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Activity, Abundance, and Diversity of Nitrifying Archaea and Denitrifying Bacteria in Sediments of a Subtropical Estuary: Bahía del Tóbari, Mexico

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Abstract Fixed nitrogen (N) removal from estuaries via coupled nitrification–denitrification plays a significant role in the global N cycle and the biogeochemistry of individual estuaries. Much of our understanding of these processes is drawn from temperate estuaries, yet tropical and subtropical estuaries may respond differently to N inputs. I tested the hypothesis that nitrification is limited within subtropical estuaries by comparing nitrification and denitrification potentials, and the abundance of archaeal ammonia monooxygenase (*amoA*) and bacterial nitrite reductase (*nirS*) genes, across five sites in Bahía del Tóbari, Mexico. Sampling was conducted when agricultural runoff supplied substantial quantities of N (ca. 20–80 μM ammonium), yet nitrification was detected at a single site. Denitrification was measured at four sites, and three displayed nitrate uptake rather than net nitrification—indicating a N sink within these sediments. Bacterial *nirS* genes uniformly outnumbered archaeal *amoA* genes (3- to 49-fold) and were more abundant in the northern part of the estuary. Patterns of community similarity among different sites were also different for *nirS* and archaeal *amoA*: similarities between sites based on *nirS* were often greater than for *amoA*, and sites were more rarely statistically different from each other. While *amoA* abundance was inversely related to temperature, neither *amoA* nor *nirS* was correlated with nitrification or denitrification potentials. My results are broadly consistent with known and proposed patterns of nitrification and denitrification in subtropical estuarine sediments, including the idea that nitrification is limited within subtropical estuarine sediments.

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Introduction

Anthropogenic nitrogen (N) inputs to coastal systems have increased dramatically since the preindustrial era (Galloway et al. 2004; Gruber and Galloway 2008; Canfield et al. 2010), leading to coastal eutrophication and the proliferation of hypoxic "dead zones" (reviewed by Diaz and Rosenberg 2008). However, coastal and estuarine ecosystems act as "filters" that remove large quantities of N through conversion to gaseous forms and attenuate the flux of N from land to sea. Globally, ca. 45 % of marine N loss occurs in estuarine and continental shelf sediments (Seitzinger et al. 2006). The processes that remove N are microbially mediated and occur under anaerobic conditions. Denitrification reduces nitrate (NO_3^-) and nitrite (NO_2^-) to N_2 gas and typically dominates N loss from coastal and estuarine systems (Kuypers et al. 2006; Francis et al. 2007; Trimmer et al. 2003; Risgaard-Petersen et al. 2004a; Rich et al. 2008). Yet relatively little is known about this process in tropical and subtropical estuaries: in a cross-system analysis of denitrification in aquatic ecosystems, Piña-Ochoa and Álvarez-Cobelas (2006) identified <10 measurements of denitrification between 30°S and 30°N latitude, and zero measurements between 1° and 25° (in both hemispheres).

At the same time, Corredor et al. (1999) hypothesize that N cycling within undersampled tropical and subtropical estuaries differs fundamentally from N cycling in the temperate zone. They argue that the habitat favorable to nitrifiers in tropical and subtropical estuarine sediments is limited from above by light and from below by low oxygen concentrations and the presence of sulfide (Morell and Corredor 1993; Corredor et al. 1999). Because nitrification is the two-stage aerobic oxidation of ammonia (NH_3) to NO_3^- via NO_2^-, mediated by ammonia-oxidizing archaea

(AOA) and bacteria (AOB) and nitrite-oxidizing bacteria, it requires oxygen. Sulfide also inhibits nitrification (Joye and Hollibaugh 1995) and is produced via sulfate reduction in sediments. Yet previous studies have detected 16S rRNA from AOB at depths of up to 40 cm in sediments (Freitag and Prosser 2003; Mortimer et al. 2004), suggesting that AOB are actively synthesizing proteins at depths where little dissolved oxygen is present. AOA and AOB may also have varying sensitivities to light, sulfide, and low oxygen, with evidence of niche specialization among different groups of AOA and AOB provided by analysis of ammonia monooxygenase (*amoA*) genes (reviewed by Erguder et al. 2009 and Hatzenpichler 2012).

Molecular techniques have been therefore proven useful to explore the ecology of AOA and AOB communities and populations. For denitrifier communities, nitrite reductase catalyzes the first committed step to generation of a gaseous product (Zumft 1997), and nitrite reductase (*nirK* or *nirS*) gene abundance and diversity has been examined in coastal, marine, and estuarine sediments (Braker et al. 2000; Nogales et al. 2002; Hannig et al. 2006; Santoro et al. 2006; Tiquia et al. 2006; Smith et al. 2007; Abell et al. 2010; Mosier and Francis 2010). A single study has examined nitrifiers and denitrifiers within the same subtropical estuary: Abell et al. (2010) found that AOA were the dominant ammonia oxidizers in Australia's Fitzroy estuary that *nirS*-type bacterial denitrifiers were the dominant denitrifiers and outnumbered AOA, and that denitrification (based on isotope-pairing measurements) was more readily detected than nitrification (based on core incubations). These data support the idea that N cycling proceeds in a fundamentally different way within tropical and subtropical estuaries, but it is unlikely that results from a single estuary can be uniformly applied to hundreds of subtropical and tropical estuaries. Of Earth's 1,200 estimated estuaries (Millennium Ecosystem Assessment 2005), ca. 400 experience hypoxia (Diaz and Rosenberg 2008): 24 hypoxic systems are located in the tropics and 53 are located between 23.4° and 30° (Diaz and Rosenberg 2008). These comparatively low numbers may reflect lack of sampling, lack of current anthropogenic impact, differential responses to nutrient enrichment and eutrophication, or a combination of the three, and deserve investigation. In particular, Galloway et al. (2008) note that although much of our knowledge on N dynamics is drawn from temperate systems, subtropical and tropical regions will receive the most dramatic increases in N inputs over the next few decades.

Mexico's estuaries represent a series of case studies for studying low-latitude N cycling, as Mexico spans ca. 14–33°N latitude, estuaries line both eastern and western coastlines, and these systems experience a range of human influence. I compared the abundance and activity of AOA and *nirS*-type denitrifying bacteria in sediments of Bahía del Tóbari (Fig. 1), a shallow, subtropical estuary located on the northwest coast of mainland Mexico. Tóbari represents an ideal experimental test

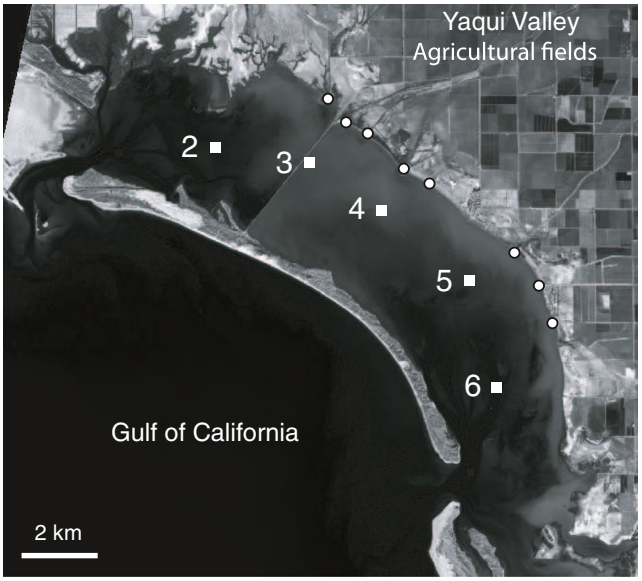

Fig. 1 Location of sampling sites within Bahía del Tóbari, Mexico are shown as *squares*. Sampling sites are plotted on 10 m resolution panchromatic data from the Advanced Land Imager satellite (courtesy of National Aeronautics and Space Administration and Greg Asner of the Carnegie Institution of Washington). Sites 2 and 6 are located in 10–12 m channels that appear darker in this image, while concentric circles show the location of agricultural drain inputs into Tóbari. Scale bar is at *lower left*

of the Corredor et al. (1999) hypothesis because it receives substantial amounts of N in agricultural runoff, predominantly in the form of dissolved ammonium (NH_4^+), following Valley-wide fertilization and irrigation events (Harrison and Matson 2003; Beman and Francis 2006; Ahrens et al. 2008; see "Materials and Methods"). My hypothesis is that N cycling in Tóbari functions differently from temperate estuaries, where coupled nitrification/denitrification serves as an important mechanism for nitrogen removal (Corredor et al. 1999), and that this is expressed through differences in nitrifier/denitrifier activity and abundance in Tóbari.

Materials and Methods

Site Description

Tóbari is a ~90-km^2 estuary located along the northwest coast of Mexico at 27.08°N, 109.96°W (Fig. 1) and is situated between the Yaqui Valley—an intensive agricultural region (Matson et al. 1998; Matson 2012)—and the Gulf of California—a productive (Zeitzschel 1969) and biodiverse (Roberts et al. 2002) marginal sea located between the Baja California peninsula and mainland Mexico. The Yaqui Valley is host area of the "Green Revolution" for wheat and remains a productive agricultural area with 225,000 ha of intensively managed, irrigated, wheat-based agriculture critical to the Mexican economy (Matson 2012). The Yaqui Valley is also representative of 43 % of wheat-producing areas throughout

the world, particularly those in the temperate-to-subtropical region. In line with global patterns, use of fertilizer N in the Valley has increased over threefold between 1968 and 1995 (from 80 to 250 kg N ha^{-1} per 6-month wheat crop), and survey results indicate substantial increases in just the past decade. While this has increased grain yields and the protein contest of the grain, intensive use of N fertilizers has produced unintended environmental consequences (Turner et al. 2003; Matson 2012).

Research in the Yaqui Valley over the past few decades has documented consistently large losses of N from agriculture fields to the atmosphere, groundwater, and surface waters of the Valley (Matson et al. 1998; Panek et al. 2000; Riley and Matson 2000; Riley et al. 2001; Harrison and Matson 2003; Beman and Francis 2006; Ahrens et al. 2008). These include some of the highest fluxes of nitrous oxide (N_2O) on record (Matson et al. 1998), and concentrations of NO_3^- and NH_4^+ in surface waters that are comparable to polluted surface waters in the USA States and Europe (Harrison and Matson 2003). These losses are driven by the internal dynamics of the Valley. Farmers typically fertilize twice with N (applied either as urea or as anhydrous ammonia) and typically apply most fertilizer several weeks prior to planting, with the remainder applied several weeks after plating. These fertilization events occur concurrently with irrigation, and two or more irrigation events subsequently follow. During the first fertilization/irrigation event (November), large amounts of N are lost as dissolved NH_4^+ because large amount of N are added while no crops are present; during the second fertilization/irrigation event (January), plant growth results in smaller losses of N; and during the following irrigation events, N is lost predominantly in its more easily leached form, NO_3^- (Ahrens et al. 2008). The fields are drained by a system of drainage canals that empty distinct sections of the Valley and discharge directly into the estuaries of the Gulf of California (Ahrens et al. 2008, 2012).

Agricultural runoff from irrigation is therefore the primary source of freshwater to Tóbari (Beman et al. 2005; Schoups et al. 2005), and Tóbari receives substantial amounts of N (Harrison and Matson 2003; Beman and Francis 2006; Ahrens et al. 2008). Discharge from the seven major drains entering Tóbari (white circles in Fig. 1) is typically low and ranges from 0 $m^3 s^{-1}$ in summer months to 2 $m^3 s^{-1}$ during irrigation events. N concentrations are high, with typical NH_4^+ concentrations of 1 to 10 mg N L^{-1}, reaching a maximum of up to 40.8 mg N L^{-1}, and typical NO_3^- concentrations of 1 to 5 mg N L^{-1}, reaching up of 21.1 mg N L^{-1} (Ahrens et al. 2012). The total flux of N to Tóbari is ca. 10^6 kg N $year^{-1}$ and inputs of N peak at the same time for different drains entering Tóbari (Ahrens et al. 2008, 2012). However, the northernmost drain (north of the bridge/causeway in Fig. 1) transports an order of magnitude more N into the estuary than the other drains, which all export similar amounts of N into Tóbari (Ahrens et al. 2012).

Circulation in Tóbari is driven primarily by wind and tides and is known to be rapid (M.E. Cruz-Colin, S.G. Monismith, A. Valle-Levinson, and J.A. Delgado-Contreras, unpublished data): the estimated residence time of 5–10 days (Ahrens et al. 2008), owing in part to the relatively shallow depths of 1.5 m in the interior, and 4–8 m at the mouths, of the estuary (Beman and Francis 2006). Many Gulf of California estuaries are characterized as "negative" estuaries, where circulation is driven by evaporative generation of warm, salty waters that flow out of the estuaries at depth and are balanced by shallow inflow. Others—including Tóbari—are "mixed" estuaries, exhibiting characteristics of both negative estuaries and "positive" estuaries in different seasons (Valle-Levinson et al. 2001). Estuarine circulation was positive at the time of my sampling due to runoff of irrigation water into Tóbari.

Sample Collection and Biogeochemical Analyses

Surface sediments (upper 10 cm) were collected from five sites in Tóbari in January 2004 (Table 1). This includes the north channel of Tóbari (site 2), through the interior (sites 3–5), to the south channel (site 6). (Sites 1 and 7 were previously sampled for biogeochemical measurements but this sampling was discontinued.) Duplicate sediment cores were collected using box cores and cut-off 5-cc syringes, frozen immediately on dry ice, and stored at −80 °C until DNA extraction. Two cores were compromised during transport (cores 4B and 6B did not remain frozen) and were not analyzed; hence, data from sites 4 and 6 are reported for single cores (see below). Salinity and temperature were measured using a YSI-85 (YSI Incorporated, Yellow Springs, OH), and water samples were collected for nutrient analyses in 10-mL Lachat tubes, immediately frozen on dry ice, and stored at −20 °C until analysis. An Alpkem Flow Solution IV autoanalyzer was used to measure combined nitrate and nitrite [NO_2^- + NO_3^-] concentrations in undiluted water samples (Technique P/N 000623) (O/I_Analytical 1999). The lower detection limit for [NO_2^- + NO_3^-] was 0.14 μM, standards ranged from 0.29 to 179 μM, and r^2=0.999. Details of NH_4^+ measurements are reported in Beman and Francis (2006).

Table 1 Average temperature, salinity, NO_3^- concentrations, and NH_4^+ concentrations in Bahía del Tóbari during sampling in January 2004

Site	Temperature (°C)	Salinity (ppt)	NO_3^- [μM]	[NH_4^+] (μM)
2	17.3	35.2	0.376	22.1
3	18.3	33.8	0.842	80.4
4	18.4	34.1	0.257	22.9
5	18.3	34.1	0.240	27.1
6	18.0	34.7	0.305	34.2

Temperature, salinity, and NH_4^+ data were previously published in Beman and Francis (2006)

Potential Nitrification and Denitrification Rates

Sediment samples for nitrification and denitrification potential measurements were collected in triplicate from each site using box cores and were transported on ice from the field to the lab in Ziploc bags. Samples were stored for 3 h before incubation. I used potentials for two primary reasons: (1) they likely provide better comparisons with DNA-based analyses, as quantitative PCR (QPCR) and sequencing of DNA target all microbes present, and therefore capture the overall abundance and diversity of the entire community rather than a subset of the community that may be active at any given time. (2) Nitrification and denitrification potentials provide maximum possible rates under ideal conditions, thereby avoiding potential issues with substrate limitation in situ.

Nitrification potentials were measured using the method of Hart et al. (1994) modified slightly for sediments. Ten cubic centimeter sediment samples were incubated in a 100-mL solution of [750 μM] $(NH_4)_2SO_4$, [700 μM] K_2HPO_4, and [300 μM] KH_2PO_4 in the dark at room temperature (23 °C). Nitrification potential rates were calculated as the least-squares regression of change in $NO_2^- + NO_3^-$ concentrations over 21 h. Regressions were tested for significance, but due to the limited number of data points, I used a P value of 0.10, as none of the regressions were significant at the 0.05 level. Denitrification potential (also referred to as "denitrification enzyme activity" in the literature) was measured using the method of Tiedje (1982) modified by Holtgrieve et al. (2006). Ten cubic centimeter sediment samples were amended with 25 mL of [1 mM] KNO_3 and [1 mM] sodium succinate in 125 mL Wheaton vials, capped and sparged with N_2 gas to remove oxygen, and acetylene (C_2H_2) was injected into the headspace to block the final step of denitrification. Sediment slurries were shaken continuously for 1 h at 23 °C and sampled at 15-min intervals. Five milliliters of headspace was analyzed by gas chromatography (Shimadzu GC8A configured with a ^{63}Ni electron capture detector; Kyoto, Japan), and denitrification potential rates were calculated as the least-squares regression of N_2O accumulation over 1 h. Regressions were tested for significance, but due to the limited number of data points, I used a P value of 0.10; regressions were significant at the 0.05 level for one incubation at sites 3, 4, and 5, and at the 0.10 level for one incubation at site 6. Chloramphenicol was not added to denitrification potentials, which may lead to an overestimation of activity because new denitrification enzymes may be produced, and is a limitation of these data.

DNA Extraction and Quantitative PCR

For QPCR analysis, DNA was extracted in triplicate from 446 to 563 mg of each sediment core using the MP Biomedicals FastDNA Spin Kit for soil (MP Biomedicals, Solon, OH, USA). This resulted in six DNA samples for sites 2, 3, and 5 (duplicate cores, with triplicate DNA samples for each) and three DNA samples from site 4 and 6 (single cores, with triplicate DNA samples for each). DNA was quantified using the PicoGreen assay and the manufacturer's protocol (Life Technologies Corporation, Carlsbad, CA, USA).

QPCR assays were performed in triplicate on a Stratagene MX3005P QPCR system (Agilent Technologies, La Jolla, CA, USA). For archaeal *amoA*, QPCR analyses were identical to Beman et al. (2012) and the standard curve, r^2=0.983. Analysis by Throbäck et al. (2004) demonstrated that the *nirS* primers used in my QPCR protocol (cd3aF and R3cd) are effective for quantifying a broad spectrum of denitrifiers; these have been successfully used by Lam et al. (2009) in marine systems and Hallin et al. (2009) for soils. I optimized *nirS* QPCR for sediments: *nirS* primers cd3aF and R3cd were added at a concentration of 0.4 μM to a 25-μL reaction mixture containing 12.5 μL SYBR Premix F, 1.25 units AmpliTaq polymerase, 40 ng μL^{-1} BSA, and 1 ng DNA. Thermocycling conditions were 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 54 °C for 45 s, and 72 °C for 60 s. The *nirS* gene from *Pseudomonas stutzeri* was synthesized at Blue Heron Biotechnologies (Bothell, WA, USA) and diluted to known concentrations for use as a QPCR standard; r^2 of standard curves were 0.966 and 0.996.

nirS PCR Amplification, Cloning and Sequencing

DNA extracts were used as templates in 50 μL PCR mixtures for amplification, cloning, and sequencing of *nirS*. I used the nirS1F and nirS6R primers described by Braker et al. (1998) and conditions described by Santoro et al. (2006). Triplicate PCR reactions were pooled to minimize PCR bias, gel-purified, and cloned using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA). Sequencing was performed using vector primers on ABI 3730xl capillary sequencers (Applied Biosystems, Foster City, CA, USA). Samples were also screened for the copper-containing nitrite reductase gene (*nirK*) gene using the primers nirK1F (Braker et al. 1998) and nirK583FdgR (Santoro et al. 2006) and conditions described by Santoro et al. (2006); however, *nirK* genes were only detected in the site 6 sample and no meaningful comparisons can be made.

Analysis of Community Composition

Clone libraries were generated from a single extract from each site and numbered 43 sequences at site 2, 41 sequences at site 3, 35 sequences at site 4, 41 sequences at site 5, and 31 sequences at site 6. Nucleotide sequences were assembled and edited using Sequencher™ v.4.2 (GeneCodes, Ann Arbor, MI, USA) and aligned using MacClade v4.08 (http://www.macclade.org; Sinauer Associates, Sunderland, MA, USA). Jukes–Cantor-corrected nucleic acid distance matrices were generated using PAUP 4.0b10 (Sinauer Associates) and abundance-based Sørensen-type similarity indices (Chao et al.

2005) and the standard error of these values were calculated using the program SONS (Schloss and Handelsman 2006). Observed differences in community composition between libraries were statistically compared using ∫-Libshuff (Schloss et al. 2004), and significant *P* values were evaluated after correcting for multiple pairwise comparisons using the Dunn–Sidák method (Sokal and Rohlf 1984). Operational taxonomic units were defined as sequence groups in which sequences differed by 5 %. The 191 *nirS* sequences generated in this study have been deposited in the GenBank database under accession numbers KC614131-KC614321.

Statistical Analyses

Data were log-transformed to achieve normality and statistical analyses were conducted in MATLAB version 7.6.0 (R2008a).

Results

Denitrification and Nitrification Potentials

Denitrification potentials were variable across transect and ranged over several orders of magnitude, from 0.028 to 0.52 nmol N h^{-1} cm^{-3} sediment (Fig. 2). For purposes of comparison, these rates translate to 6 to 342 mmol N m^{-2} $year^{-1}$ (assuming denitrification occurs over a 1–3-cm depth range; Jenkins and Kemp 1984). There was a general trend towards higher denitrification potential values in the interior of the estuary at sites 3, 4, and 5, with lower potential at site 6, and no significant denitrification potential measured at site 2. Nitrification was only detected at site 6 and was 37 times the measured denitrification potential (1.02±0.46 nmol N h^{-1} cm^{-3} sediment; Fig. 2). At sites 3, 4, and 5, nitrate concentrations decreased significantly within sediment slurries during the incubation, resulting in a "negative" nitrification potential. The rate of uptake was similar to measured denitrification potentials at the same sites: uptake of 0.54 nmol N h^{-1} cm^{-3} vs. denitrification potential of 0.24 nmol N h^{-1} cm^{-3} at site 3, 0.49 vs. 0.52 nmol N h^{-1} cm^{-3} at site 4, and 0.65 vs. 0.45 nmol N h^{-1} cm^{-3} at site 5.

amoA and *nirS* Abundance

AOA and *nirS*-type denitrifying bacteria were quantified based on the abundance of *amoA* and *nirS* genes within sediments. Significant differences between the two genes and between different sites were observed: overall *nirS* was substantially more abundant than *amoA*, ranging from 3 to 49 times greater in each core. *amoA* was not amplified in four DNA samples (one DNA sample from core 2B, two samples from core 3B, one sample from core 5A), whereas *nirS* genes were present in all DNA samples. In addition, bacterial *amoA* was amplified only from sites 2 and 4 in a previous study (Beman and Francis 2006) and *nirK* was amplified only from site 6 in this study. Averaged across all samples, archaeal *amoA* genes numbered 2.27×10^5 genes g^{-1} sediment, whereas bacterial *nirS* averaged 39.5×10^5 g^{-1} sediment (ANOVA $P<0.0001$). *amoA* ranged from 9.17 to 59.7×10^5 genes g^{-1}, and *nirS* ranged from 27.2 to 882×10^5 genes g^{-1}.

amoA and *nirS* were statistically compared at the core level rather than the site level (due to the lack of replicate cores from sites 4 and 6); however, variation within cores lead to no significant difference in *amoA* abundance between cores ($P=0.173$). *nirS*-type denitrifiers did display significant

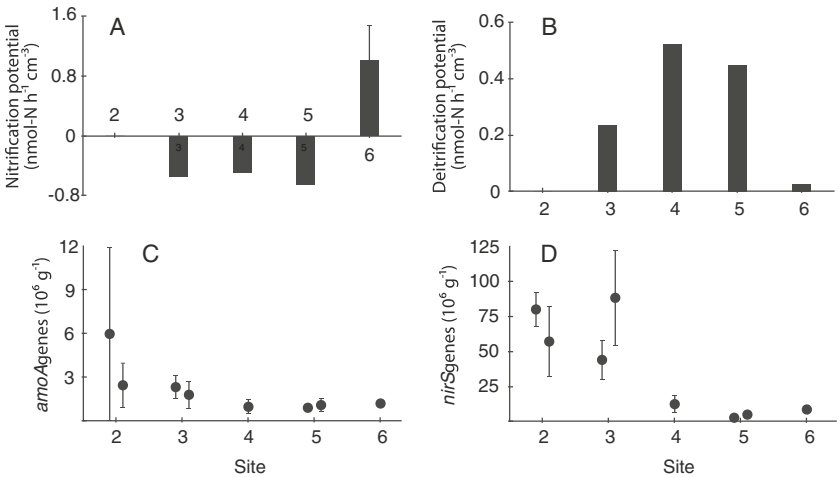

Fig. 2 Nitrification (**a**) and denitrification (**b**) potentials, and abundances of archaeal *amoA* (**c**) and bacterial *nirS* (**d**) genes across sampling sites in Bahía del Tóbari. In **a** and **b**, nitrification potential for site 6 represents the mean of the two replicates displaying significant changes during the incubation; other data represent single replicates with significant changes (see text). Denitrification potentials are based on quadruplicate measurements of N_2O accumulation in sample vials. Panels **c** and **d** show average values for individual sediment cores based on triplicate DNA samples. *Error bars* denote one standard deviation

spatial variation in abundance throughout the estuary (ANOVA $P<0.0001$). Post hoc tests showed that replicate cores from site 2 had higher *nirS* abundance than cores from sites 5 and 6, and one replicate was also significantly different from the core collected at site 4 (Fig. 2). One of the replicate cores from site 3 also contained significantly more *nirS* genes than sites 4, 5, and 6, whereas the other site 3 replicate was not significantly different from any other samples. Cores from sites 2 and 3 were not significantly different from one another, and cores from sites 4, 5, and 6 were likewise statistically indistinguishable. Across the estuary, both genes were most abundant at site 2, followed by site 3; *amoA* was least abundant at site 4, while *nirS* was least abundant at site 5, and site 6 exhibited moderate levels of both genes.

Community Composition and Variation Based on *amoA* and *nirS*

Similarities and differences among denitrifier communities were determined by cloning and sequencing of *nirS* genes, and analyzed using abundance-based Sørensen's similarity indices (L_{abd}) (Schloss and Handelsman 2006). The most similar libraries were those from sites 4 and 5 ($L_{abd}=0.71$ at 5 % nucleotide difference level; Fig. 3); the least similar libraries were sites 5 and 6 ($L_{abd}=0$) and sites 4 and 6 ($L_{abd}=0.04$). Libraries from the channel sites were generally dissimilar from other libraries: similarities for the site 6 library ranged from 0 to 0.19 and were slightly higher for the site 2 library ($L_{abd}=0.10$–0.64). Whereas similarities in *nirS* libraries graded smoothly from site to site, previous analysis of archaeal *amoA* gene libraries showed a "checkered" pattern, with high similarities among some libraries and no similarity among others (Beman and Francis 2006). For example,

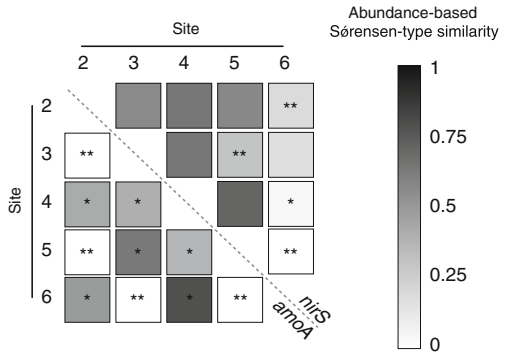

Fig. 3 Similarities among *nirS* libraries (*above the diagonal*) and archaeal *amoA* libraries (*below the diagonal*) from Bahía del Tóbari. For a given site along the horizontal axis compared with another site along the vertical axis, *gray shading* indicates the abundance-based Sørensen's index (L_{abd}) between libraries for a 5 % nucleotide difference. *Asterisks* denote the significance of this comparison based on ∫-Libshuff, where a *single asterisk* denotes at least one comparison between libraries was significant ($P<0.05$) and they are likely representative of different in situ populations; *double asterisks* indicate that both comparisons between X and Y, and Y and X were significant and indicative of different in situ populations

reading across the site 3 row for *nirS*, L_{abd} declines from 0.64 to 0.30 to 0.17 from site 4 to 5 to 6. Reading down the January site 3 column for *amoA*, $L_{abd}>0.38$ for sites 4 and 5—but for sites 2 and 6, $L_{abd}=0$.

I tested whether lower similarity values are indicative of significantly different *nirS*-type denitrifier or AOA communities (Schloss et al. 2004). Consistent with the low similarities above, nearly all of the significant ($P<0.05$) differences among *nirS* libraries involved libraries from site 6: three of four comparisons between the site 6, the library, and the other four libraries were significantly different, while libraries from sites 3 and 5 were also distinct—the only case involving libraries other than those drawn from site 6 (Fig. 3). In contrast, most *amoA* libraries were significantly different from one another, and in no case were *nirS* libraries significantly different when *amoA* libraries were not.

Discussion

Denitrification and nitrification potentials and gene abundance data from Bahía del Tóbari broadly support the Corredor et al. (1999) hypothesis: at the estuary level, ammonia oxidizers are less active and less abundant than denitrifiers, with site 6 as a sole exception where potential nitrification rates were high while denitrification potential was low. Denitrification potentials (6 to 342 mmol N m^{-2} $year^{-1}$) were consistent with mean annual denitrification rates measured in ten different estuaries using five different methods, which ranged from 20 to 1,100 mmol N m^{-2} $year^{-1}$ (compiled in Piña-Ochoa and Álvarez-Cobelas 2006). They also fall within the wide range reported from other subtropical estuaries: from 3–80 mmol N m^{-2} $year^{-1}$ throughout the Fitzroy estuary, to 70–260 mmol N m^{-2} $year^{-1}$ in Corpus Christi Bay (Bernot et al. 2003), to a mean annual rate of 547 mmol N m^{-2} $year^{-1}$ in Apalachicola Bay (Mortazavi et al. 2000). Temperature can directly influence denitrification rates (Dawson and Murphy 1972) but was not significantly related to denitrification potentials in Tóbari ($r^2=0.707$, ANOVA $P=0.108$). [NH_4^+], [NO_3^-], and temperature were all poor predictors of denitrification and nitrification potentials (all $r^2=0.008$–0.209, all ANOVA $P>0.146$).

At the single site where nitrification potential was detected, it was 37 times greater than denitrification potential. This suggests that nitrifier communities are able to rapidly metabolize NH_4^+ at site 6. "Negative" nitrification potentials observed at sites 3, 4, and 5 may also obscure active nitrification (if NH_4^+ is rapidly nitrified but NO_3^- is subsequently consumed). However, I specifically sampled during agricultural runoff and high NH_4^+ flux, and potential measurements likely overestimate nitrification under in situ conditions. This suggests that nitrification is limited within Tóbari sediments—or at least occurs at slower rates than N uptake—at all but site 6. At

sites 3–5, there are at least three possible explanations for the uptake of N measured in nitrification potentials: (1) assimilation and immobilization of N by microbial communities, (2) assimilation by photosynthetic organisms, or (3) removal of nitrate on anaerobic microsites within sediment particles (e.g., Woebken et al. 2007). Neither of the first two possibilities was directly examined, but microbial N assimilation was observed in mangrove sediments in Terminos Lagoon, Mexico (Rivera-Monroy and Twilley 1996), as well as in meta-analysis of estuaries and other ecosystems (Taylor and Townsend 2010). Taylor and Townsend (2010) found that availability and accumulation of dissolved N is uniformly controlled by the nutrient requirements of microorganisms across different types of ecosystems, including estuaries. Benthic microalgae have also been shown to outcompete AOB for N in fjord sediments (Risgaard-Petersen et al. 2004b). An interesting point is that the rate of uptake was similar to measured denitrification potentials at the same sites (0.24–0.65 nmol N h^{-1} cm^{-3}), suggesting that the nitrate removal is due to denitrification on anaerobic microsites. Regardless of the mechanism, this implies measurable N demand within Tóbari sediments.

nirS abundance was substantially greater than archaeal *amoA* in Tóbari sediments, *nirK* was amplified in a single sample, and bacterial *amoA* was amplified in two samples. The fact that denitrifiers outnumber AOA is consistent with the Corredor et al. (1999) hypothesis, while the dominance of *nirS*-type denitrifiers and AOA is consistent with observations from the Fitzroy estuary (Abell et al. 2010). These results are further consistent with AOA outnumbering AOB in many ecosystems (reviewed by Francis et al. 2007 and Erguder et al. 2009), as well as with *nirS*-type denitrifiers being more widespread than *nirK*-type denitrifiers (Zumft 1997; Santoro et al. 2006; Mosier and Francis 2010). This likely reflects differences in ecological strategies or niches among these groups. In contrast to previous studies that identified salinity as a strong control on AOA abundance (Santoro et al. 2008; Mosier and Francis 2008), salinity was not significantly related to AOA abundance (r^2=0.457, ANOVA P=0.210). This may reflect the narrow variations in salinity observed in this estuary and could be a distinguishing factor affecting microbial communities in subtropical estuaries. Temperature was significantly and inversely related to archaeal *amoA* abundance (r^2=0.790, ANOVA P<0.042). Overall, AOA abundances (10^5–10^6 *amoA* genes g^{-1}) were lower than those observed in the Fitzroy estuary (10^7–10^8 *amoA* genes g^{-1}) but comparable to those in San Francisco Bay (10^4–10^7 *amoA* genes g^{-1}), and Huntington Beach, CA, USA (10^4–10^6 *amoA* genes g^{-1}). While drawn from a limited number of estuaries, this indicates that within-estuary variations in AOA abundance are often greater than cross-system variations and likely reflects the fact that estuaries are complex systems. Given wide variations in salinity, sulfide, nutrient concentrations, sediment texture, and concentration and lability of organic matter across relatively short distances, estuaries may encompass a series of distinct habitats for microorganisms rather than a continuum.

Previous studies have reported qualitative and quantitative relationships between nitrifier and/or denitrifier community structure—composition or abundance—and N cycling, e.g., biogeochemical shifts, potential denitrification rates, or in situ denitrification rates (reviewed by Wallenstein et al. 2006; Erguder et al. 2009). Based on *amoA* genes, AOA abundance correlates with nitrification rates (Caffrey et al. 2007; Lam et al. 2007; Beman et al. 2008), and based on *nirS*/*nirK* genes, denitrifier abundance has been identified as a correlate of denitrification rates (O'Connor et al. 2006; Hallin et al. 2009; Mosier and Francis 2010). There was no significant relationship between the abundance of archaeal *amoA* and bacterial *nirS* and nitrification and denitrification potentials in Tóbari at the time of our sampling (all ANOVA P>0.05). The clearest example of this was site 2, where *amoA* and *nirS* genes were most numerous but no significant rates of nitrification or denitrification were observed (N_2O concentrations did increase within a denitrification potential incubation, but the regression P=0.15). However, site 2 is also a clear example of the difficulty of linking microbial community ecology and activity in estuaries: due to the heterogeneity of estuarine sediments, there was greater variation within cores collected at site 2 than across the entire estuary. One promising approach is to use estuarine sediment core "transplants" to isolate the effects of composition and environment on microbial activity. In the Nonesuch River, Reed and Martiny (2013) demonstrated that microbial community composition has direct effects on function that are independent from the effects of environmental differences alone.

Reed and Martiny's (2013) measurements included nitrification, and their findings indicate that different microbial communities exposed to the same environmental change do not respond identically. Understanding these responses in additional tropical and subtropical coastal and estuarine systems is crucial, as these regions will receive the most dramatic increases in N inputs over the next few decades (Galloway et al. 2008). In the case of Tóbari, large amounts of N fertilizer are applied to Yaqui fields, a large proportion of applied N is lost (Matson et al. 1998), and a relatively small proportion of lost N reaches Tóbari (Ahrens et al. 2008, 2012). This N arrives predominantly as NH_4^+ in discrete pulses and high concentrations, when it may be cycled or assimilated by microbial communities, but first must be nitrified before it may be removed via denitrification. AOA and bacterial denitrifiers displayed distinct patterns within Tóbari, including different rates and patterns of activity, contrasting patterns in community structure, and different levels of abundance. The fact phytoplankton blooms occur offshore in the Gulf of California following fertilization/irrigation in the Yaqui Valley (Beman et al. 2005) provides further support for the Corredor et al. (1999) hypothesis that nitrification is inhibited in tropical and subtropical estuaries, as lack of N removal in

Tóbari would allow more N to be exported to the Gulf of California. In Apalachicola Bay, denitrification rates estimated from nutrient budgets were variable and sometimes quite high, yet total N removal was estimated at only 9 % of annual N input (Mortazavi et al. 2000). In the Fitzroy estuary, denitrification rates were generally lower than other estuaries and were attributed to a short residence time and low organic matter loading, among other factors (Abell et al. 2010). Along with my findings, these studies indicate that for subtropical and tropical estuaries, the removal of N by coupled nitrification–denitrification differs in fundamental ways from patterns observed in temperate systems.

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