concern in coastal marine ecosystems because these ecosystems serve as nursery grounds for many commercially and recreationally important marine resources (Howarth et al. 2011). Eutrophication has been mainly attributed to increases in nutrient concentrations in surface waters; however, there is increasing evidence for submarine groundwater discharge (SGD) as an im-

portant nutrient source contributing to high productivity (Carruthers et al. 2005, Santos et al. 2013) and eutrophication in estuarine waters.

Once assimilated by primary producers, nutrients delivered to estuaries can be utilized by higher trophic levels, exported, and/or remineralized. Remineralization returns nutrients to the ecosystem and can contribute to intensification of eutrophication. Alternatively, processes in the sediments, such as denitrification, a microbially-mediated reduction of nitrate

(NO_3^-) to N_2 gas under anoxic conditions, can remove nitrogen (N) and reduce the available N to primary producers. Denitrification, by removing 50 to 75% of estuarine N loading, offsets the effects of nutrient over-enrichment (Burgin & Hamilton 2007). Understanding the spatial and temporal variability of benthic N cycling and particularly denitrification is critical for understanding the benthos' influence on estuarine productivity.

Factors such as the availability of organic matter (OM) and NO_3^- , temperature, and the abundance and activity of denitrifying microbes influence denitrification (Knowles 1982). Given abundant OM, NO_3^- , and an active microbial community, temperature exerts a strong control on denitrification rates (Seitzinger 1988). Summertime rates of denitrification in estuaries along the eastern seaboard of the United States can be as high as $400 \mu\text{mol N m}^{-2} \text{h}^{-1}$ (LaMontagne et al. 2002), and can exceed winter rates. This seasonal pattern of high denitrification rates in the summer and low rates in the winter holds for temperate estuaries as well (Ferguson & Eyre 2007). In sub-tropical estuaries, however, there appears to be no consistent relationship between temperature and denitrification rates. While in some systems, denitrification rates were higher during warmer months (An & Gardner 2002) and exceeded winter rates, in other systems, the opposite pattern has been observed (Gardner & McCarthy 2009). This indicates that in some sub-tropical systems, other biogeochemical factors such as OM availability, NO_3^- and/or hydrogen sulfide (H_2S) concentration (Joye & Hollibaugh 1995), or the abundance and activity of denitrifying microbes, rather than temperature influence denitrification rates.

Relatively few studies have investigated denitrification in SGD dominated ecosystems or the relative importance of SGD-derived nutrient fluxes compared to benthic nutrient fluxes. Typically, studies investigating SGD focus on discharge or seepage estimates, nutrient concentrations, or tracer techniques used to partition allochthonous inputs (Slomp & Van Cappellen 2004, Cole et al. 2006) but omit comparisons to benthic fluxes. Studies investigating benthic fluxes and denitrification seldom include a SGD-derived nutrient flux component (Eyre & Ferguson 2002, Gardner & McCarthy 2009, Mortazavi et al. 2012). The concurrence of the 2 is especially important to understand in estuaries where SGD is a dominant nutrient source, and in those that lack riverine input.

We measured rates of potential benthic N-fixation and denitrification, net N_2 flux, sediment oxygen demand, sediment sulfide profiles, a suite of key

functional genes, and SGD-derived nutrient fluxes in Little Lagoon, a groundwater-influenced subtropical coastal lagoon. Located in an agricultural region that experiences high population growth, groundwater in the area has elevated NO_3^- (Dowling et al. 2004), and the lagoon experiences episodic harmful algal blooms (MacIntyre et al. 2010). Our objectives were (1) to examine the magnitude and seasonality of benthic fluxes and denitrification, and (2) to determine the relative importance of benthic versus SGD N fluxes. We hypothesized that denitrification rates would be higher in the warmer summer months, and rates of SGD-derived N would exceed benthic fluxes.

MATERIALS AND METHODS

Site description

Little Lagoon, Alabama ($30^\circ 15' \text{N}$, $87^\circ 45' \text{W}$), is a shallow sub-tropical, groundwater-influenced system connected to the Gulf of Mexico by a narrow inlet. It is approximately 12.6 km long, over 1 km wide at its widest point, has an average water depth of ~ 1.5 m, a mean water residence time of approximately 10 d (Su et al. 2012), and sandy sediments.

Sample collection

Starting in November 2010, 3 sites (East, Mouth, and West) were sampled nearly monthly for 1 yr. Temperature and salinity were measured with a 556 multiprobe system (YSI). Water column samples were filtered ($0.7 \mu\text{m}$) and frozen prior to dissolved inorganic nitrogen (DIN: NH_4^+ , NO_2^- , and NO_3^-) and PO_4^{3-} analysis with a Skalar SAN⁺ autoanalyzer (precision: $\text{NO}_x = 0.005$, $\text{NO}_2^- = 0.004$, $\text{NH}_4^+ = 0.13$, $\text{PO}_4^{3-} = 0.03$; detection: NO_x and $\text{NH}_4^+ = 0.20$, $\text{NO}_2^- = 0.03$, $\text{PO}_4^{3-} = 0.07 \mu\text{M}$). Chlorophyll a (chl a) content from the top 1 cm of sediments was determined in triplicate with a Turner Designs 700 fluorometer after 90% acetone cold extraction for 24 h (Welschmeyer 1994).

Net N_2 and benthic fluxes

Triplicate intact polycarbonate sediment cores (33×9.5 cm i.d.) were collected from each site, capped, and set up in a flow through system under darkness in an environmental chamber at *in situ* temperature.

Filtered (0.7 μm) site inflow water flowed at a rate of 0.08 l h⁻¹ to the 5 cm of overlying water above the sediment surface and exited through an outflow tube. These cores were equilibrated over a 24 h period prior to sample collection. Triplicate discrete inflow and outflow samples were filtered and frozen immediately, and analyzed as described above for nutrient analyses. Total dissolved N (TDN) was measured as NO₃⁻ after persulfate oxidation (Valderrama 1981).

Separate triplicate samples were collected per core by overflowing 12 ml Exetainer vials by 2 tube-volumes, and adding 50% (w/v) ZnCl₂ solution prior to dissolved N₂ and Ar measurements by membrane inlet mass spectrometry (MIMS) (Kana et al. 1994). Atmosphere equilibrated standard water was run every 9 samples, and standard gas concentrations were determined according to Hamme & Emerson (2004). The MIMS was equipped with a copper reduction column set at 600°C to remove oxygen (Eyre et al. 2002). Positive N₂ fluxes indicate denitrification rates exceed N fixation, and negative N₂ fluxes indicate N fixation is greater than denitrification (An & Joye 2001). Error was determined as standard error of the triplicate core measurements. The reported denitrification estimates could include the contribution of anammox to N₂ production. Although anammox was not directly measured during this study, it is expected to be a minor contributor to overall N₂ production in the lagoon, as observed in tropical shallow water systems (Dong et al. 2011). Recent simultaneous measurements of anammox and denitrification in Little Lagoon have indicated that anammox contributes to <5% of the overall N₂ flux (R. J. Bernard unpubl. data).

Sediment oxygen demand (SOD) was measured from oxygen concentrations in inflow and outflow water analyzed with a microelectrode and a Unisense® multimeter analyzer. Benthic fluxes ($\mu\text{mol m}^{-2} \text{h}^{-1}$) were determined from the flow rate and normalized to sediment surface area (Lavrentyev et al. 2000); positive fluxes indicate release from the sediments and negative fluxes indicate uptake by the sediments.

Oxygen and hydrogen sulfide profiles

Oxygen (O₂) and H₂S microelectrodes were calibrated according to the manufacturer's instructions and were used to determine the depth of the oxic layer and the concentration of H₂S (measured as HS⁻ concentration, [HS⁻]) from duplicate intact sediment cores (17.5 × 9.5 cm i.d.) collected at each site.

DNA extraction and quantitative PCR

DNA was extracted from ~1 g of sediment from samples collected in April, August, September and November 2011 following a modified phenol:chloroform method (Wilson 1987). Gene copy numbers were determined with an Agilent Stratagene MX3500P quantitative PCR instrument and GoTaq qPCR Master Mix (Promega). All qPCR reactions were performed in 20 μl reactions, which contained 1 μl template, 0.5 μM of each primer, 0.25 mg ml⁻¹ BSA and an additional 1.25 mM MgCl. Genes quantified included bacterial *amoA* (Rotthauwe et al. 1997; normal 3 steps, 95°C 5 min, 40 cycles of 95°C 30 s, 57°C 1 min, 72°C 1 min), archaeal *amoA* (Francis et al. 2005; normal 3 steps, 95°C 5 min, 40 cycles of 95°C 30 s, 53°C 1 min, 72°C 1 min), *nirK* (Hallin & Lindgren 1999; normal 3 steps, 95°C 5 min, 40 cycles of 95°C 30 s, 58°C 1 min, 72°C 1 min), *nirS* (Throback et al. 2004; normal 3 steps, 95°C 5 min, 40 cycles of 95°C 30 s, 57°C 1 min, 72°C 1 min), *dsrB* (Wagner et al. 1998; fast 2 steps, 95°C 5 min, 40 cycles of 95°C 30 s, 58°C 40 s), *nifH* (Poly et al. 2001; normal 3 steps, 95°C 5 min, 40 cycles of 95°C 30 s, 61°C 1 min, 72°C 1 min), and *nrfA* (Mohan et al. 2004; normal 3 steps, 95°C 5 min, 40 cycles of 95°C 30 s, 60°C 1 min, 72°C 1 min). For each gene, a standard curve was generated from purified plasmids containing the target of interest. The standard curve was included on each plate in triplicate, and each sample was also analyzed in triplicate. Amplification efficiencies for the qPCR analyses ranged from 77.2% for *dsrB* to 94.3% for the bacterial *amoA* (mean = 86.3% ± 6.8). The R² of the standard curves ranged from 0.987 to 0.997 (mean = 0.994 ± 0.004 [SE]). For each sample, gene numbers were converted to copies g⁻¹ wet sediment, and the average copy number was calculated from 3 technical replicates. For each assay, the dissociation curve was checked to ensure that only signals from targets were being detected. No non-specific amplification was observed for any of the genes quantified.

Experiments for potential rates of N₂ fixation, nitrification, and denitrification

During September and November 2011, potential rates for N₂ fixation and denitrification were measured in triplicate using 20 g homogenized sediment from the top 5 cm and filtered site water (headspace to sample ratio: 15:55 ml and 25:45 ml for potential denitrification and N₂ fixation, respectively). Potential N₂ fixation rates were measured as ethylene

(C₂H₄) production from acetylene (C₂H₂) reduction (Welsh et al. 1996). Rates of N₂ fixation by sulfate reducing bacteria (SRB) were determined after the addition of sodium molybdate as a specific inhibitor of the sulfate reduction process (Capone 1993). After C₂H₄ analysis on a Shimadzu gas chromatograph (GC-2014) with flame ionization detection (GC-FID), production rates of C₂H₄ were converted to potential N₂ fixation rates using a C₂H₂:N₂ reduction ratio of 3:1 (Capone 1993).

Potential nitrification rates were determined according to Henriksen et al. (1981). Potential denitrification rates were measured in filtered site water amended with potassium nitrate (KNO₃⁻) to a final concentration of 100 µM using the acetylene block technique (Sørensen 1978). After the addition of C₂H₂ (10% v/v) and a 1 h incubation, gas samples were injected into evacuated 12 ml Exetainer vials and N₂O production was quantified with a Shimadzu GC-2014 with an electron capture detector (GC-ECD) within 24 h. C₂H₂ reduction assays are simple to carry out and allow large numbers of samples to be run, which is necessary to constrain the high spatial and temporal variability of denitrification rates. Two major problems associated with this method include inhibition of nitrification, and incomplete inhibition of N₂O reduction, which are both time dependent (Seitzinger et al. 1993). Incubations were held to 1 h to minimize these effects. While it is recognized that C₂H₂ reduction assays may underestimate rates of N₂ fixation, and the inhibition of nitrification can lead to underestimates of denitrification, these potential rates can still be informative (Caffrey et al. 1993).

Radioisotope measurements

Radon in the water was analyzed over a 3 yr period (2010 to 2012) using an automated system (Burnett et al. 2001, Dulaiova et al. 2005). Surveys were conducted from a pontoon boat while maintaining underway speeds of less than 5 km h⁻¹. The multi-detector system used measured ²²²Rn from a constant stream of water passing through an air–water exchanger that distributes radon from the running water to a closed air loop. The air stream is fed to 3 commercial radon-in-air monitors (RAD-7, DurrIDGE) that measures the activity of ²²²Rn in the air stream via measurement of the short-lived (*t*_{1/2} = 3 min) α-emitting daughter, ²¹⁸Po. The radon activity in water was then calculated from the temperature and salinity-dependent solubility coefficient (Schubert et al. 2012). Continuous temperature and salinity meas-

urements were recorded with a Van Essen Instruments CTD Diver attached to the submersible pump used for delivering water to the air–water exchanger for radon analysis. These probes were calibrated prior to deployment for each field excursion.

Surface water samples were collected for radium isotope and nutrient groundwater analysis in Little Lagoon, and monitoring wells around the lagoon during several periods from 2010 to 2012 (Moore 2000). Radium isotopes were extracted onto Mn-fibers by standard techniques, analyzed for short-lived ²²³Ra and ²²⁴Ra via a RaDeCC (radium delayed coincidence counter) system (Moore & Arnold 1996), and analyzed for long-lived ²²⁶Ra and ²²⁸Ra via gamma spectrometry (Dulaiova et al. 2005). Data listings and additional details concerning the radioisotope analyses can be found in Su et al. (2012).

Submarine groundwater discharge evaluations

SGD was estimated from the radioisotope data using a 1-dimensional advection–diffusion model (Cable et al. 1997). The model was applied using relevant environmental parameters (water temperature, wind speed, etc.), and the measured radon and radium inventories, along with groundwater radioisotope activities from the wells were used to estimate isotope fluxes, and ultimately discharge. Assuming steady-state conditions, the inputs of radon and radium to the lagoon should originate as a combination of diffusion from sediments and advection of groundwater (or pore water). Losses, other than radioactive decay, should be dominated by atmospheric evasion for radon in an almost closed system such as Little Lagoon. Mixing losses were estimated by radium isotopic techniques (Moore 2000) and a tidal prism model (Monsen et al. 2002) and shown to be small relative to the other loss terms. After determining the radioisotope fluxes needed to balance the observed inventories and calculated losses, groundwater fluxes were assessed by dividing these fluxes by the measured groundwater end-member isotope activities.

Statistical analysis

All data were tested for ANOVA assumptions, and if data could not be transformed to meet assumptions, nonparametric tests were used. Abiotic factors, net N₂ and benthic fluxes, and potential rates were analyzed by 2-way ANOVA with month and site as inde-

pendent variables. When significant differences occurred, the Holm-Sidak multiple comparison post hoc-test was used to determine significant interactions. Correlation analysis was used to examine the relationship between biogeochemical parameters and net N_2 flux. Statistical significance of the data sets was determined at $p < 0.05$, and error is reported as standard error. All statistical analyses were prepared with SigmaPlot 12 (Systat Software).

RESULTS

Water column parameters

Water temperature and salinity exhibited significant temporal differences ($p < 0.001$ for both). January and August 2011 were the coldest and warmest months (mean 6.8 and 33.0°C, respectively) (Fig. 1A). Salinity ranged from 25 PSU in November 2010 to 31 in August (Fig. 1B). Temperature and salinity were similar at the West and East sites ($p = 0.128$ and $p = 0.120$, respectively). However, temperatures at the Mouth were lower than at East and West sites in the lagoon ($p = 0.042$ and $p = 0.002$, respectively). The salinity at the Mouth and the West sites were similar ($p = 0.090$), while salinity at the East was lower than at the West ($p = 0.005$). Water column NH_4^+ concentrations ranged from 0.38 ± 0.10 to $7.80 \pm 2.90 \mu\text{M}$, but were not significantly different between sites ($p = 0.402$) or seasons ($p = 0.087$) (Fig. 1C). Water column NO_3^- concentration varied from 0.04 ± 0.01 to $0.59 \pm 0.15 \mu\text{M}$, with lower concentrations measured during summer (Fig. 1D) than winter ($p = 0.004$). In contrast, water column PO_4^{3-} concentrations were higher during summer than winter ($p = 0.050$) (Fig. 1E).

Benthic chl *a*

Benthic chl *a* content ranged from 30.2 ± 2.4 to $168.5 \pm 6.2 \text{ mg m}^{-2}$ (Fig. 1F) and was similar at the West and East Sites ($p = 0.322$) but lower at the Mouth ($p = 0.028$ and $p = 0.005$, respectively).

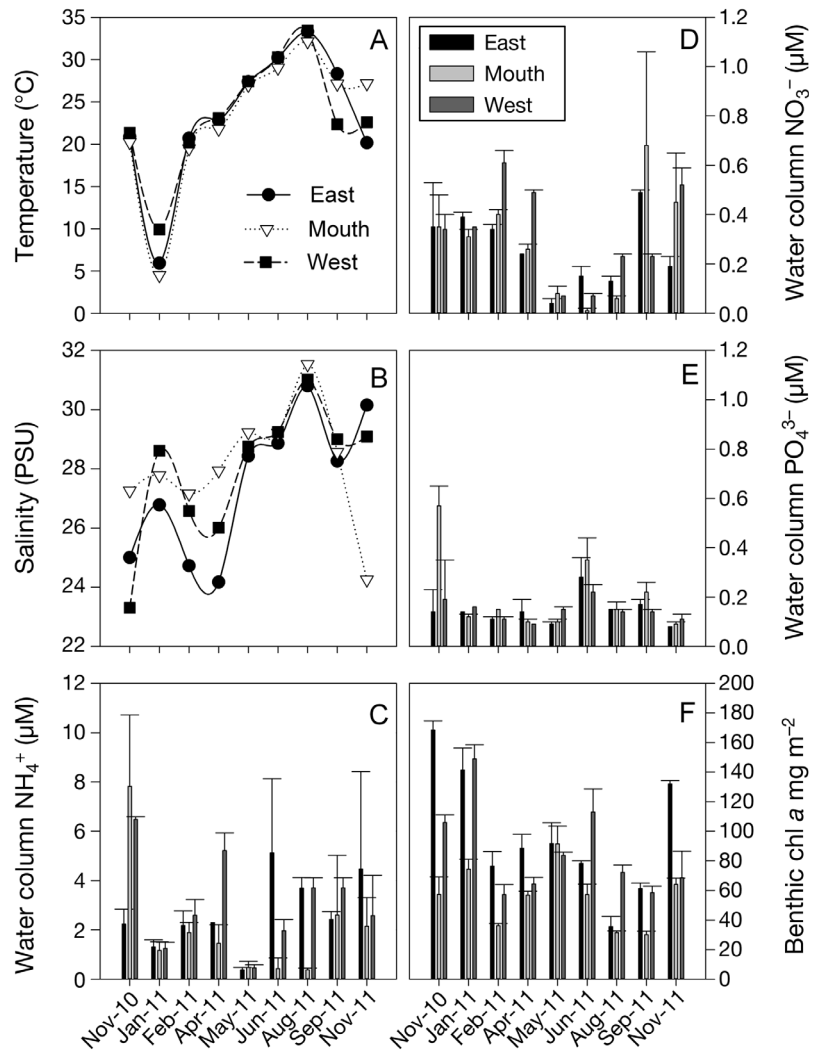


Fig. 1. (A) Temperature, (B) salinity, (C) water column ammonium, (D) nitrate, (E) phosphate concentrations, and (F) benthic chl *a* content, from November 2010 to November 2011 in Little Lagoon. Means are shown \pm SE

Higher benthic chl *a* values were observed during November 2010 and January 2011 (mean = 116.0 mg m^{-2}) and were higher ($p = 0.011$) than the values observed in August and September (mean = 48.2 mg m^{-2}). There was a negative correlation between chl *a* and temperature ($\rho = -0.322$), but only at $p = 0.0519$.

Sediment oxygen demand

SOD averaged in the lagoon ranged from 86 ± 34 to $780 \pm 150 \mu\text{mol O}_2 \text{ m}^{-2} \text{ h}^{-1}$ (Fig. 2) and exhibited significant ($p < 0.001$) temporal variation. At the East and Mouth sites SOD was highest in February,

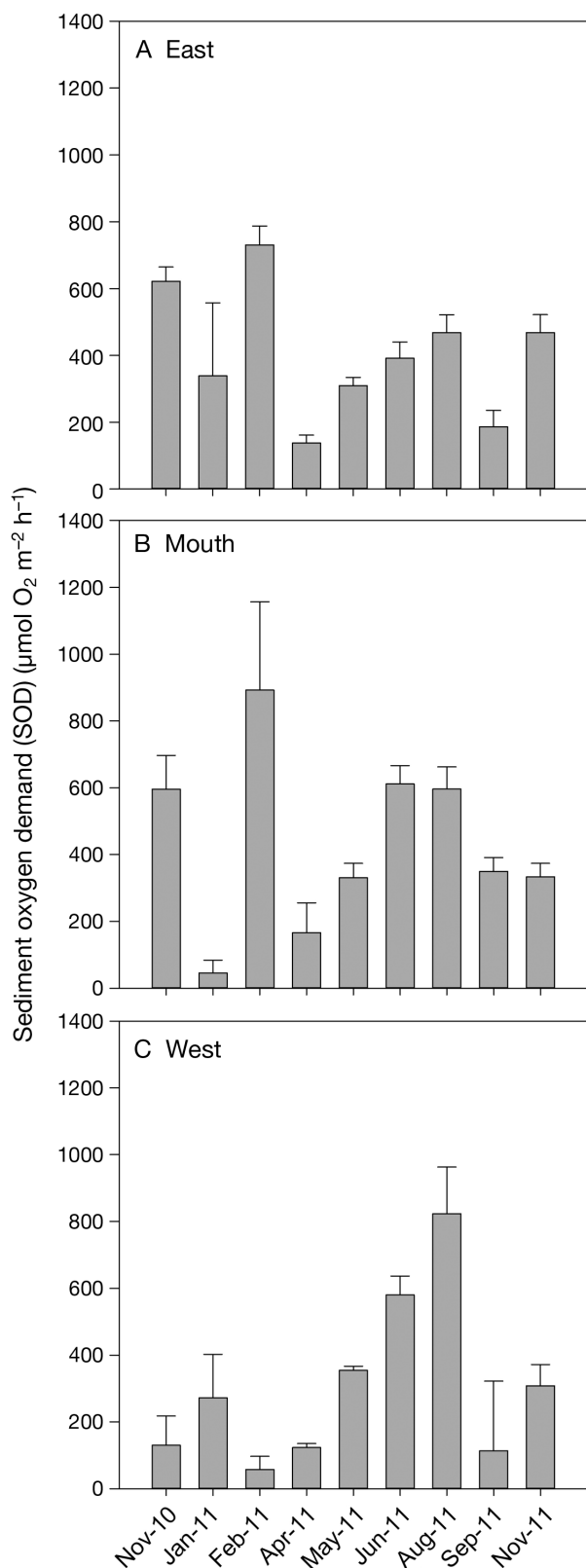


Fig. 2. Rates of sediment oxygen demand (SOD) ($\mu\text{mol O}_2 \text{ m}^{-2} \text{ h}^{-1} \pm \text{SE}$) at (A) East, (B), Mouth, and (C) West sites in Little Lagoon from November 2010 to November 2011

while at the West site SOD was highest during August. The lowest SOD occurred during April at the East and Mouth sites and during February at the West site. Although SOD did not differ between sites ($p = 0.128$), a significant interaction was found between month and site ($p < 0.001$), with greater SOD occurring when temperatures were low ($\rho = -0.464$, $p = 0.004$). SOD was positively correlated with water column NO_3^- concentration ($\rho = 0.578$, $p = 0.0002$). Integrated over the course of a year, the SOD in Little Lagoon was $102.5 \pm 6.5 \text{ g O}_2 \text{ m}^{-2} \text{ yr}^{-1}$ (Table 1).

Benthic fluxes of DIN, DON, and PO_4^{3-}

Dissolved organic nitrogen (DON) flux averaged at the sites ranged from -100.9 ± 20.0 to $404.8 \pm 179.5 \mu\text{mol N m}^{-2} \text{ h}^{-1}$ (Fig. 3A) and did not differ among sites ($p = 0.481$) or seasons ($p = 0.106$). Annually, the sediments were a DON source to the water column ($7.0 \pm 2.9 \text{ g m}^{-2} \text{ yr}^{-1}$) (Table 1), and were largely driven by the June 2011 flux. NH_4^+ was the largest component of the DIN flux, and ranged from -53.8 ± 13.0 to $202.0 \pm 87.2 \mu\text{mol N m}^{-2} \text{ h}^{-1}$ (Fig. 3B) averaged across the lagoon, but did not differ between sites ($p = 0.775$). NH_4^+ fluxes exhibited temporal variability ($p = 0.001$) with larger fluxes in the warm months and the greatest efflux overall in August. The benthic $\text{NO}_2^- + \text{NO}_3^-$ flux exhibited significant temporal variability ($p < 0.001$) and ranged from -62.0 ± 10.3 to $9.3 \pm 5.9 \mu\text{mol N m}^{-2} \text{ h}^{-1}$ (Fig. 3C). There were no differences in $\text{NO}_2^- + \text{NO}_3^-$ flux between the sites ($p = 0.085$). Generally $\text{NO}_2^- + \text{NO}_3^-$ efflux was observed during winter and uptake was observed during summer.

Benthic PO_4^{3-} fluxes were similar between sites ($p = 0.111$) and ranged from -2.40 ± 0.05 to $0.85 \pm 0.63 \mu\text{mol P m}^{-2} \text{ h}^{-1}$ (Fig. 3D). The PO_4^{3-} flux exhibited temporal variability ($p < 0.001$) with the largest efflux and uptake occurring during May and June, respectively. The June PO_4^{3-} uptake was different than the other measured fluxes ($p < 0.001$ all months).

Sediment hydrogen sulfide profiles

The lowest $[\text{HS}^-]$ in the sediments were observed in January at the East and Mouth sites ($0 \mu\text{M}$ to a depth of 10 mm for both sites) and in November 2010 at the West site ($289.32 \pm 0.22 \mu\text{M}$ at 10 mm) (Fig. 4). $[\text{HS}^-]$ was generally lower at the East site than elsewhere. The $[\text{HS}^-]$ increased with increasing temper-

Table 1. Yearly-integrated benthic exchange fluxes and submarine groundwater discharge (SGD) nutrient fluxes and concentrations (averages \pm standard error) from shallow (<3 m; $n = 32$) and deep (5 to 55 m, all but one >20 m; $n = 18$) wells around Little Lagoon (area = $10.52 \times 10^6 \text{ m}^2$). SGD estimates were based on data reported in Su et al. (2012) of 0.6 to $2.9 \text{ m}^3 \text{ s}^{-1}$. In order to calculate fluxes via SGD, nutrient concentrations were weighted 42% shallow, 58% deep based on estimates in Su et al. (2012) for relative amounts of each discharging into Little Lagoon. nd = no data. For N species and net N_2 , units are g N. For P and O_2 , units are g P and g O_2 , respectively. TDN: total dissolved N; DON: dissolved organic N; DIN: dissolved inorganic N; SOD: sediment oxygen demand

Parameter	NO_2	NO_3	NH_4	TDN	DON	DIN	PO_4	Net N_2	SOD
Benthic exchange fluxes ($\text{g m}^{-2} \text{ yr}^{-1}$)	-0.04 ± 0.004	-1.8 ± 0.14	3.7 ± 1.5	6.8 ± 3.4	7.0 ± 2.9	-0.17 ± 1.2	0.07 ± 0.05	-0.67 ± 0.74	102.52 ± 6.5
Range of SGD nutrient fluxes ($\text{g m}^{-2} \text{ yr}^{-1}$)	0.003–0.01	1–5	2–10	60–300	50–300	3–20	0.2–1	nd	nd
Concentration: shallow wells (μM)	0.18 ± 0.01	12 ± 1.3	82 ± 3	271 ± 6	177 ± 5	95 ± 3	5.5 ± 0.2	nd	nd
Concentration: deep wells (μM)	0.07 ± 0.01	61.2 ± 2.5	97.5 ± 2.8	3750 ± 520	3600 ± 510	159 ± 5	2.59 ± 0.19	nd	nd

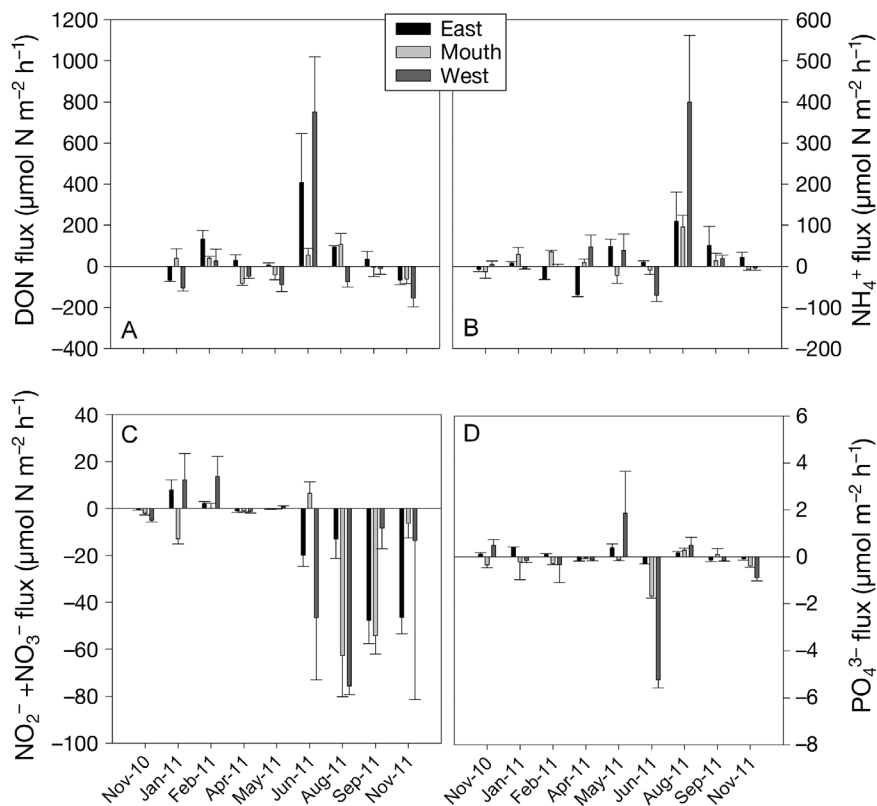


Fig. 3. Rates ($\mu\text{mol N m}^{-2} \text{ h}^{-1} \pm \text{SE}$) of (A) dissolved organic nitrogen (DON) flux, (B) ammonium flux, (C) nitrate plus nitrite flux, and (D) phosphate flux from November 2010 to November 2011 in Little Lagoon

atures ($\rho = 0.367$, $p = 0.0257$), and the highest $[\text{HS}^-]$ were observed in November 2010 at the East site ($1275.33 \pm 0.57 \mu\text{M}$ at 10 mm) and August 2011 at the Mouth ($>3000 \mu\text{M}$ at 10 mm) and West sites ($2712.6 \pm 2.96 \mu\text{M}$ at 10 mm) (Fig. 4). During the summer, O_2 typically was depleted by 1 mm at all sites but was present from 2 to 10 mm during the winter (data not shown).

Net N_2 fluxes

The average benthic net N_2 flux ranged from -61.7 ± 4.5 to $51.6 \pm 4.9 \mu\text{mol N m}^{-2} \text{ h}^{-1}$ (Fig. 5). Net N_2 fluxes were similar between the sites ($p = 0.559$) but differed temporally ($p < 0.001$). Generally, when the water column DIN and DIP N:P ratios were $>16:1$, net N_2 efflux occurred, while N_2 fixation was predominant when the ratios were $<16:1$ (Fig. 5). At the East site, net N_2 efflux occurred in November 2010, and in April and September 2011. The greatest efflux and uptake were 80.1 ± 7.7 and $-55.1 \pm 0.6 \mu\text{mol N m}^{-2} \text{ h}^{-1}$, respectively, and occurred during April and May. In the remaining months, N_2 uptake (i.e. net N_2 fixation) occurred (Fig. 5A). At the Mouth site, net N_2 fixation was prevalent for the majority of the study period with the exception of November 2010, when a net N_2 efflux occurred (Fig. 5B). The greatest N_2 efflux and uptake were 50.3 ± 3.1 and $-52.3 \pm 11.3 \mu\text{mol N m}^{-2} \text{ h}^{-1}$, respectively, and occurred during November 2010 and August 2011. The West site displayed the most temporal variability, with a net N_2 efflux occurring during January through April and September and November 2011, while N_2 fixation occurred during May through August as well as November 2010 (Fig. 5C). The greatest N_2 efflux and uptake were 55.2 ± 32.7 and $-77.8 \pm 1.5 \mu\text{mol N m}^{-2} \text{ h}^{-1}$, respectively, and occurred during April and June. In Little Lagoon, net N_2 fluxes were higher

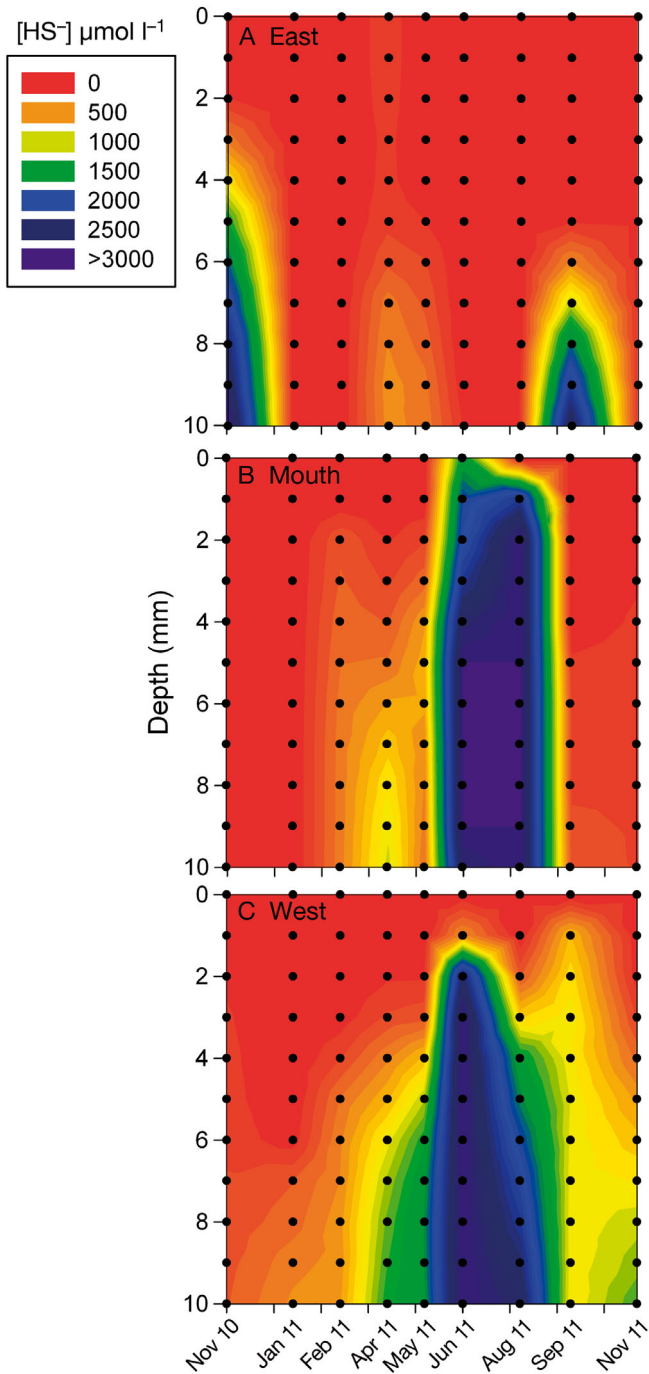


Fig. 4. Profiles of hydrogen sulfide concentration (measured as HS^-) at (A) East, (B) Mouth, and (C) West sites in Little Lagoon from November 2010 to November 2011. The standard curve is linear to 3000 μM . Dots are sediment probe sampling points

when water column NO_3^- concentrations and SOD were higher ($\rho = 0.477$, $p = 0.005$ and $\rho = 0.530$, $p = 0.001$, respectively). However, no relationship was found between net N_2 flux and $[\text{HS}^-]$, temperature or benthic chl *a* ($p = 0.541$, $p = 0.421$, $p = 0.423$,

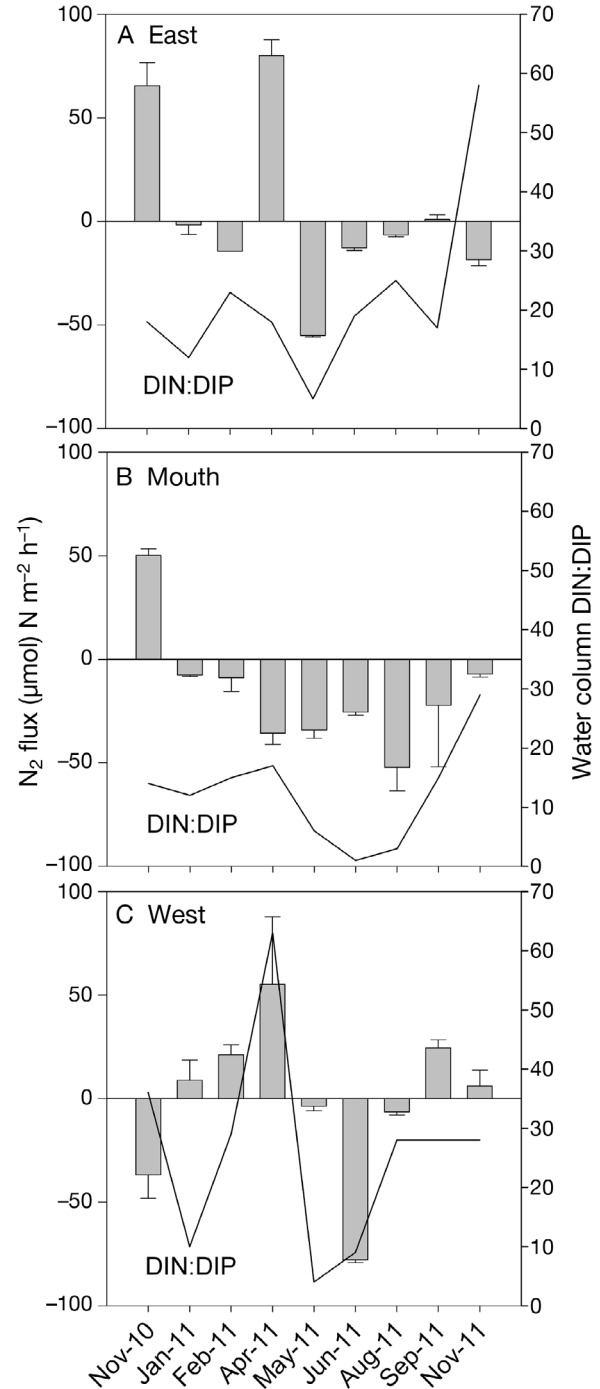


Fig. 5. Benthic flux of N_2 ($\mu\text{mol N m}^{-2} \text{h}^{-1} \pm \text{SE}$) at (A) East, (B) Mouth, and (C) West sites from November 2010 to November 2011 in Little Lagoon. Solid line is the DIN:DIP ratio. DIN: dissolved inorganic N; DIP: dissolved inorganic P

respectively). Bioturbation associated with macroinfaunal communities can have a strong spatial effect on benthic nutrient fluxes (Ferguson & Eyre 2007); however, bioturbation rates were not measured in this study.

Gene numbers

Gene copy numbers indicated that *nifH*, *dsrB* and *nrfA* markers for N_2 fixation, sulfate reduction, and dissimilatory nitrate reduction to ammonium (DNRA) were more abundant than *nirS*, *nirK* and bacterial *amoA* markers for denitrification and nitrification (Fig. 6), especially at the Mouth and West sites. However, *nirS* and *nirK* genes were more abundant at the East site than elsewhere in the lagoon. The bacterial *amoA* genes were the least abundant of all the genes measured, and archaeal *amoA* genes were below the detection limit. The abundances of *dsrB* were lower when *nirS* and *nirK* genes were higher ($\rho = -0.601$, $p = 0.0359$ and $\rho = -0.587$, $p = 0.0416$, respectively) and *nirS* and *nirK* were correlated ($\rho = 0.692$, $p = 0.0113$). There was a strong linear relationship between *dsrB* and *nifH* ($r^2 = 0.59$), suggesting that a fraction of N_2 fixation in Little Lagoon is potentially performed by SRB. This contention is further supported by potential N_2 fixation assay results (see next section). Gene copies for *dsrB*, *nrfA*, and bacterial *amoA* increased when *nifH* increased ($\rho = 0.923$, $p < 0.001$; $\rho = 0.676$, $p = 0.0139$; and $\rho = 0.648$, $p = 0.0203$, respectively). The *nrfA* abundances also increased when *dsrB* increased ($\rho = 0.685$, $p = 0.0126$). Gene markers for *nifH*, *dsrB*, bacterial *amoA*, and *nrfA* were positively correlated with temperature ($\rho = 0.692$, $p = 0.0113$; $\rho = 0.669$, $p = 0.0154$; $\rho = 0.701$, $p = 0.0101$; and $\rho = 0.687$, $p = 0.0126$, respectively). There was no correlation between benthic chl *a* and any of the gene markers.

Potential rates of N_2 fixation, denitrification, and net nitrification

The average benthic N_2 fixation rate increased from $0.02 \text{ nmol N g}^{-1} \text{ h}^{-1}$ in September to $0.09 \text{ nmol N g}^{-1} \text{ h}^{-1}$ in November 2011 (Fig. 7A) but did not display temporal ($p = 0.318$) or spatial ($p = 0.380$) variability. The activity of SRB accounted for 60 to 65% of total N_2 fixation at the 3 sites in September. In November, N_2 fixation by SRB increased, representing 67 and 78% at the Mouth and West sites, while at the East site it decreased to 12% (Fig. 7A). Mean potential denitrification rates increased from $0.02 \text{ nmol N g}^{-1} \text{ h}^{-1}$ in September to $0.04 \text{ nmol N g}^{-1} \text{ h}^{-1}$ in November 2011 (Fig. 7B) and displayed significant temporal ($p < 0.001$), but no spatial ($p = 0.916$) variability. Net nitrification rates were undetectable (data not shown).

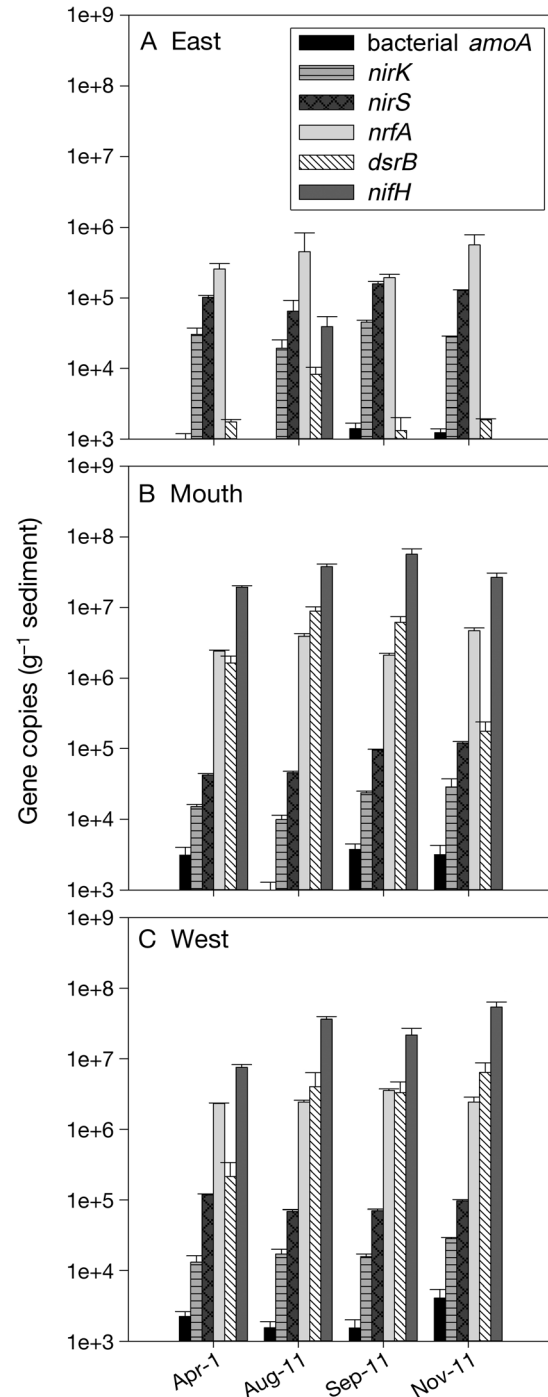


Fig. 6. The abundance of genes (copies g^{-1} sediment \pm SE) measured in triplicate at (A) East, (B) Mouth, and (C) West sites in Little Lagoon in April, August, September and November 2011

Nutrient fluxes via SGD

Based on radium isotopic results from monitoring wells, groundwater sources to the lagoon are split between a surficial and deeper aquifer that provide

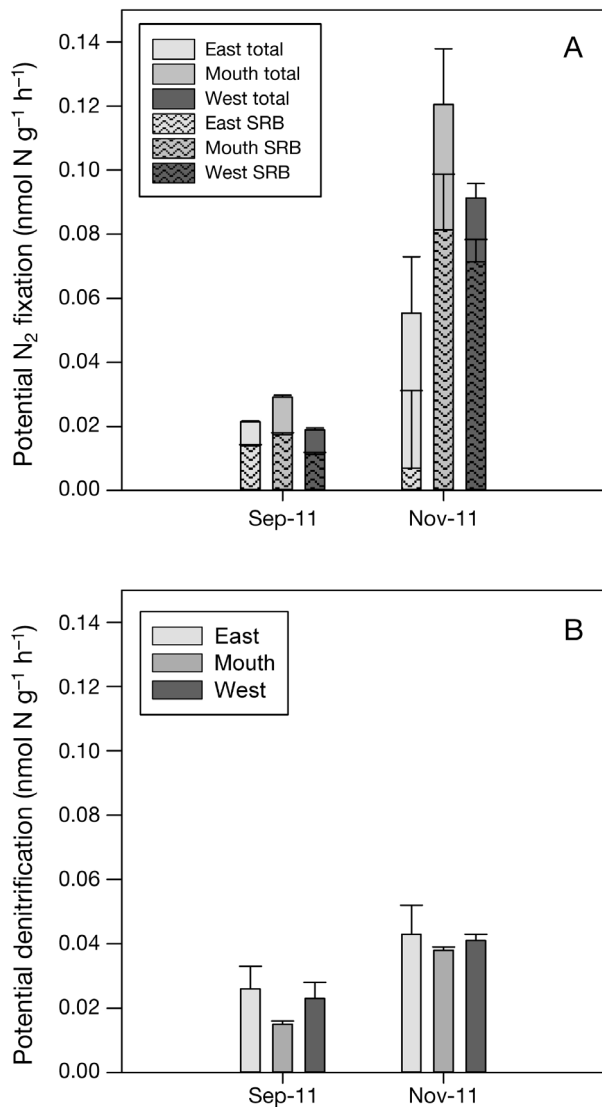


Fig. 7. Potential rates ($\mu\text{mol N g}^{-1} \text{h}^{-1} \pm \text{SE}$) of (A) N_2 fixation and (B) denitrification at Little Lagoon in September and November 2011. In panel A, pattern fill indicates sulfate reducing bacteria (SRB), while plain gray shading is total N_2 fixation

42 and 58% of the groundwater, respectively. Continuous radon and salinity surveys of the entire lagoon on 6 occasions showed that most of the groundwater enters from the eastern end of the lagoon (Su et al. 2012). The total SGD rates based on a radon mass balance approach were found to vary over the period studied from 0.60 to 2.9 $\text{m}^3 \text{s}^{-1}$, with higher rates occurring earlier in the study when the water table was higher. Significant correlations were found between radium (groundwater-derived) and nutrients when groundwater discharges were high, but not at low discharges (Su et al. 2012).

In order to estimate the nutrient fluxes into Little Lagoon via SGD, the low ($0.6 \text{ m}^3 \text{ s}^{-1}$) and high ($2.9 \text{ m}^3 \text{ s}^{-1}$) SGD rates from Su et al. (2012) were multiplied by the average groundwater nutrient concentrations. The concentrations used were weighted to account for the 42–58% split between the surficial and deeper aquifer. SGD N fluxes were up to 42 times larger than those estimated by benthic exchange, except for NH_4^+ (which were similar) (Table 1). DON dominated the SGD-derived N fluxes. P input via SGD was also greater than sediment fluxes (Table 1).

DISCUSSION

Balance between N fixation and denitrification determines net N_2 flux

When temperatures were high (Fig. 1A), $[\text{HS}^-]$ was high (Fig. 4), net N_2 flux was negative (i.e. N_2 fixation) (Fig. 5), the sediments consumed NO_3^- (Fig. 3C) and released NH_4^+ (Fig. 3B). This pattern can be explained by remineralization releasing NH_4^+ , NO_3^- uptake fueling denitrification, and N_2 fixation exceeding N_2 loss through denitrification resulting in a net N_2 uptake by the sediments. Evidence for limited denitrification in the summer was concurrent with high abundances of gene markers for N fixation, sulfate reduction, and DNRA, indicating that rates of processes associated with these bacteria could significantly contribute to benthic N cycling in Little Lagoon. Based on gene copy numbers, N fixation, sulfate reduction, and DNRA could potentially be more prevalent in this system than nitrification and denitrification, especially during the warmer summer months. Gene copy numbers based on DNA need to be interpreted carefully, as they do not necessarily represent community activity at the time of sampling; however, increases and decreases in gene numbers over time indicate increasing or decreasing populations of organisms containing the genes of interest. Furthermore, the potential rates provide evidence for measurable activity by the microbial community associated with N_2 fixation and denitrification (Fig. 7). The patterns observed in Little Lagoon are contrary to sub-tropical estuaries that have higher rates of denitrification and higher *nirS* gene abundances during the summer months (Francis et al. 2013). In the Gulf of Mexico region, low summertime denitrification rates and high DNRA rates have been found in Texas estuaries that maintained sulfidic sediments (An & Gardner 2002), suggesting the patterns of N cycling in Little Lagoon could be similar to other Gulf of Mexico estuaries.

Although there was no significant relationship between temperature and net N_2 flux, generally, at the East and Mouth sites, N was fixed at a higher rate than it was denitrified regardless of the season. However, at the West site during low temperature periods, denitrification exceeded N_2 fixation (Fig. 5C). Higher net denitrification during periods of cooler temperatures (Fig. 5) does not support our hypothesis of higher denitrification rates during summer months. Potential denitrification measurements also showed higher rates when $[HS^-]$ and temperatures were low (Figs. 4 & 7). Higher denitrification in winter compared to summer has also been observed in other sub-tropical estuaries (Gardner & McCarthy 2009) and is contrary to temperate estuaries where denitrification peaked in the summer (Ferguson & Eyre 2007).

Higher denitrification in Little Lagoon, when it did occur, may be more dependent on water column NO_3^- than coupled nitrification-denitrification. Sulfidic sediments in Little Lagoon during the warmer months (Fig. 4) likely interfere with nitrification and limit NO_3^- supply (Joye & Hollibaugh 1995). On such occasions, N_2 fixation rates could exceed denitrification rates (Fig. 5). Based on Redfield stoichiometry and average SOD ($382.7 \mu\text{mol O}_2 \text{ m}^{-2} \text{ h}^{-1}$), the NH_4^+ flux ($26.1 \mu\text{mol N m}^{-2} \text{ h}^{-1}$) is lower than expected ($57.8 \mu\text{mol N m}^{-2} \text{ h}^{-1}$), indicating that some NH_4^+ is nitrified (Twilley et al. 1999). However, the lack of measurable potential nitrification rates as well as high $[HS^-]$, low bacterial *amoA* genes, and undetectable archaeal *amoA* genes suggests that nitrification is a likely minor process in Little Lagoon.

Since coupled nitrification-denitrification is likely hampered in Little Lagoon, water column NO_3^- concentration exerts an influence on the balance between N_2 fixation and denitrification, as has been reported in other estuaries (Fulweiler & Nixon 2012). On multiple occasions, when the DIN:DIP ratio was $>16:1$ (i.e. P limitation), denitrification exceeded N_2 fixation. However, at the Mouth site, where the DIN:DIP ratio was primarily $<16:1$ (indicating N limitation), N_2 fixation was greater than denitrification. While the data is consistent with this interpretation in many instances, in other instances it deviates from the explanation that DIN:DIP is driving the net N_2 fluxes, indicating that during these occasions, other biogeochemical factor(s) drive the net N_2 flux rates. Examples where denitrification is mainly fueled by water column NO_3^- (Fulweiler & Nixon 2012) are much less common than denitrification primarily supported by coupled nitrification-denitrification (An & Joye 2001). During periods when N is not limiting (i.e. high N:P ratio), Little

Lagoon shows a similar trend to other estuaries where higher water column NO_3^- concentrations is concurrent with greater denitrification.

Both the *in situ* cores and slurry incubations showed the predominance of N_2 fixation (Figs. 5 & 7). Though not statistically significant because of the limited data set, N_2 fixation appeared to increase from summer to fall (Fig. 7A). The SRB were ubiquitous and present in high numbers (Figs. 6 & 7) and between 60 and 78% of N_2 fixation was accounted for by the activity of the SRB. Generally, SRB contribution to nitrogenase activity tends to be lower (30 to 60%) (Welsh et al. 1996) and N_2 fixation rates tend to be higher (0.5 to $4.0 \mu\text{mol N m}^{-2} \text{ h}^{-1}$) than those measured in Little Lagoon. N fixation is not traditionally regarded as a significant process in estuaries; however, N_2 fixation by SRB may represent an important N source to the system (Fulweiler et al. 2013), especially during periods of low DIN:DIP (Fig. 5).

The SOD in Little Lagoon ($102.5 \pm 6.5 \text{ g O}_2 \text{ m}^{-2} \text{ yr}^{-1}$) is on the lower end of the range reported for temperate and sub-tropical estuaries (36 to $1132 \text{ g O}_2 \text{ m}^{-2} \text{ yr}^{-1}$) (Cowan et al. 1996, Cowan & Boynton 1996). Generally, high SOD rates are likely because of high OM remineralization and nitrification rates (Dunn et al. 2012); however, the prevalence of sulfides in the sediments in Little Lagoon imply that a large fraction of SOD is driven by the chemical oxygen demand attributed to reoxidation of reduced sulfur originating from sulfate reduction. Given the ubiquitous presence of sulfides in the sediments regardless of the season, and O_2 concentration that was $<150 \mu\text{M}$ by 2 mm in the winter and undetectable by 1 mm in the summer (data not shown), SR appears to be a dominant pathway for OM remineralization in Little Lagoon. The weak relationship between SOD and net N_2 fluxes in Little Lagoon may likely result from the influence of SR, low NO_3^- availability, and/or the predominance of N_2 fixation rather than denitrification.

Sediment chl *a* content, a proxy for labile OM, has been shown to influence denitrification rates (Fulweiler et al. 2013); however, in Little Lagoon chl *a* content did not correlate with net N_2 fluxes, indicating that OM availability is not a predominant factor contributing to the balance between N fixation and denitrification. Taken together, these lines of evidence suggest water column NO_3^- concentration rather than temperature, or sediment OM availability, has a large impact on denitrification rates and influences the balance between N fixation and denitrification and the overall net N_2 fluxes in Little Lagoon.

Benthic and SGD N fluxes

The benthic fluxes were episodic and variable in magnitude over the course of the study (Fig. 3), with only DON lacking significant temporal variability. This is in agreement with other investigations from tropical to temperate ecosystems that generally show a markedly seasonal pattern in benthic nutrient fluxes (Cowan & Boynton 1996, Fulweiler & Nixon 2012). Yearly-integrated fluxes show overall consumption of NO_3^- , and release of PO_4^{3-} , NH_4^+ , and DON (Table 1). Similar to Texas estuaries where N_2 sources and sinks are nearly balanced (Gardner et al. 2006), annually, the sediments in Little Lagoon were neither a source nor a sink for N_2 . Benthic fluxes for NH_4^+ were similar, while NO_3^- and PO_4^{3-} fluxes were lower than those for Gulf of Mexico estuaries (Cowan et al. 1996, Gardner et al. 2006). The ubiquitous presence of $[\text{HS}^-]$, *dsrB*, and *nrfA* during the summer indicate DNRA could potentially contribute to the NH_4^+ release; however, DNRA rate measurements are needed to support this contention. These measurements will be important because DNRA retains fixed N in the system (An & Gardner 2002, Gardner & McCarthy 2009), which can potentially exacerbate the effects of eutrophication.

SGD nutrient fluxes in Little Lagoon are 7 to 42 times larger than benthic fluxes (Table 1) and, similar to the benthic N fluxes, DON dominates the SGD N input (Table 1). Nutrient concentrations in groundwater and SGD in Little Lagoon are higher than reported for other sub-tropical estuaries (Carruthers et al. 2005, Kim et al. 2013), likely because of excess nutrients in the groundwater in nearby agricultural landscapes (Dowling et al. 2004). The larger magnitude of SGD nutrient fluxes compared to benthic fluxes suggests that SGD can exert a large influence on the primary producers' nutrient demand and influence productivity, as shown in other systems (Carruthers et al. 2005).

Both the benthos and SGD are important DON sources to the water column in Little Lagoon. Episodic but high DON flux rates (Fig. 3A) dominate the benthic N fluxes with ranges similar to values reported for other sub-tropical systems (Eyre & Ferguson 2002) in which DON was also a major fraction of TDN flux. However, the SGD-DON flux is higher in magnitude than what has been reported in other systems (Kim et al. 2013, Santos et al. 2013). Typically, DON only represents a small fraction of the total water column inventory of macronutrients in estuaries, yet it is an important intermediary in microbial metabolism. DON fluxes may support the

episodic algae blooms occurring in Little Lagoon (MacIntyre et al. 2010). Further investigation of the input and DON bioavailability to Little Lagoon and its contribution to primary productivity and occurrences of harmful algae blooms is required.

CONCLUSIONS

Unlike many estuarine systems where denitrification is coupled to NO_3^- production from nitrification, multiple lines of evidence suggest that denitrification in Little Lagoon, when it does occur, is primarily supported by water column NO_3^- . Net N_2 fixation generally occurred when the water column NO_3^- was low, the DIN:DIP ratio was <16:1, and sediment $[\text{HS}^-]$ was high. Benthic N_2 fluxes indicated that annually, the sediments were neither a source nor a sink for N_2 . Both the benthos and SGD were a DON source to the water column; however, the SGD nutrient inputs were disproportionately higher than benthic fluxes. In SGD dominated systems such as Little Lagoon, where the contribution of nutrients from SGD dominate the N fluxes, SGD may play an important role influencing primary producer community composition and abundance.

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