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Biogeochemical processes in a small California estuary. 2. Nitrification activity, community structure and role in nitrogen budgets

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ABSTRACT: Nitrification rates, bacterial abundance and productivity, and the diversity of ammonia-oxidizing bacteria were measured at 5 sites in Elkhorn Slough, a small estuary in central California, between 1997 and 1999. Of the sampling sites, 2 received high nutrient runoff from agricultural fields and other non-point sources, and 3 received runoff from grasslands and woodlands. The diversity of ammonia-oxidizing bacteria in terms of DNA sequences was investigated at 3 of the sites in August 1998. Both 16S and *amoA* sequences from sediment samples were more closely related to those of *Nitrosomonas marina* than to any other cultured nitrifier, but there was considerable diversity within the group, and site-specific patterns were not detected. Potential nitrification was seasonally and spatially variable, with the highest rates occurring at the head of Elkhorn Slough in late winter and fall. Bacterial productivity was highest during the summer and generally higher at a site adjacent to grasslands compared to the 2 agricultural sites. The variability in nitrification rates was not directly correlated with hydrographic and environmental variables. Physical factors may affect nitrification rates indirectly by controlling salinity and bottom-water oxygen concentrations. Potential nitrification rates were positively correlated with pore water NH_4^+ concentrations under well flushed conditions but were negligible in the presence of high NH_4^+ concentrations under poorly flushed conditions, due to low oxygen availability, hypersaline conditions or both. Sediment nitrogen budgets for 3 of the sites suggested that denitrification removed about 25% of the mineralized nitrogen at the poorly flushed site, but 50% or more at well flushed sites. Poorly flushed systems appear to be less efficient at removing high nitrogen inputs than well flushed systems because of the low rates of coupled nitrification-denitrification in the former, implying that physical factors such as the flushing regime or residence time can significantly affect nitrogen removal by denitrification.

KEY WORDS: Estuary · Nitrification · Nitrifying bacterial diversity · Eutrophication · Sediment

INTRODUCTION

Nitrification is the sequential oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) and then to nitrate (NO_3^-), predominantly by chemolithotrophic bacteria. Nitrification represents an important link in the nitrogen cycle between the mineralization of organic matter (production of NH_4^+) and the loss of fixed nitrogen by denitrification. Although molecular oxygen is required for both NH_4^+ and NO_2^- oxidation, nitrifiers are often reported to tolerate, and actively grow under, low oxygen conditions. In estuarine sediments, nitrification is restricted to the oxygenated zone at the sediment-water interface. NO_2^- and NO_3^- produced during nitrification can diffuse into the water and be used by phytoplankton or, more likely, be denitrified in underlying, anoxic sediments. In many estuarine sediments, NO_3^- production by nitrification controls rates of denitrification (Jenkins & Kemp 1984, Kemp et al. 1990, Rysgaard et al. 1999). In estuaries with water column

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hypoxia or anoxia, nitrification may be eliminated by the lack of oxygen (Kemp et al. 1990) or as a result of sulfide inhibition of nitrification (Joye & Hollibaugh 1995).

Nitrifying bacteria comprise 2 functionally distinct groups in the Proteobacteria: ammonia oxidizers and nitrite oxidizers. Approximately 25 ammonia-oxidizing and 8 nitrite-oxidizing strains are in culture (Koops & Pommerening-Roser 2001), but a greater diversity of strains has been detected in various soil and aquatic environments using molecular ecological approaches to characterize DNA sequences obtained from the environment. In this study, both 16S rRNA genes and the functional gene, ammonia monooxygenase (*amoA*), were used to characterize the ammonia-oxidizing bacteria (AOB) at sites where nitrification was measured directly. This approach should make it possible to link community composition and microbial diversity to the ecosystem processes performed by a particular functional group; in this case, nitrifiers.

Our study site, Elkhorn Slough, is a small estuary extending inland for 11.4 km from Monterey Bay on the central California coast. Seasonal and spatial distributions of nutrients, benthic nutrient fluxes and hydrography were reported for 5 sites representing different nutrient inputs and flushing regimes in Elkhorn Slough by Caffrey et al. (2002). We expected that patterns in potential and *in situ* nitrification rates should vary along the estuarine gradient in response to oxygen and ammonium concentrations. We hypothesized that different groups of nitrifiers might be associated with different environments within the slough and that diversity might relate to measured rate patterns. Here we report nitrification rates, bacterial abundance and bacterial production rates, and AOB diversity at the same sites (Caffrey et al. 2002). We interpret the spatial and temporal variation in rates in relation to chemical and physical factors, as well as in relation to nutrient runoff and fluxes at these sites and in similar regimes. A simple budget for 3 of the sites was computed in order to link the measured distributions, processes and fluxes.

MATERIALS AND METHODS

Site description. Sampling was carried out at 5 sites in Elkhorn Slough, California, an estuary with seasonal freshwater inputs and a seawater connection to Monterey Bay (Fig. 1). Two of the sites (Lower Azevedo Pond and Hudson's Landing) receive runoff from agricultural fields and other non-point-source runoff, and are characterized by high dissolved inorganic nitrogen (DIN) concentrations (Table 1). Lower Azevedo Pond (referred to in the text as Azevedo Pond) is non-tidal, while the Hudson's Landing site is a tidal creek near the head of Elkhorn Slough. The other 3 sites (Hidden Pond, Hummingbird Island, Vierra Mudflat) do not receive direct, non-point-source runoff and have lower DIN concentrations (Table 1). Hidden Pond, which has minimal tidal flushing, and Hummingbird Island are located in the Elkhorn Slough National Estuarine Research Reserve (ESNERR), while Vierra Mudflat is near the mouth of the slough. Hummingbird Island and Vierra Mudflat were sampled less intensively and were included in order to represent the main flow regime of the estuary.

Sampling. Samples for nitrification potential and bacterial abundance and production were collected monthly from November 1997 to November 1998 at Hidden Pond, Azevedo Pond and Hudson's Landing. Additional nitrification potential samples were collected in July 1999 at Hidden Pond and Azevedo Pond, while Hudson's Landing, Hummingbird Island and Vierra Mudflat were sampled in February, March, July and December of 1999. The slough experiences a mixed semidiurnal tide. Sampling was performed at lower low tide whenever possible, but in 8 cases (e.g. when lower low tide occurred at night), sampling was performed at higher low tide. Shallow cores (3 cm deep, 12 cm in diameter) were collected and returned to the lab in coolers for determination of potential nitrification and bacterial abundance and productivity. Triplicate cores (6 cm diameter) were collected at 2 stations for the intact-core nitrification measurements. During removal and transport, careful handling of the cores ensured that the structure of the sediment was preserved. Samples were processed promptly at the lab (within 2 h of collection), except for the intact-core nitrification experiments. The overlying water was gently aerated while these cores were held in a controlled temperature room at ambient temperature for

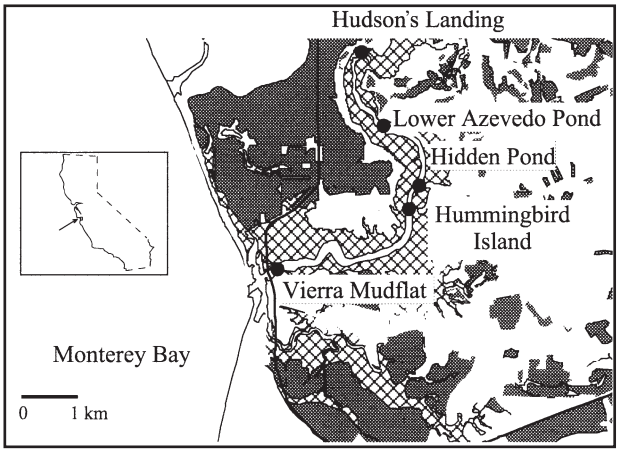

Fig. 1. Map of Elkhorn Slough showing location of sites, marsh (crosshatch) and agricultural lands (stippled)

24 h until experiments were begun. Overlying or nearby water was collected during sampling for use in experiments and for nutrient analyses. Temperature and salinity data were obtained at the time of sampling using a YSI salinometer.

Potential nitrification rate. The method of Henriksen et al. (1981) was adapted to measure the nitrifying capacity of the sediment microbial community. Cores were subsampled to a depth of 5 mm using a cut-off beveled glass syringe corer. On 2 occasions (March and July 1999), 5 to 10 and 10 to 20 mm depth intervals were assayed for potential nitrification activity. Five replicates were incubated for each site in 1997 to 1998; 3 replicates were incubated for each site in 1999. Sediments (0.3 to 0.5 g wet wt) were added to either seawater or slough water amended with 0.5 mM NH_4Cl in a total volume of 100 ml (November 1997 to March 1998) or 50 ml (April 1998 to December 1999). Slurries were incubated in the dark at ambient temperatures and shaken at 200 rpm. After 30 min and 24 h, aliquots were filtered through a GFC filter and frozen for later nutrient analysis. Potential nitrification rates are expressed in terms of nmol NO_2^- cm^{-3} h^{-1} after accounting for the bulk density of the sediment.

Intact-core nitrification rate. The labor-intensive nature of these assays precluded making them at all stations or on the monthly schedule that was followed for the potential nitrification rates. Incubations were performed at Hudson's Landing on 7 occasions (March, April, May and November 1998, and March, July and November 1999), at Azevedo Pond in 1998 and at Vierra Mudflat in 1999.

Nitrification in cores was measured using acetylene, a nitrification inhibitor (Sloth et al. 1992, Caffrey & Miller 1995). The overlying water was replaced with the collected water before beginning the experiments, and cores were sealed with caps equipped with a magnetic stirring system and sampling ports. Cores were incubated in the dark at ambient temperatures. Samples for determination of NH_4^+ concentration were taken every hour for 8 h and frozen for later analysis. After 4 h, acetylene was added to the cores to inhibit nitrification, and a headspace was established to minimize changes in O_2 concentrations in cores during the second half of the incubation. Samples for O_2 were taken throughout the incubation to monitor oxygen concentrations in the water overlying the sediment, and analyzed by Winkler titration. Fluxes were calculated by linear regression of the NH_4^+ accumulation in the overlying water in individual cores before and after inhibition. Nitrification rates in individual cores were calculated as the difference between NH_4^+ fluxes measured before and after inhibition.

Bacterial productivity. In the lab, cores were subsampled with beveled 5 ml glass syringes to a depth of 5 mm. Sediments (5 replicates from each site, 0.35 to 0.5 g wet wt) were placed in 15 ml conical tubes and 1 ml of 0.2 µm filtered seawater or slough water was added. Bacterial productivity was measured using the protocol of Hogan & Ward (1998), which was optimized for Elkhorn Slough sediments by adaptation from the method described by Moriarty & Pollard (1990), using 2 h incubations and total thymidine additions of 30 nM. ^{3}H-thymidine incorporation was converted to mg C m^{-2} h^{-1} using the conversion factors for similar environments given by Austin & Findlay (1989).

Bacterial abundance. The top 5 mm of sediment was subsampled and preserved for staining with DAPI (Porter & Fieg 1980). The protocol was modified for staining of sediment samples by suspending the sediments in 10 ml of 0.2 µm filtered phosphate buffered saline (PBS: NaCl, 8 g; KCl, 0.2 g; Na_2HPO_4, 1.15 g; KH_2PO_4, 0.2 g; distilled water, 1.0 l; pH = 7.3). The suspended sediment was preserved with formalin (2% final concentration, filtered and sodium borate-buffered) and stored at 4°C until staining (within 1 wk). The slurry was diluted (200 ml into 0.8 ml PBS) and DAPI was added to a final concentration of 25 µg ml^{-1} and allowed to react for 5 min in darkness. Samples were filtered onto polycarbonate filters. Bacteria on slides were counted at 1600× on a Zeiss Axioscope epifluorescence microscope.

Table 1. Characteristics and average water column DIN concentration of sampling sites in Elkhorn Slough

Site	Depth of overlying water	Flushing regime	Land-use adjacent to study site	Average water column DIN concentration[a] (µM)
Azevedo Pond	0 to 30 cm	Non-tidal	Strawberry fields	47.0
Hudson's Landing	Intertidal	Tidal flushing	Strawberry fields, flowers, truck crops, cattle pasture, town	98.8
Hidden Pond	Shallow subtidal (several cm)	Muted tidal flushing	Grassland	12.6
Hummingbird Island	Intertidal	Tidal flushing	Grassland, woodland	14.1
Vierra Mudflat	Intertidal	Tidal flushing	Grassland, woodland	12.0

[a]From Caffrey et al. (2002)

Ammonia-oxidizing bacterial diversity. Reference strains of AOB were maintained in pure culture as described by Ward & Carlucci (1985), using freshwater (Soriano & Walker 1968) or seawater (Watson 1965) medium as appropriate. High molecular weight bacterial genomic DNA was obtained from cells harvested from 1 l cultures and purified as described by Voytek & Ward (1995).

Using sterile cut-off glass syringes, 3 replicate cores were obtained from each of the 3 main sites (Hidden Pond, Azevedo Pond, and Hudson's Landing). Syringes containing the cores were frozen on dry ice in the field and transported to the lab for extraction of nucleic acids. The upper 5 mm of each core was suspended in 0.8 ml 0.5 M Na_2EDTA, and stored at −80°C. DNA was extracted from the sediment cores and purified using lysozyme, SDS and proteinase K treatment and phenol/chloroform extraction, followed by CsCl equilibrium density gradient centrifugation (DeLong 1992, DeLong et al. 1993). CsCl purification was necessary to obtain PCR quality DNA due to the high organic/humic content of the sediments.

PCR amplification and sequence analysis of 16S rRNA genes and the gene for ammonia-monooxygenase (*amoA*) were used to evaluate the diversity of ammonia-oxidizing bacteria in sediment samples. The PCR primer sequences are given in Table 2. Two primer sets targeting the 16S rRNA genes, the NitAB primers (Voytek & Ward 1995) and the β-Amo-f/Nso-r primers (McCaig et al. 1994, Mobarry et al. 1996), were designed to be specific for all 9 described ammonia oxidizers within the β-subdivision of the Proteobacteria (Woese et al. 1984, Head et al. 1993). General bacterial primers (GM5f, Muyzer et al. 1993; and DS907r, Teske et al. 1996) were used to verify that sample DNA was of PCR amplification quality. The primers of Rotthauwe et al. (1995) AmoA-1f and AmoA-2r were used to amplify a portion of the *amoA* gene. Amplification conditions for all of these primer sets were optimized according to the method of Cobb & Clarkson (1994), resulting in only slight modifications of the PCR protocols recommended by the authors cited above. Amplification was performed on 1 µl (50 to 100 ng DNA) of purified DNA extracts.

DNA from all 3 sites in August 1998 was analyzed using the β-Amo-f/Nso-r primers. DNA from Azevedo Pond in August was also analyzed using the NitAB and the AmoA-1f/AmoA-2r primers. For every PCR experiment, both positive (*Nitrosomonas europaea*) and negative (no DNA) controls were included. PCR amplified fragments were resolved by electrophoresis of the reaction mixture on 1% (wt vol) horizontal agarose minigels in 1× TAE buffer.

PCR products from NitAB, β-Amo-f/Nso-r and AmoA-1f/AmoA-2r amplifications were cloned into the pCR2.1 vector according to the manufacturer's instructions (Invitrogen). Plasmid DNAs containing inserts were isolated for sequencing by alkaline lysis procedures (QIAprep spin columns, Qiagen) or by direct PCR of recombinant clones using vector specific primers (Invitrogen). Plasmid templates were sequenced using forward and reverse vector primers (T7, M13) with an ABI 310 DNA sequencer (Big Dye-Terminator Cycle Sequencing Ready Reaction FS kit, PE Applied Biosystems).

Sequences were initially compared to the available databases using the BLAST network service (Altschul et al. 1990) to determine phylogenetic affiliation and orientation. The program Check_Chimera (RDP, Maidak et al. 1999) was used to analyze sequences for the presence of chimeric PCR artifacts. Sequences were then compiled in Sequence Navigator (PE Applied Biosystems) and aligned with representative nitrifier sequences from the Ribosomal Database Project. Evolutionary distance, parsimony and maximum likelihood analyses were performed on the aligned sequences using PHYLIP 3.5 (Felsenstein 1989). Neighbor-joining phylogenetic trees were constructed, and bootstrap values from 100 pseudoreplicates were obtained for the consensus trees. Nucleotide sequence accession numbers: The partial 16S rRNA and *amoA* gene sequences from the environmental samples were deposited in GenBank with accession numbers AY186207–AY186227 and AY186228–AY186238 respectively.

Table 2. Table of primers for 16S rRNA genes and ammonium mono-oxygenase (*amoA*) gene used in PCR amplification

Primer	*Escherichia coli* position	Sequence (5'-3')	Target sequence	Source
GM5f	341–357	CCTACGGGAGGCAGCAG	Bacterial 16S	Muyzer et al. (1993)
DS907r	908–929	CCCCGTCAATTCCTTTGAGTTT	Bacterial 16S	Teske et al. (1996)
NitA	136–158	CTTAAGTGGGGAATAACGGCATCG	β-AOB 16S	Voytek & Ward (1995)
NitB	1216–1236	TTACGTGTGAAGCCCTACCCA	β-AOB 16S	Voytek & Ward (1995)
β-amo-f	143–162	TGGGGRATAACGCAYCGAAAG	β-AOB 16S	McCaig et al. (1994)
Nso-r	1228–1246	CGCCATTGTATTACGTGTGA	β-AOB 16S	Mobarry et al. (1996)
AmoA-1f	–	GGGGTTTCTACTGGTGGT	amoA	Rotthauwe et al. (1997)
AmoA-2r	–	CCCCTCKGSAAAGCCTTCTTC	amoA	Rotthauwe et al. (1997)

Analytical techniques. Nutrient analyses (NO_2^- and NO_3^-) were performed on samples from November 1997 to March 1998 using a manual cadmium reduction method (Jones 1984), and an unreduced fraction was analyzed for nitrite (Strickland & Parsons 1972). For nitrification assays from April 1998 to December 1999, NO_2^- and NO_3^- were analyzed with a Lachat auto analyzer using standard seawater methods provided by Lachat. Ambient water samples from each site were also analyzed for NH_4^+, NO_2^- and NO_3^- by auto analyzer. Oxygen samples were fixed with Winkler reagents and stored in 7 ml glass bottles. Oxygen concentrations were measured within 4 h of collection using the starch endpoint titration method (APHA 1985). Analytical methods for some additional variables were reported previously (Caffrey et al. 2002) and some of those results are incorporated into the discussion here.

Analysis of variance was used to investigate the relationships among sites and dates. Pearson's correlation coefficient was used to examine relationships among rates and constituents such as temperature, salinity, overlying water and pore water nutrient concentrations (from Caffrey et al. 2002). Microsoft Excel and Systat were used to do the calculations.

RESULTS

Hydrography

Water temperatures at the sampling sites ranged from 7°C in December to 30°C in July (Fig. 2a). Salinity showed considerable site-to-site differences, although consistent seasonal trends occurred at all sites (Fig. 2b). Salinity was generally lowest and near 0 during the winter rainy season and highest in the late summer and fall before the onset of winter rains (Fig. 2b). A peak salinity of 35 occurred in October 1998 at Hudson's Landing near the head of Elkhorn Slough. Azevedo Pond and Hidden Pond also exhibited salinity maxima in October 1998, at 66 and 36.5 respectively. The salinity minima observed at all sites in February 1998 were associated with extreme rainfall and runoff events caused by El Niño storms at that time. Because of the sampling schedule for access to the sites, salinity was measured at low tide, the period of greatest freshwater influence. Tidal variations in salinity can be extreme during the rainy season, ranging up to 15 following rain events, although the summer tidal variations in salinity rarely exceed 2 to 3 (Wenner et al. 2001).

Nitrification

Potential nitrification rates were most variable and usually highest at the head of the slough, Hudson's Landing, with peak rates occurring in February and September 1998 (Fig. 3a). Rates at the other agricultural site, Azevedo Pond, were consistently low, sometimes as much as 100-fold lower than the maximum rates observed at Hudson's Landing. Rates at Hidden Pond, an ESNERR site, were highest in winter and spring of 1998 and relatively low the rest of the time. In 1999, potential nitrification was much lower than in 1998, with highest rates occurring at ESNERR sites (Hummingbird Island and Hidden Pond) (Fig. 3a). As in 1998, rates at Hudson's Landing were variable, although much lower in 1999. Periods of peak nitrification might have been missed in 1999, given the

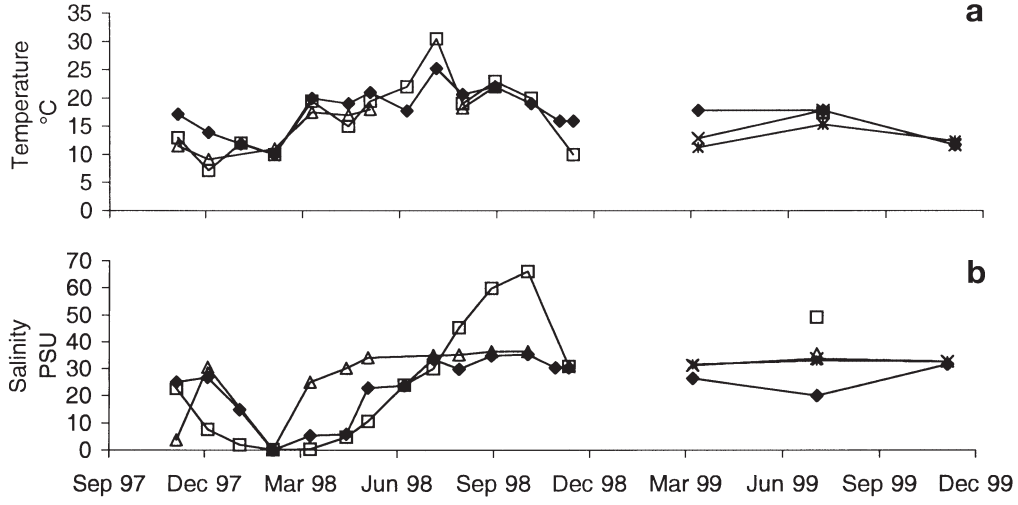

Fig. 2. (a) Temperature and (b) salinity at Elkhorn Slough sampling sites between November 1997 and December 1999. Azevedo Pond (□), Hudson's Landing (◆), Hidden Pond (△), Hummingbird Island (×), Vierra Mudflat (✳)

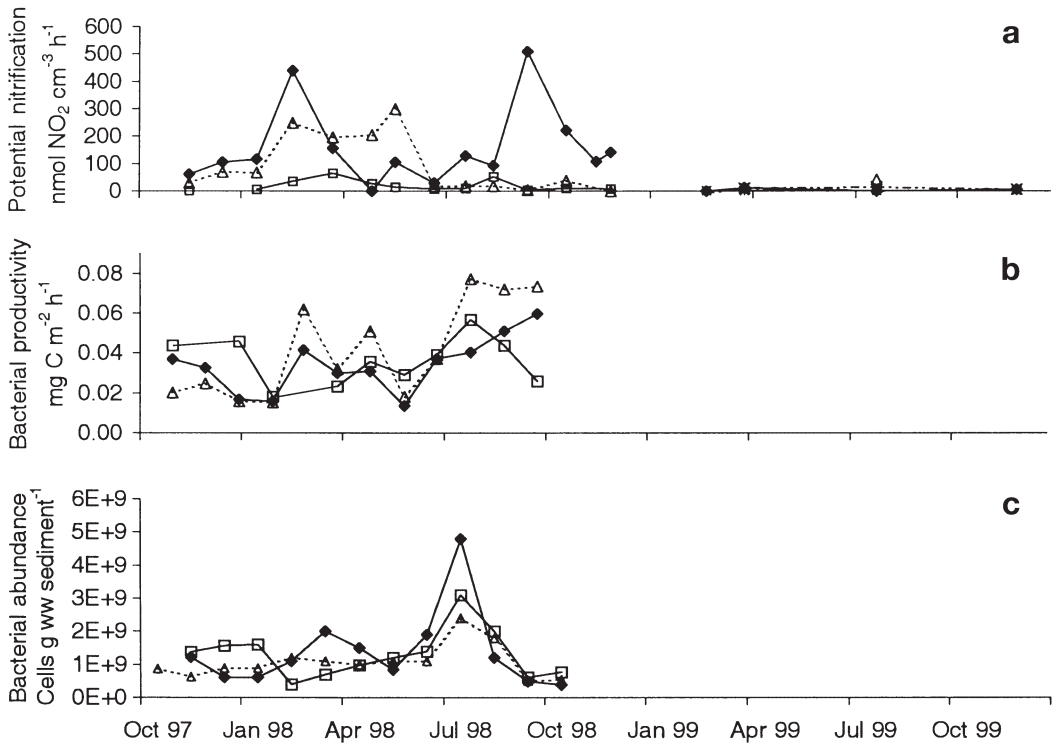

Fig. 3. (a) Potential nitrification (nmol NO_2^- cm^{-3} h^{-1}), (b) bacterial productivity (nmol ^{3}Hthy g wet wt^{-1} h^{-1}) and (c) bacterial abundance (cells g wet wt^{-1}) at Elkhorn Slough sampling sites between November 1997 and December 1999. Azevedo Pond (□), Hudson's Landing (◆), Hidden Pond (△), Hummingbird Island (×), Vierra Mudflat (✳)

reduced sampling frequency compared to the monthly measurements made in 1998. In addition, interannual climatic variations may also have contributed to the observed differences, with 1998 being very wet and relatively warm compared to 1999 (Fig. 2a). Consistently low rates were measured near the mouth of the slough (Vierra Mudflat). An ANOVA showed that potential nitrification rates differed significantly among sites and dates ($p < 0.001$). On the 2 occasions when depth profiles of potential nitrification were measured, there was no significant difference ($p = 0.2$) in rates at the different depths (data not shown). There was no significant correlation between potential nitrification rates and temperature, salinity, or pore water NH_4^+ concentration at any of the sites.

Because of the different sampling schedules, direct comparisons between sites of potential and intact core nitrification measurements are limited, but where comparisons are possible, results are largely consistent. Nitrification rates measured in intact-core incubations were highest at the head of the slough (Hudson's Landing), compared to the other agricultural site (Azevedo Pond) or the mouth of the slough (Vierra Mudflat) (Table 3). Both potential and intact-core nitrification rates were negligible at Hudson's Landing in April 1998, and both rates at Hudson's Landing exceeded those at Azevedo Pond. In contrast to the potential nitrification measurements at Vierra Mudflat, measurements in intact cores were variable at this site, with the highest rate occurring in March 1999 and rates decreasing over subsequent sampling periods. Intact-core rates at Azevedo Pond and Vierra Mudflat cannot be compared directly because they were not measured at the same time.

Table 3. Nitrification (mmol m^{-2} d^{-1}) in intact cores at Azevedo Pond, Hudson's Landing, and Vierra mudflat. Mean (SE), n = 3, nd = no data

Date	Azevedo Pond	Hudson's Landing	Vierra Mudflat
1998			
March	0.0	1.7 (1.1)	nd
April	0.0	0.0	nd
May	0.4 (0.4)	1.7 (3.1)	nd
November	nd	5.5 (1.1)	
1999			
March	nd	4.1 (3.9)	2.9 (0.1)
July	nd	9.0 (6.4)	0.4 (0.5)
November	nd	2.5 (2.0)	0.0 (0.1)

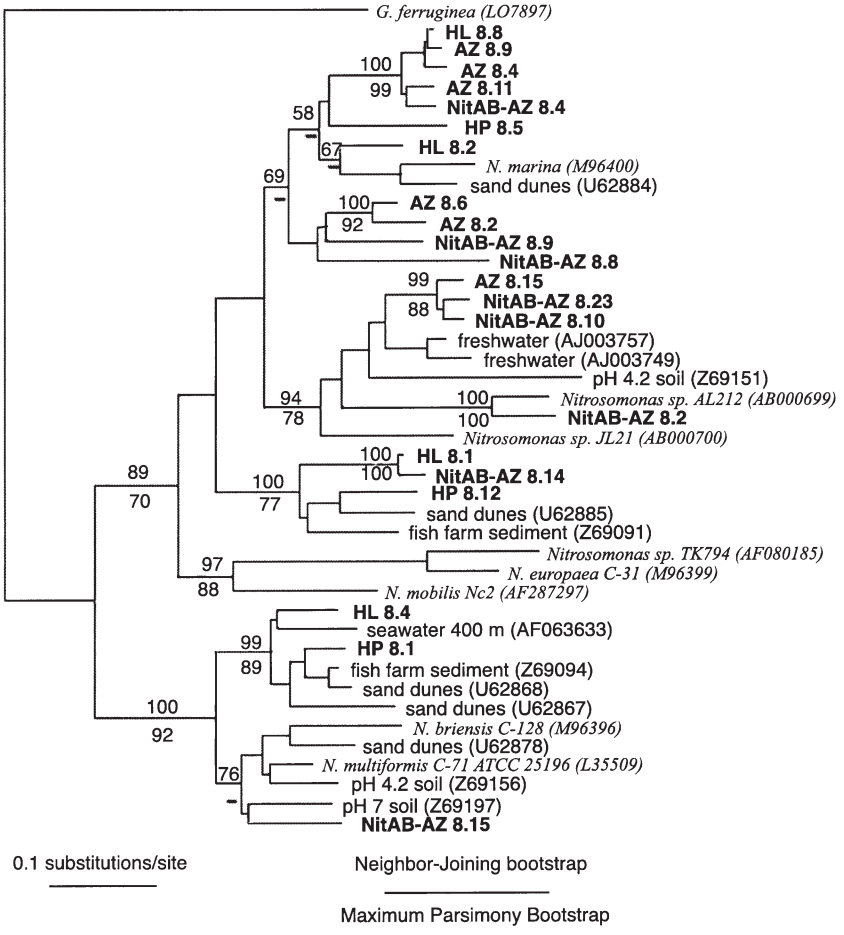

Fig. 4. Neighbor-joining distance tree based on 16S rDNA analysis (1062 bp). Clones obtained with β-Amo-f/Nso-r primers: HL = Hudson's Landing; AZ = Azevedo Pond; HP = Hidden Pond. Clones obtained with NitAB primers: NitAB-AZ. Accession numbers for sequences obtained from GenBank in parentheses. Bootstrap values greater than 50% shown

Bacterial production and abundance

Hidden Pond exhibited the most seasonal variability in bacterial production, with a minimum in winter and high rates in spring and late summer (Fig. 3b). Rates at all 3 sites were minimal in Feb 1998, when relatively high potential nitrification rates were observed. Bacterial production rates exhibited another minimum in June 1998, when nitrification potentials at all 3 sites were also very low.

The main feature of the bacterial abundance distribution is a peak in July 1998 (Fig. 3c). Abundances were overall relatively high, $>10^9$ cells g^{-1} of sediment (wet wt), reaching a maximum of nearly 5×10^9 g^{-1} at Hudson's Landing in July and decreasing to 5×10^8 g^{-1} by October.

When bacterial production rates are normalized to cell number, the variability in rates is minimized for most of the year, and the main feature of the distribution is a maximum in per cell rates in the late summer/fall of 1998. The maximum represents an approximately 3-fold increase at all 3 sites and is due mostly to the disproportionately greater increase in total production rates compared to cell numbers.

Diversity of ammonia-oxidizing bacteria

Sixty 16S rRNA clones (15 obtained from amplification with the β-Amo-f/Nso-r primers from each of the sites in August 1998, and 15 cloned NitAB fragments from Azevedo Pond in August 1998) were screened by sequencing the first 350 bp of each clone from each direction. Thirty unique clones were chosen for complete sequence analysis. Three of these clones were rejected as non-β group species and 6 were rejected as potential chimeric sequences based on uncertain positioning within the tree in conjunction with Check_Chimera results. Fifteen *amoA* clones from Azevedo Pond in August 1998 were sequenced in their entirety. A total of 21 unique 16S rDNA sequences (approximately 1100 bp) and 11 unique *amoA* sequences (449 bp) were obtained.

On the basis of 16S rDNA sequences (1062 bp), most of the ammonia-oxidizer sequences detected in Elkhorn Slough fell into a cluster that includes the cultured *Nitrosomonas* strains, and of all the cultured strains appeared to be most closely related to *Nitrosomonas marina* (Fig. 4).

Three of the sequences grouped in a cluster containing *Nitrosolobus multiformis* and *Nitrosospina briensis*. Thirteen of the sequences were obtained with the β-Amo-f/Nso-r primers and 8 with the NitAB primers; both sets of sequences were distributed similarly throughout the *Nitrosomonas* and *Nitrosolobus* clusters.

AmoA sequences obtained from Azevedo Pond exhibited phylogenetic affinities that were consistent with those deduced from the 16S sequences. Three of the Azevedo Pond *amoA* sequences clustered with *Nitrosospira* and *Nitrosospina*, and with environmental sequences obtained from rice roots (Rotthauwe et al. 1997). The remaining 8 *amoA* sequences were most closely related to the *amoA* sequence from *Nitrosomonas marina* (Fig. 5).

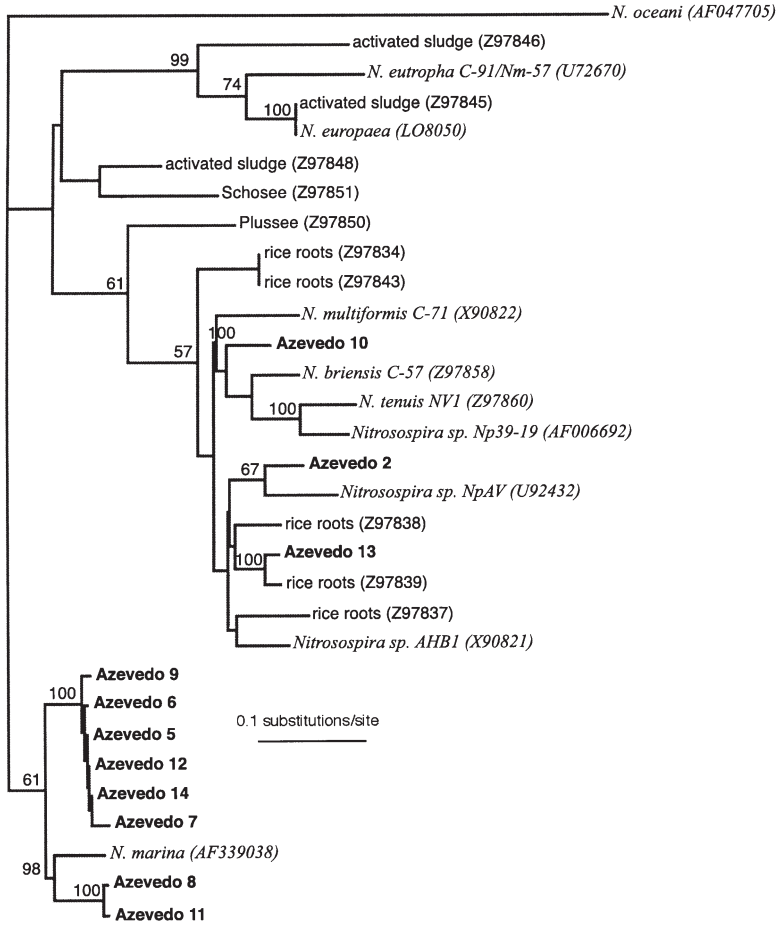

Fig. 5. Neighbor-joining distance tree based on *amoA* DNA analysis (330 bp). Clones obtained with AmoA-1f/AmoA-2r primers from Azevedo Pond are designated Azevedo (bold). Accession numbers for sequences obtained from GenBank in parentheses. Bootstrap values (neighbor-joining only) greater than 50% shown

DISCUSSION

Bacterial productivity was positively correlated with temperature at all sites (r = 0.72 to 0.82), which could simply be related to the positive effect of temperature on productivity and metabolic processes in general. Temperature was found to be a controlling factor for heterotrophic bacterial production in Chesapeake Bay at temperatures <20°C (Shiah & Ducklow 1994). Above 20°C, productivity was not well correlated with temperature and it was suggested that productivity was then regulated by substrate supply. Temperature exceeded 20°C at Elkhorn Slough for most of the summer, so temperature could be limiting in the winter months (December to February), when it was well below 20°C. Bacterial productivity was not correlated with total organic carbon content (Harrington 1999), perhaps because a large portion of this carbon is recalcitrant. Measurements of dissolved organic carbon might be more useful in determining whether productivity is governed by carbon availability.

Factors controlling the abundance of bacteria in sediments are poorly understood. Abundance may in part be determined by the amount of pore water in the sediments. Schmidt et al. (1998) found that bacterial abundances in sediments were consistent over a variety of sites when normalized to the pore-water volume. The bacterial densities found in this study are similar to those found in other sediments (Schmidt et al. 1998) and reported previously in sediments of Elkhorn Slough (Hogan & Ward 1998, Golet & Ward 2001).

Bacterial abundance and bacterial productivity were not correlated over the course of the year. There was a striking opposite relationship between productivity and abundance in the late summer, when numbers decreased and productivity increased, indicating a large increase in activity per cell (Fig. 3b,c). Such a shift could imply an increase in bactivory, which reduced the population but allowed increased activity on the part of the remaining, but rapidly recycling, cells. In a study of a sandy-sediment microbial community, grazing was not found to significantly effect bacterial populations but productivity did rise to keep pace with grazing pressure (Sundbäck et al. 1996).

The change in productivity per cell could also reflect a major change in the bacterial assemblage composition. Many obligate anaerobic bacteria (including some sulfate reducers, photosynthetic purple sulfur bacteria and methanogens) and most autotrophs (including ammonium oxidizers and nitrite oxidizers) do not take up thymidine for incorporation into DNA (Johnstone & Jones 1989, Wellsbury et al. 1993). These groups could contribute importantly to sediment bacterial assemblages in the oxic/anoxic gradient environments at these sites. A steep reduction in these populations with a smaller increase in the aerobic heterotrophic assemblage could account for this increase in productivity and simultaneous decrease in bacterial abundance. We do not have oxygen data for the sediments throughout the study, but sulfide concentrations were highest and closest to the sediment-water interface at all 3 sites in July (Caffrey et al. 2002). Thus, we would expect the relative abundance of anaerobic bacteria to be greatest at this time, a shift in the opposite direction to that required for this explanation of the change in productivity

per cell. The thymidine method is probably a minimal and variable measure of the activity of the anaerobic component (Winding 1992), and variable oxygen conditions probably contribute to the observed patterns of productivity in Elkhorn Slough.

Diversity of ammonia-oxidizing bacteria

The AOB 16S rDNA sequences obtained from all 3 sites using the β-Amo/Nso primers were most closely related to *Nitrosomonas marina* (Fig. 4). While the freshwater/terrestrial strains (e.g. *Nitrosomonas europaea*) were well separated from our clones in the tree, our clones clustered with other cloned sequences obtained by others from freshwater-lake and sediment environments (Speksnijder et al. 1998). Some of the Elkhorn Slough clones also clustered with sequences derived from sand dunes (Kowalchuk et al. 1997) and activated sludge (Suwa et al. 1997). Thus, even within the cluster related to *N. marina*, considerable diversity is present among the Elkhorn Slough AOB assemblage.

The 3 sequences (HL 8.4, HP 8.1, NitAB-AZ 8.15) that clustered outside the *Nitrosomonas* group were most closely related to strains derived from seawater, marine sediments and soil. It is interesting that no sequences closely related to *Nitrosomonas europaea* were retrieved from this site, especially in light of previous findings (Ward et al. 2000) that several sequences obtained with the NitAB primers derived from Mono Lake, an alkaline saline lake, were closely related to *N. europaea*. These inter-site differences appear therefore to reflect different populations, rather than variability associated with choice of primers.

For the Azevedo Pond site, AOB sequences were obtained using both sets of 16S rRNA primers. The NitAB primers yielded 8, and the β-Amo-f/Nso-r primers yielded 6, unique sequences respectively. Only the NitAB primers yielded clones (NitAB-AZ 8.14 & NitAB-AZ 8.15) that clustered outside of the *Nitrosomonas marina* cluster for Azevedo Pond, but related clones were obtained from the β-Amo-f/Nso-r primers from both Hidden Pond and Hudson's Landing. Thus, the 2 primer sets performed similarly within the constraints of this relatively small comparison.

AmoA phylogenies were compared on the basis of both amino acid (not shown) and DNA sequences. The topology of the 2 trees was almost identical and DNA provides higher resolution (Rotthauwe et al. 1997). The *amoA* sequences correlated well with the 16S rDNA sequences from the same site, in that all but 3 are most closely related to the *Nitrosomonas marina* sequence. Of the other 3, 1 clustered with *Nitrosolobus multiformis*, as did 1 of the 16S sequences; 1 clustered with sequences derived from rice roots. The smaller database of environmental *amoA* sequences precludes a direct comparison of the diversity detected by the 16S and *amoA* genes; the most striking result is their concurrence that organisms related to *N. marina* are probably important in this environment. Neither 16S rRNA nor *amoA* primers used here were capable of detecting *Nitrosococcus oceani*, the only known gamma-subdivision AOB, which is considered to be restricted to the marine environment and might well be present in the slough. Thus, the total AOB community is very likely more diverse than detected in this study.

AOB sequences from Elkhorn Slough reflect a relatively diverse community that was present at a site characterized at the time of sampling by low oxygen concentration and salinity greater than that of normal seawater (Fig. 2b). Further investigation should show whether organisms represented by these sequences are important throughout the year. The large seasonal and temporal salinity gradient among the 3 sites suggests that salinity fluctuations and frequently hypersaline conditions may be important in determining nitrifier community structure. *Nitrosomonas marina* includes strains that are restricted to growth at salinities substantially below that of full strength seawater. Several other isolates, which are nearly identical to *N. marina* in 16S rDNA sequence, are cultivated in full-strength seawater (Ward & Carlucci 1985, Voytek 1996). Several sequences from the database which are very similar to *N. marina* were derived from freshwater and soil sites, but it is not possible at this point to draw conclusions about the halotolerance of organisms associated with particular cloned sequences.

Although the number of clones retrieved was not an exhaustive sampling of the diversity, the fact that only half of the 60 clones initially sequenced were unique implies relatively limited diversity in this environment. There were no discernible patterns among sites. The clones did not cluster by site in terms of 16S rDNA sequences, implying that some of the strains are probably widespread within the slough. Unfortunately, we did not obtain sufficient information on the redundant sequences to perform a rigorous quantification of diversity. In terms of our initial hypothesis that we might find different groups associated with different sites or suites of environmental conditions, it is interesting that we did not find site-specific dominant groups. Lowest nitrification rates were observed at Azevedo Pond, but the assemblage at this site yielded sequences over the same range of diversity as obtained from the other sites. This may imply that nitrifiers can persist under adverse conditions at low activity in the environment, as they are well known to do in culture. It may be that different members of the assemblage are variably active under different conditions. However,

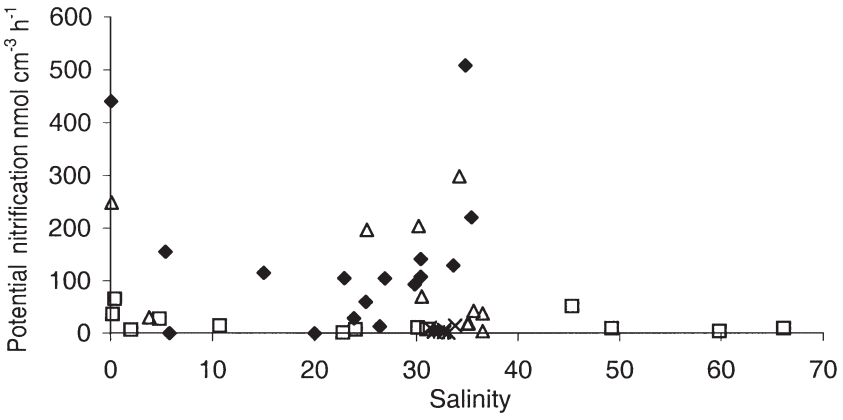

Fig. 6. Potential nitrification (nmol NO_2^- cm^{-3} h^{-1}) versus salinity at Elkhorn Slough sampling sites. Azevedo Pond (□), Hudson's Landing (◆), Hidden Pond (△), Hummingbird Island (×), Vierra Mudflat (✳)

it appears that a diverse assemblage of organisms is responsible for the observed nitrification rates at all 3 sites, with the major groups most closely related to *Nitrosomonas marina*. Environmental factors, which may regulate the activity of individual cells or strains, may exert control on nitrification rates by favoring the activity of different members of the assemblage.

Factors controlling nitrification

Potential nitrification rates differed significantly among the 3 sites ($p \leq 0.001$) and were highly variable both spatially and temporally. No single factor such as temperature, salinity, pore water NH_4^+ or sulfide concentrations explained the seasonal and site variations (ANOVA). However, a combination of these factors may be responsible for the observed patterns. These interactions may obscure the simple dependence on ammonium and oxygen concentrations, which we initially hypothesized would control nitrification rates. Elkhorn Slough experiences a wide variation in salinity over tidal and annual cycles. Extreme salinities occur in tidal ponds with restricted circulation, such as Azevedo Pond, where salinities reached 66. Highest potential nitrification rates in the slough occurred at either 0 or 34 PSU (Fig. 6). Previous studies with estuarine sediments and *Nitrosomonas* isolates from freshwater and estuarine sediments reported optimal nitrification rates at 0 PSU (Rysgaard et al. 1999), at 5 to 10 PSU (Jones & Hood 1980) and at 10 to 25 PSU (Helder & DeVries 1983). Nitrification was inhibited at salinities >25 PSU (Jones & Hood 1980, Helder & De Vries 1983). The bimodal peak in potential nitrification rates in Elkhorn Slough may suggest different community composition at different salinities, i.e. a seasonal succession. Extension of our molecular diversity investigation to additional months in the time series could address this possibility.

In addition to the high salinity during summer and fall, oxygen concentrations may fall too low in the overlying water during this period in Azevedo Pond and Hidden Pond and may limit nitrification at this time of year. In Azevedo Pond, which exhibited the lowest rates consistently, water column dissolved oxygen concentrations are frequently <2 mg l^{-1} (Caffrey et al. 1997), and sulfide was often detected by smell. In a nearby pond, which also receives agricultural runoff, oxygen was essentially undetectable for periods of several hours during certain tidal conditions (Beck & Bruland 2000). In contrast, at Hudson's Landing at the head of the slough, salinity never exceeded 36, and no water column dissolved oxygen concentrations <2 mg l^{-1} have been observed (Caffrey et al. 1997). The conditions within sediments, which differed importantly from those in the overlying water, may be controlling nitrification rates here. High pore water sulfide and low pore water dissolved oxygen concentrations in the sediments (Caffrey et al. 2002) (Table 4) may be responsible for the low nitrification rates observed during the summer. While low oxygen may have influenced rates, it was not enough to prevent the occurrence of a diverse nitrifying assemblage, even at Azevedo Pond. It would be worthwhile to investigate the relative oxygen tolerance of different

Table 4. Sediment characteristics of sampling sites in Elkhorn Slough

Site	Sediment organic content[a] (%LOI)	Porewater NH_4^+ concentration in 0 to 4 cm depth layer[a] (µM)	Depth of oxygen penetration in sediment[a] (mm)	Sediment water content (%)
Hidden Pond	14.1	330	2.12	68
Azevedo Pond	17.4	1135	0.9	76
Hudson's Landing	13.5	990	0.9	70
Hummingbird Island	7.1	190	6.0	36
Vierra Mudflat	0.5	98	Not measured	20

[a]From Caffrey et al. (2002)

strains of nitrifying bacteria and to determine the role of this variable in influencing their distribution.

Nitrification rates in Azevedo Pond and Hudson's Landing may also be affected by agricultural runoff, which may contain pesticides capable of inhibiting nitrification (Martins & Bremer 1997, Pell et al. 1998, Harrington 1999). Thiram, a fungicide that inhibits both ammonia- and nitrite-oxidation (Martins & Bremner 1997), was detected in pore waters of Elkhorn Slough in 1998 (Harrington 1999), but its presence and concentration were not correlated with potential nitrification rates. Considering the large number of agricultural chemicals applied in the area, it would be difficult to ascribe even a significant effect on soil microbiology to a particular component by simple correlation. The 2 agricultural sites in this study exhibited either the lowest (Azevedo Pond) or the highest (Hudson's Landing) potential nitrification rates, so there is no consistent evidence for the effect of agricultural practices or chemicals on the process here.

Potential nitrification represents the activity that nitrifiers are capable of under optimum substrate conditions, while rates measured in intact cores represent the activity of nitrifiers under *in situ* substrate conditions. No relationship was detected between *in situ* and potential nitrification rates in the Elkhorn Slough study, although other studies (Henriksen et al. 1981, Kemp et al. 1990, Henriksen et al. 1993) have found positive correlations between potential and intact-core rates. This lack of correlation occurs in part because intact core rates were higher than potential rates on 3 occasions at Hudson's Landing and once at Vierra Mudflat. If nitrification were confined to a thin zone of very active nitrification near the surface (perhaps the top ca. 2 mm), slurrying the top 5 mm may have diluted out that active zone or released reduced components into the nitrification zone that then inhibited nitrification in the thin oxidized layer. In contrast, potential rates in Azevedo Pond were always greater than measurements in intact cores, perhaps because slurrying allowed aeration of otherwise very reducing sediments, the opposite of the effect suspected at Hudson's Landing. Although the potential measurements and the identification of several nitrifier DNA sequences clearly indicate the presence of nitrifying bacteria in Azevedo Pond, the intact-core measurements indicate that conditions were rarely favorable for nitrification to occur.

Nitrification rates in intact cores at Hudson's Landing at the head of Elkhorn Slough are among the highest reported in the literature (Henriksen et al. 1981, Kemp et al. 1990, Henriksen et al. 1993, Caffrey & Miller 1995, Lohse et al. 1995). The high intact-core rates were surprising given the generally high concentrations of pore water sulfides and shallow depth of oxygen penetration in the sediments at this location (Caffrey et al. 2002) (Table 4). Tidal pumping, which has been shown to increase oxygen availability in sediment and to increase exchange of pore water NH_4^+, enhancing the potential for nitrification deeper in sediments (Rocha & Cabral 1998), may explain the high nitrification rates at Hudson's Landing. The interaction between oxygen and NH_4^+ availability may be central to controlling rates of nitrification. Across a variety of estuarine and coastal systems (including some sites in Elkhorn Slough), potential nitrification rates increase as pore water NH_4^+ concentrations increase ($r = 0.76$, $p < 0.01$, Fig. 7). The relationship breaks down in systems that undergo periodic anoxia or hypoxia, such as parts of Elkhorn Slough and mid Chesapeake Bay (Fig. 7). This suggests that physical factors such as stratification and tidal flushing exert an indirect control on nitrification rates by their control of bottom-water oxygen concentrations (Kemp et al. 1990). Thus, high nutrient inputs by themselves do not directly drive nitrification rates. High nutrient inputs act by increasing water column primary production, leading to increased organic deposition to sediments. The resulting increased remineralization and high pore water NH_4^+ concentrations may lead to enhanced nitrification rates under well flushed conditions (enhanced oxygen

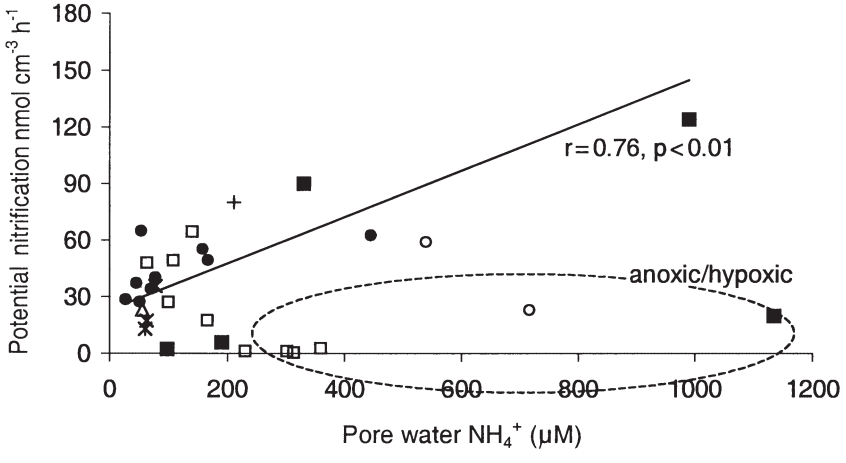

Fig. 7. Potential nitrification (nmol NO_2^- cm^{-3} h^{-1}) versus pore water NH_4^+ (µM) in 0 to 4 cm layer from Elkhorn Slough (this study, ■, Caffrey unpubl. data, □), Chesapeake Bay (Kemp et al. 1990, ○), San Francisco Bay (Caffrey 1995 and J. M. Caffrey unpubl. data, ●), Bering Sea (Henriksen et al. 1993, ✳), Aarhus Bay (J. M. Caffrey unpubl. data, 0 to 2 cm layer, △), Sado estuary (Rocha & Cabral 1998, +). Correlation line (solid) excludes sites which periodically go anoxic or hypoxic (dashed oval)

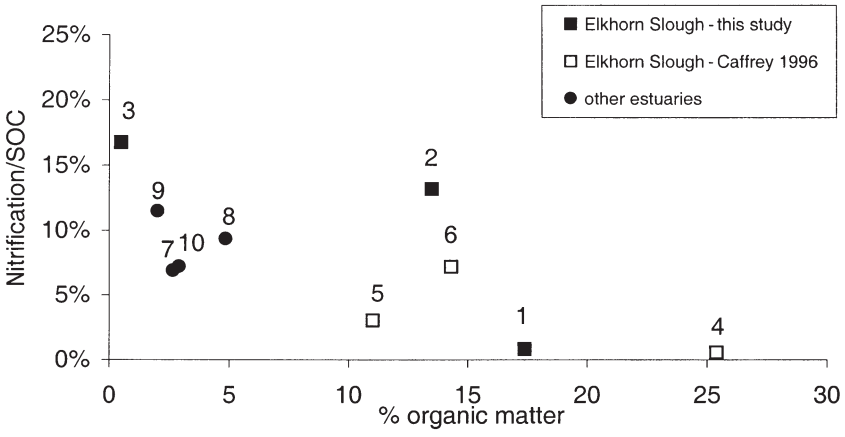

Fig. 8. Percentage of sediment oxygen consumption from nitrification (assuming 2:1 O:N molar ratio) versus percent organic matter in Elkhorn Slough and other estuaries: Elkhorn Slough (this study, ■), 1 = Azevedo Pond, 2 = Hudson's Landing, 3 = Vierra Mudflat; Elkhorn Slough (Caffrey unpubl. data, □), 4 = agricultural sites, 5 = grazing sites, 6 = reserve sites; other studies (●), 7 = San Francisco Bay (Caffrey & Miller 1995, Caffrey et al. 1996), 8 = Chesapeake Bay (Kemp et al. 1990, Kemp & Boynton 1992), 9 = Bering Sea (Henriksen et al. 1993), 10 = Aarhus Bay (Jørgensen 1996, J. M. Caffrey unpubl. data)

concentrations), as we observed at Hudson's Landing, or negligible nitrification under poorly flushed conditions (reduced oxygen concentrations), as at Azevedo Pond.

Nitrification plays a central role in linking organic matter mineralization (ammonification) and nitrogen removal through denitrification in Elkhorn Slough, as it does in many other estuarine systems (e.g. Jenkins & Kemp 1984, Henriksen & Kemp 1988, Kemp et al. 1990, Rysgaard et al. 1999). Nitrification is an important component of sediment oxygen consumption, and has been reported to be responsible for 1 to 35% of sediment oxygen consumption, depending on location (Henriksen & Kemp 1988). Across a variety of estuaries, nitrification accounts for a low percentage of sediment oxygen consumption (assuming a 2:1 molar O:N ratio) at sites with high sediment organic contents (Fig. 8). In Elkhorn Slough, nitrification represents <1% of sediment oxygen consumption at Azevedo Pond, the organic-rich, poorly flushed agricultural site, but up to 45% of sediment oxygen consumption at Vierra Mudflat, the organic-poor, well flushed site at the mouth of the slough (average 17%, range 0 to 45%).

Nitrogen budgets

Sediment nitrogen budgets for Hudson's Landing, Azevedo Pond and Vierra Mudflat were constructed based on nitrification rates (this study) and benthic fluxes (Caffrey et al. 2002) (Fig. 9). Nitrogen mineralization and denitrification were calculated by difference (mineralization = NH_4^+ flux + nitrification, denitrification = nitrification – NO_3^- flux) and assume that nitrate ammonification was negligible and that pore water NH_4^+ or NO_3^- pools were not changing over the course of the flux measurements. Nitrogen mineralization rates were highest (9.2 mmol m^{-2} d^{-1}) at the head of Elkhorn Slough (Hudson's Landing) and lowest (1.4 mmol m^{-2} d^{-1}) at the mouth (Vierra Mudflat). The trend in denitrification rates across the 3 sites was similar with highest rates, 4.6 mmol m^{-2} d^{-1}, at the head of Elkhorn Slough, the lowest rates, 0.8 mmol m^{-2} d^{-1}, at the mouth, and intermediate rates, 1.4 mmol m^{-2} d^{-1}, in Azevedo Pond. Relatively low estimated denitrification rates in Azevedo Pond are a result of the low nitrifica-

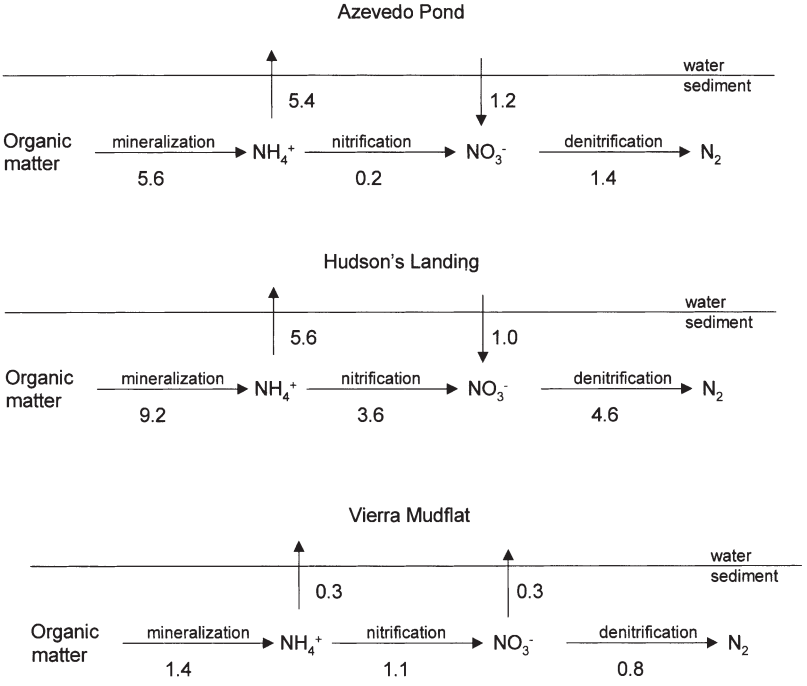

Fig. 9. Sediment nitrogen budget at Azevedo Pond, Hudson's Landing, Vierra Mudflat. Benthic flux measured in Caffrey et al. (2002). Nitrogen mineralization and denitrification were calculated by difference (rates in mmol m^{-2} d^{-1})

tion rates. Low nitrification rates are most likely due to hypersaline and low oxygen conditions observed during the dry season due to the lack of flushing in the pond. Thus, the coupling between nitrification and denitrification was low at this site, with only 25% of the mineralized nitrogen being denitrified. In contrast, 50 to 60% of the mineralized nitrogen was denitrified at the well flushed mouth (Vierra Mudflat) and head (Hudson's Landing) of the slough, indicating a more efficient coupling of nitrification and denitrification at these sites than at the poorly flushed Azevedo Pond. A similar breakdown of coupled nitrification and denitrification has been observed in stratified estuaries such as Chesapeake Bay (Kemp et al. 1990). Thus, the physical factors such as flushing regime and residence time can significantly affect the removal of nitrogen by denitrification (Nixon et al. 1996). One management implication of this work is that control of nutrient inputs in a poorly flushed system may be more important than in a well flushed system because in well flushed systems coupled nitrification and denitrification is more likely to occur and to result in more efficient nitrogen removal.

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