

## Interactions between Methane Oxidation and Nitrification in Coastal Sediments

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*Galveston Bay sediments exhibit substantial spatial and seasonal variability in rates of nitrification and aerobic methane oxidation. We examined the biogeochemical and microbiological controls on these processes using aerobic enrichment slurries. Potential aerobic methane and ammonia oxidation rates from unamended control slurries were compared to rates in slurries amended with methane, ammonium, or methane + ammonium. Bacterial community composition was monitored using denaturing gradient gel electrophoresis (DGGE) analysis of PCR amplified ribosomal and functional gene DNA. Potential methane and ammonia oxidation rates increased over time in sediments amended with methane and ammonium, respectively. The highest potential methane oxidation rates occurred in treatments receiving both ammonium and methane suggesting that methanotrophs in the enrichment cultures were nitrogen limited. The highest ammonia oxidation rates occurred in treatments amended with ammonium only. Treatments receiving both ammonium and methane exhibited ammonia oxidation rates and pore-water ammonium concentrations similar to those measured in the unamended control suggesting that methanotrophs may have inhibited ammonia oxidation by sequestering available ammonia. Sequence analysis revealed a decrease in general bacterial community diversity over time and a shift in ammonia-oxidizing bacterial composition corresponding with methane availability. However, methanotroph community composition similarities between treatments with different relative methane oxidation rates suggest that changes in physiological activity, as well as shifts in community composition, contributed to the observed patterns in potential rates.*

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### Introduction

Methane and ammonia oxidizing bacteria play central roles in the biogeochemical cycles of carbon and nitrogen. Methanotrophic bacteria consume up 90% of the methane produced in

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freshwater and marine soils and sediments (Reeburgh et al. 1993). By converting methane to biomass or carbon dioxide, methanotrophs decrease the flux of methane, a radiative trace gas, to the atmosphere (Topp and Hanson 1991). Nitrifying bacteria oxidize ammonia to nitrite (ammonia oxidizers) and then to nitrate (nitrite oxidizers). In sediments, denitrifying bacteria consume this nitrate in a process called “coupled denitrification.” Coupled denitrification removes fixed nitrogen from the environment, possibly mitigating anthropogenic nitrogen (N) inputs in eutrophic systems or exacerbating N limitation in systems where fixed N stocks are in short supply (Jenkins and Kemp 1984; Blackburn and Sørensen 1988).

Physiological, biochemical, and ecological similarities between aerobic methane- and ammonia-oxidizing bacteria promote interactions that may significantly affect methane and ammonia oxidation rates. Both methane and ammonia oxidizing bacteria are obligate, aerobic chemoautotrophs. Methane-oxidizing bacteria are distinguished by their ability to use methane as their sole source of structural carbon and energy. Ammonia-oxidizers derive energy from the conversion of ammonia to nitrite—the first step in the process of nitrification. Ammonia-oxidizing bacteria comprise at least seven phylogenetically distinct clusters (Stephen et al. 1998) in the  $\beta$ - and  $\gamma$ -subdivisions of the Proteobacteria (Head et al. 1993). Methane oxidizing bacteria have traditionally been classified into two groups based primarily on the mechanism of carbon assimilation employed. Type I methanotrophs utilize the ribulose monophosphate (RuMP) pathway for formaldehyde assimilation while type II methanotrophs assimilate formaldehyde via the serine pathway (Bowman et al. 1993). These groups form two distinct clusters within the  $\gamma$ - and  $\alpha$ -subdivisions of the Proteobacteria (Hanson and Hanson 1996).

The monooxygenase systems of methane and ammonia oxidizers are evolutionarily related (Holmes et al. 1995). Methane- and ammonia-oxidation co-occur in sediments near the oxic-anoxic boundary where both oxygen and reduced substrate are available. Neither group can survive solely by oxidizing the other's substrate but methanotrophs as well as ammonia oxidizers can oxidize either methane or ammonia (Bedard and Knowles 1989; Bodelier and Frenzel 1999). However, a consistent pattern of interaction between these processes in the environment has failed to emerge, with reports of both inhibition (King and Schnell 1994a) and stimulation (Bodelier et al. 2000) of methane oxidation by ammonia availability. Similarly, methanotroph activity may contribute to (Bodelier and Frenzel 1999) or suppress nitrification (Megraw and Knowles 1987; Roy and Knowles 1994).

Molecular techniques, combined with measurements of methane and ammonia oxidation rates, may help elucidate controls on these processes. Denaturing gradient gel electrophoresis (DGGE) has been used to compare different community “fingerprints” as well as to obtain specific phylogenetic information by sequencing excised bands (Muyzer et al. 1997; Murrell et al. 1998). Overall community analysis via DGGE traditionally employs PCR primers that target the variable 3 region of the 16S bacterial rDNA (Muyzer et al. 1993). Group specific diversity has been investigated by nesting the variable 3 primer set within template from primer pairs designed to target group specific regions of 16S rDNA such as nitAB for ammonia oxidizers (Voytek and Ward 1995; Bano and Hollibaugh 2000) or MethT1/T2 for methanotrophs (Wise et al. 1999). More recently, primer pairs targeting functional genes such as particulate methane monooxygenase (*pmo*) have been employed to examine group specific diversity (McDonald and Murrell 1997; Henckel et al. 1999).

Here, we report the result of laboratory experiments aimed at unraveling interactions between methane and ammonia oxidation in Galveston Bay sediments. We used ammonium and/or methane enrichments to examine the impact of relative substrate availability on rates of ammonia and methane oxidation and on patterns of bacterial community composition. We hypothesized that shifts in overall community structure would correlate with changes in potential ammonia or methane oxidation rates. Molecular techniques enabled us to examine

whether group specific diversity was affected by enrichment conditions. The molecular data, in conjunction with rate data, allowed us to evaluate whether the response of the community was to increase overall activity (and maintain diversity) or shift towards fewer species, which were adapted to higher substrate concentrations.

## Materials and Methods

### Study Site

The Galveston Bay estuarine ecosystem is shallow (average depth is about 4 m), productive, and slightly eutrophic (Joye and An 1999). As part of a three-year study, rates of benthic nitrogen and carbon cycling were determined at four to eight stations three to six times per year (An and Joye 2001). In order to refine our understanding of the controls on methane and ammonia oxidation in this system, we employed laboratory enrichment experiments so that we could vary substrate concentrations and evaluate the response of microorganisms over time under controlled conditions.

Surface sediment (0–5 cm) and overlying water were collected July 22, 1999, from Station 4 at the mouth of the Trinity River (Joye and An 1999). The overlying water was warm (29 C), saline (12.2‰), oxygenated (4.6 mg L<sup>-1</sup> O<sub>2</sub>), and had a pH of 7.5. Nutrient concentrations were determined using standard colorimetric methods (An and Joye 2001). Nutrient concentrations in the overlying water were 8.6 μM ammonium (NH<sub>4</sub><sup>+</sup>), 0.32 μM nitrite (NO<sub>2</sub><sup>-</sup>) and there was no detectable nitrate (NO<sub>3</sub><sup>-</sup>) or methane (CH<sub>4</sub>). The sediment consisted predominantly of sand with moderate pore water concentrations of nutrients (NH<sub>4</sub><sup>+</sup>: 250 μM; NO<sub>3</sub><sup>-</sup> + NO<sub>2</sub><sup>-</sup>: 10 μM; PO<sub>4</sub><sup>3-</sup>: 2.5 μM) and dissolved CH<sub>4</sub> (100 μM). Sediment was sieved to remove macrofauna and a sediment slurry was prepared by combining sediment and 0.2 μm-filtered bottom water to achieve a porosity of 0.8. A 100-mL volume of slurry was placed in each of twelve 250 mL serum bottles. The slurries were subjected to four treatment regimes (*n* = 3 each): no treatment (control), CH<sub>4</sub>-only (2 mmols L<sup>-1</sup> headspace CH<sub>4</sub> = 80 μM dissolved porewater CH<sub>4</sub>), NH<sub>4</sub><sup>+</sup>-only (300 μM porewater NH<sub>4</sub><sup>+</sup> as NH<sub>4</sub>Cl), and CH<sub>4</sub> + NH<sub>4</sub><sup>+</sup> (80 μM dissolved CH<sub>4</sub> + 300 μM NH<sub>4</sub><sup>+</sup>). The slurries were mixed by gentle shaking (75 rpm) and maintained at 25 C. Amendments of CH<sub>4</sub> (daily) and NH<sub>4</sub><sup>+</sup> (every other day) were made following sampling and aeration (via bubbling with water saturated air for 20 min). Porewater concentrations of NH<sub>4</sub><sup>+</sup>, NO<sub>2</sub><sup>-</sup>, and NO<sub>3</sub><sup>-</sup> were monitored over the course of the incubation. Time series subsamples from each treatment were analyzed for potential methane and ammonia oxidation rates. Concurrent samples from each slurry were extracted for DNA, which was amplified by the PCR, analyzed by DGGE, and sequenced (select bands only).

### Rate Measurements

Ammonia oxidation rates were estimated by quantifying the changes in slurry NO<sub>3</sub><sup>-</sup> + NO<sub>2</sub><sup>-</sup> concentration following a 24-h incubation (Joye and Hollibaugh 1995). One mL subsamples of slurry from each enrichment bottle were transferred to a 38 mL serum bottle containing 25 mL sterile bottom water to which 300 μM NH<sub>4</sub><sup>+</sup> had been added. A bottle from each treatment was sacrificed immediately so that the initial NO<sub>3</sub><sup>-</sup> + NO<sub>2</sub><sup>-</sup> concentration could be determined. Bottles were shaken at 75 rpm and maintained at 25°C during the incubation. Initial and final porewater NO<sub>3</sub><sup>-</sup> + NO<sub>2</sub><sup>-</sup> concentration was determined using cadmium reduction and diazotization followed by colorimetric quantification on an Alpkem autoanalyzer (OI Analytical). Ammonia oxidation rates were calculated as the difference between final and initial NO<sub>3</sub><sup>-</sup> + NO<sub>2</sub><sup>-</sup> concentration.

Potential methane oxidation rates were determined by monitoring the change in headspace  $\text{CH}_4$  concentration over time. One mL subsamples of sediment from each bottle were transferred to a 25 mL serum bottle containing 1 mL sterile bottom water. The bottle was sealed with a hungate stopper and methane was added to the headspace to achieve a final concentration of  $300 \mu\text{M CH}_4$ . An initial sample was analyzed after a 12-h equilibration period (to avoid solubility induced concentration changes) and a second sample was analyzed 24 h later. Bottles were shaken at 75 rpm and maintained at  $25^\circ\text{C}$  during the incubation. Methane concentrations were determined using a Shimadzu 14-A gas chromatograph equipped with a Porpak T column and a flame ionization detector (Joye et al. 1999). Methane oxidation rates were calculated by quantifying the decrease in headspace methane over time.

For field samples, 1 cc subsamples ( $n = 3$  each for potential nitrification and for potential methane oxidation) were collected from cores at cm depth intervals in the upper 20 cm. Each subcore was transferred to a serum vial and assays were conducted as described above.

### **DNA Extraction**

DNA was extracted from sediments using bead-mill homogenization (method adapted from the MO BIO Ultraclean Soil DNA kit protocol). A  $450 \mu\text{L}$  aliquot of sediment was combined with glass beads ( $100 \mu\text{L}$ ) and  $750 \mu\text{L}$  of extraction buffer (1.5 M NaCl, 5 mM  $\text{MgCl}_2$ , 100 mM Tris HCl (pH 7), 100 mM EDTA (pH 8), 100 mM  $\text{Na}_2\text{HPO}_4$  (pH 8), 1% SDS, and proteinase K) and vortexed at high speed for 10 min. After centrifugation ( $12,000 \times g$  for 5 min), the supernatant was treated with  $250 \mu\text{L}$  potassium acetate (4M, pH 5) and centrifuged (at  $12,000 \times g$ ) for 5 more min. Next, the supernatant was added to  $750 \mu\text{L}$  of 6M guanidine thiocyanate and 1 mg sterile diatomaceous earth and mixed by inversion. The diatomaceous earth-bound DNA was trapped on a GF/F filter, washed twice with 0.5 mL guanidine thiocyanate (6M), twice with 0.5 mL isopropanol solution (25% isopropanol, 25% ethanol, 100 mM NaCl, and 10 mM Tris HCl, pH 8), once with 0.5 mL ethanol, and eluted with  $60 \mu\text{l}$  warm 1X TE buffer. The DNA obtained was suitable for the PCR and no subsequent purification was necessary.

### **PCR Amplification of Bacterial Community DNA**

Previously characterized oligonucleotide primers were chosen to examine patterns of overall and group specific community composition (Table 1). The primer pair 341f and 534r (*E. coli* position) was used to amplify the variable 3 region of ribosomal DNA (Muyzer et al. 1993). Ammonia-oxidizers were detected using a nested approach: EUB1/EUB2 (Liesack et al. 1991) was used to amplify total bacterial rDNA and the product was subjected to a second amplification using Nit-A/Nit-B (nitAB; Voytek and Ward 1995). NitAB is internal to the EUB primers and targets a  $\beta$ -subdivision ammonia oxidizing bacteria-specific region of 16S rDNA. Methane oxidizing bacteria were detected using MethT1dF/MethT1bR and F27/MethT2b, which target type I and II methane-oxidizer specific regions of 16S rDNA, respectively (Wise et al. 1999). Methanotrophs were also detected using primers targeting the particulate methane mono-oxygenase gene (*pmo*) via nested PCR employing the primer pairs pmof1/pmof2 and pmof2/pmof1 (Cheng et al. 1998). Primers used to produce amplicons for subsequent DGGE analysis were modified with a GC clamp and labeled with fluorescein (Table 1).

A circulation free chamber was used to prepare a "master mix" containing sterile water, 1X buffer (Promega), 2.5 mM  $\text{MgCl}_2$ , 2  $\mu\text{M}$  forward and reverse primers, and 200  $\mu\text{M}$  dNTPs. One hundred  $\mu\text{L}$  of the master mix was then dispensed into individual 0.2 mL reaction tubes. Template DNA (4–10 ng) was added in a separate circulation free chamber.

TABLE 1 PCR primers used in this study

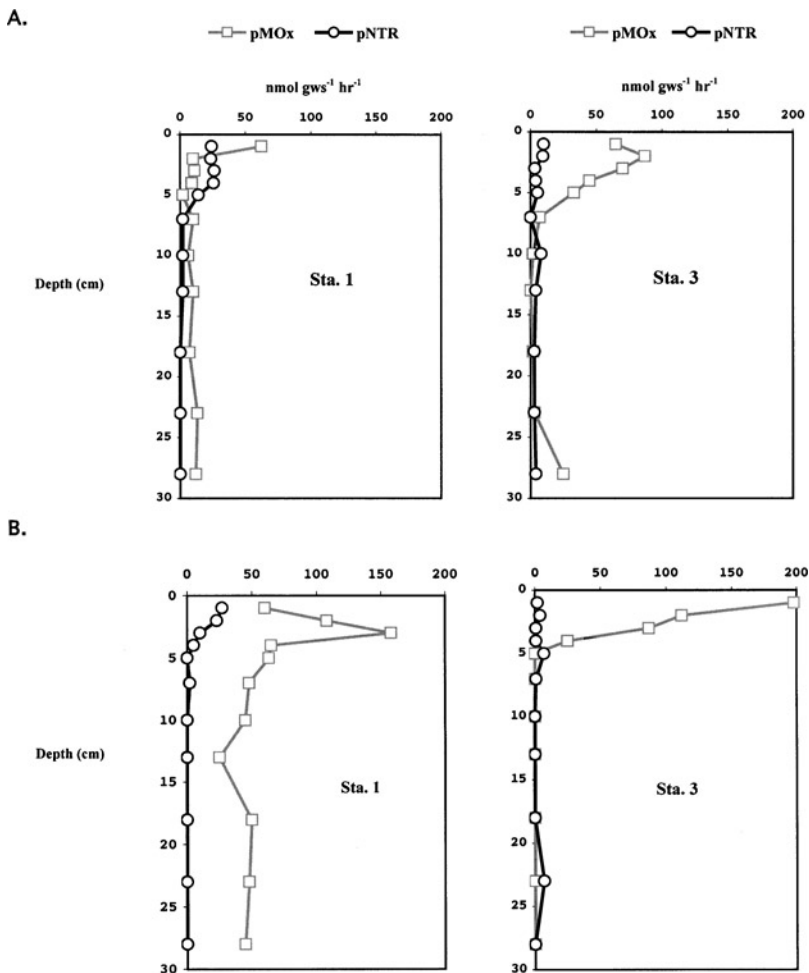
Primer	<i>E. coli</i> position	Sequence (5'-3')	Target	Product size (bp)	Source	Primer annealing temperature (°C)
Eub1	27	GAGTTTGATCCTGGCTCAG	Universal eubacterial 16S rDNA	1515	1	59
Eub2	1542	AGAAAGAGGTGATCCAGCC	AOB specific region of 16S rDNA	1097	2	55
nitA	137	CTTAAAGTGGGAATAACGCATCG	16S rDNA of type I methanotrophs	904	3	56
nitB	1234	TTACGTGTGAAGCCCTACCCCA	16S rDNA of type II methanotrophs	990	3	56
MethT1dF	102	CC TTCGGMGCYGACGAGT	Eubacterial 16S rDNA	193	4	65–55
MethT1bR	1006	GATTCYMTGSATGTCAAGG	Universal 16S rDNA	330	5	(–0.5°/cycle) 56
F27	27	AGAGTTTGATCMTGGCTCAG	<i>pmoA</i> gene of methylootrophs	178	5	56
MethT2R	1017	CATCTCTGRCSAYCATACCCGG	NA	NA	6	NA
341f	341	CTACGGGAGGCAGCAG	NA	NA	6	NA
534r	534	*ATTACCGGGCTGCTGG	NA	NA	6	NA
pmof1	NA <sup>2</sup>	GGGGGA ACTTCTGGGGTTGGAC	NA	NA	6	NA
pmor	NA	GGGGGRCIACGTCITTACCCGAA	NA	NA	6	NA
pmof2	NA	TTCTAYCCDRRCAACTGGCC	NA	NA	6	NA
pmor	NA	*GGGGGRCIACGTCITTACCCGAA	NA	NA	6	NA
GC clamp	NA	CGCCCGCCCGCCCGGCCCGT CCCCGCCGCCCGCCCGCC	NA	NA	6	NA

<sup>1</sup>Leisack et al. (1991).  
<sup>2</sup>Voytek and Ward (1995).  
<sup>3</sup>Wise et al. (1999).  
<sup>4</sup>Muyzer et al. (1993).  
<sup>5</sup>Cheng et al. (1999).  
<sup>6</sup>Murray et al. (1996).  
<sup>7</sup>NA, not applicable.  
<sup>\*</sup>Position of fluorescein.  
<sup>√</sup>Position of GC clamp.

After 5 min at 95°C in the thermocycler (MJ Research DNA Engine), 2.5 units of Taq DNA polymerase was added while the reaction tubes were held at 80°C. A “touchdown” thermal cycling program (Don et al. 1991), optimized for initial and final temperature (Table 1), was used for each of the primer pairs and each PCR was run for 30 cycles. All reactions were run with appropriate positive and negative controls and the PCR product was verified for amplification and fragment size by agarose gel electrophoresis.

### DGGE and Sequencing

PCR products were separated using DGGE (CBS Scientific). Denaturing gels were 6.5% polyacrylamide with a denaturant gradient ranging from 40 to 70%. The denaturants in a 100% solution were 40% deionized formamide and 7M urea. PCR products were concentrated by ethanol precipitation and resuspended. Samples (250 ng DNA) were mixed 1:1 with neutral loading dye and loaded onto the gel. Gels were run at 80 V for 15 h in a 60°C, 1X TAE buffer and visualized on a Hitachi FMBIO III scanner equipped for fluorescein detection. Individual bands were excised, the DNA extracted from the gel via diffusion into sterile water,



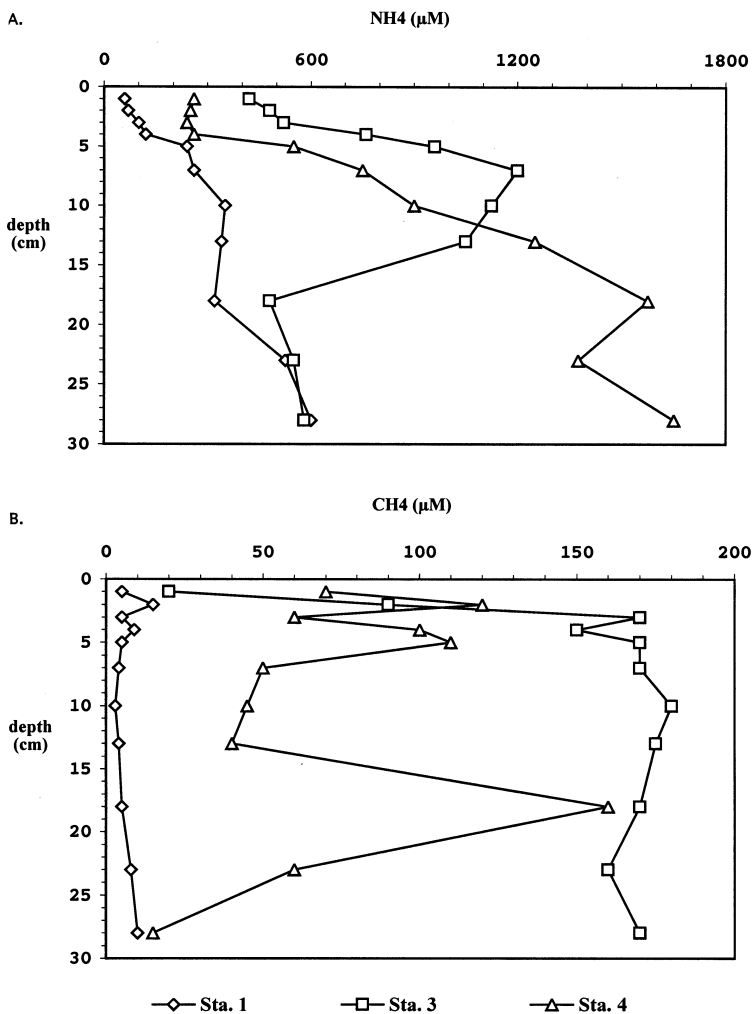
**FIGURE 1** Seasonal and spatial variation in potential ammonia (pNTR) and methane oxidation (pMOx) rates in Galveston Bay sediments: (A) August 1998 and (B) November 1998.

and then re-amplified using the original primer set. Re-amplified PCR products were verified by DGGE, purified using a Wizard PCR prep kit, and then sequenced using the original forward primer. DNA sequencing was carried out by the Molecular Genetics Instrumentation Facility at the University of Georgia. Sequence analysis and homology searches (BLAST) were conducted at the network server of the National Center for Biotechnology Information.

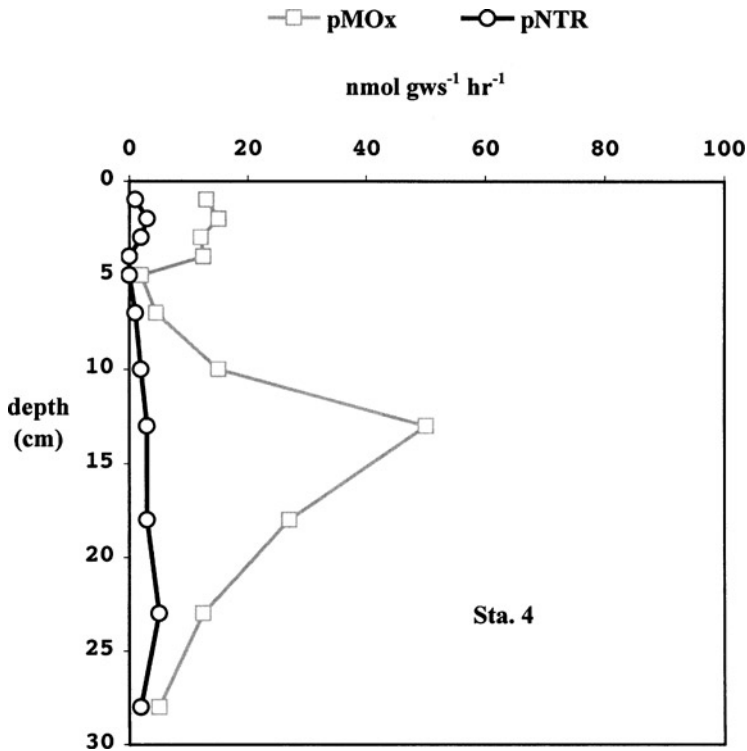
## Results

### *Patterns of Activity and Substrate Concentrations in the Field*

In Galveston Bay sediments, rates of methane and ammonia oxidation exhibit seasonal and spatial (between sites and over depth) variability (Figure 1). In these shallow, bioturbated sediments, pore water inventories of nutrients are dynamic and concentrations of  $\text{CH}_4$  rarely exceed  $200 \mu\text{M}$  (Figure 2; Joye and An 1999; An and Joye 2001). Rates of potential



**FIGURE 2** Concentration versus depth profiles of ammonium (A) and methane (B) from three Trinity River stations in Galveston Bay. The salinity at station 1 was approximately 5‰ while the salinity at the estuarine most station (3) was >10‰.



**FIGURE 3** Potential ammonia (pNTR) and methane oxidation (pMOx) rates from station 4, near the mouth of the Trinity River in Galveston Bay, in August 1998.

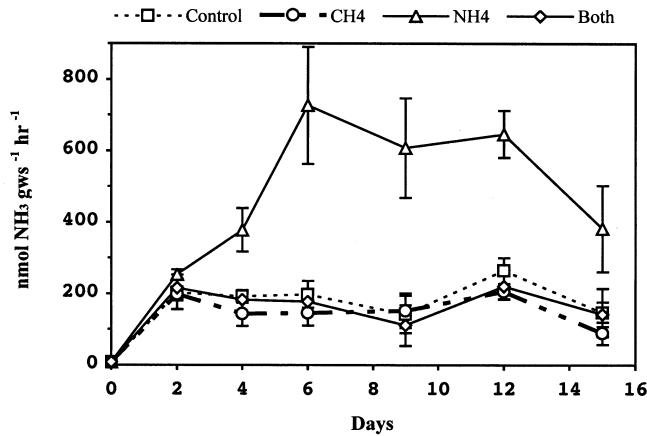
methane oxidation and nitrification at Station 4 (Figure 3) typify activity observed in the sandy, organic poor sediments that cover over 75% of the bay bottom (Joye and An 1999). Potential methane oxidation rates commonly exceeded potential nitrification in field samples and most activity of both processes was restricted to the upper 10 cm of sediment (Figure 1). Surface maxima were observed frequently and within this layer, the relative concentrations of  $\text{CH}_4$  and  $\text{NH}_4$  dissolved in the porewater were similar (ca. 75–150  $\mu\text{M}$ ). Because these organic poor sands are a common feature of the Galveston Bay benthos, we collected material from this site for use in laboratory enrichments.

### **Laboratory Enrichments**

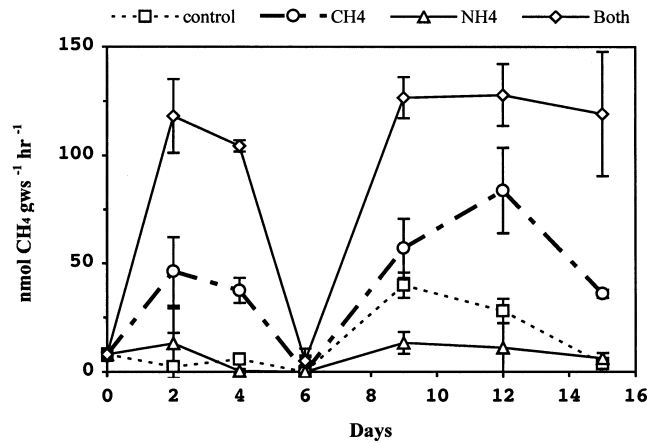
In the enrichment experiments, potential ammonia oxidation rates increased rapidly and exhibited differences between treatments by day 4. Rates presented in the following sections represent the average of the three replicates within a treatment and the value in parenthesis reflects the standard deviation around the mean. Ammonia oxidation rates in samples from slurries amended with  $\text{NH}_4^+$ -only increased from 8.2 ( $\pm 1.4$ ) to 377.9 ( $\pm 32.2$ )  $\text{nmol NH}_3 \text{ gws}^{-1} \text{ h}^{-1}$  (Figure 4A). Ammonia oxidation rates in the other treatments (including the control) also increased from 8.2 to close to 200  $\text{nmol NH}_3 \text{ gws}^{-1} \text{ h}^{-1}$  by day 4 but did not vary over the remainder of the incubation. In contrast, ammonia oxidation rates in the  $\text{NH}_4^+$ -only amended treatments increased to a maximum ( $726.5 \pm 52.6 \text{ nmol NH}_3 \text{ gws}^{-1} \text{ h}^{-1}$ ) at day 6.

Potential methane oxidation rates exhibited differences between treatments within the first two days of enrichment. Rates of methane oxidation in  $\text{CH}_4$ -only enriched sediments

A.



B.

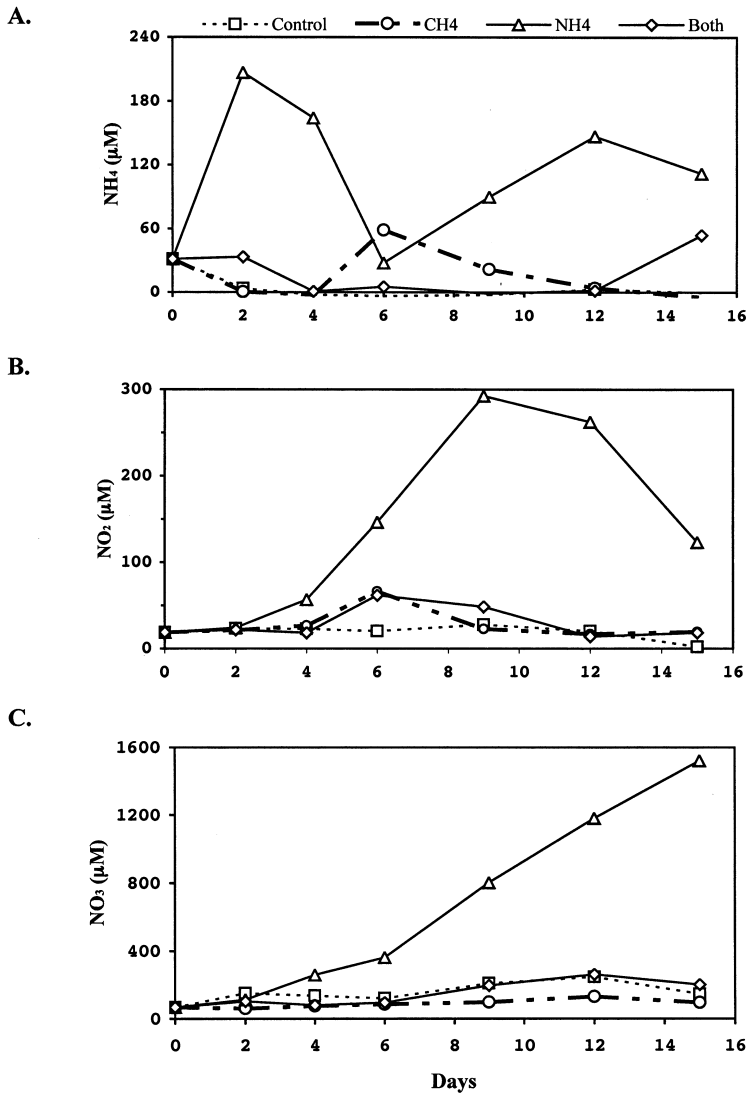


**FIGURE 4** Time course of potential (A) ammonia and (B) methane oxidation rates during the laboratory enrichment experiment. Rates are in nmol substrate oxidized  $\text{gws}^{-1} \text{h}^{-1}$ .

increased from  $8.0 (\pm 1.3)$  to  $43.6 (\pm 8.2)$  nmol  $\text{CH}_4 \text{ gws}^{-1} \text{h}^{-1}$  while rates in samples amended with  $\text{CH}_4 + \text{NH}_4^+$  increased from  $8.0 (\pm 1.3)$  to  $118.1 (\pm 26.2)$  nmol  $\text{CH}_4 \text{ gws}^{-1} \text{h}^{-1}$  (Figure 4B). Potential methane oxidation rates remained either constant or decreased in the control and  $\text{NH}_4^+$ -only amended treatments. Rates in all treatments exhibited a minimum on day 6 corresponding to a transient accumulation of nitrite in the slurry porewater (Figure 5B). Potential rates increased to their highest levels between days 9 and 12, maintaining the initially observed difference between treatments.

#### Slurry Nitrogen Concentrations

Ammonium concentrations in the  $\text{NH}_4^+$ -only amended treatment increased from 38 to  $219 \mu\text{M}$  by day 2 (Figure 5A). Ammonium concentrations in all other treatments decreased below the detection limit by day 4 and remained nondetectable for the remainder of the experiment with the exception of a small, transient concentration increase in the control on day 6. An ammonium minimum was recorded in the  $\text{NH}_4^+$ -only amended treatment



**FIGURE 5** Slurry porewater concentrations of (A) ammonium, (B) nitrite, and (C) nitrate during the enrichment experiment. Concentrations are in  $\mu\text{molar}$ .

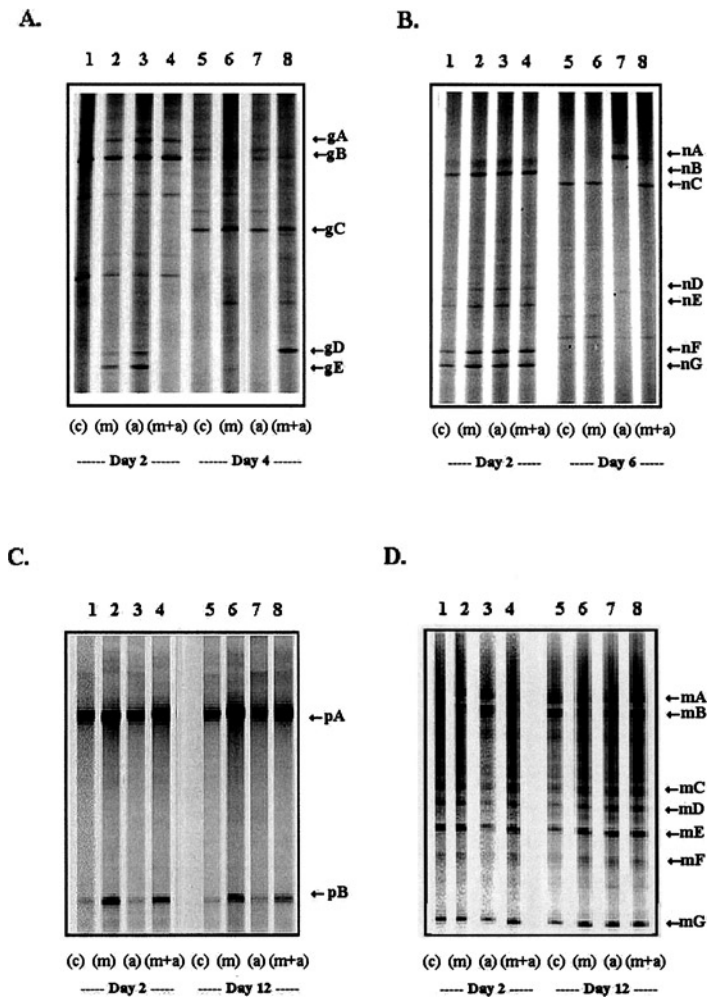
on day 6, followed by increasing concentrations through the end of the incubation. Nitrite concentrations in the  $\text{NH}_4^+$ -only treatment increased to  $292 \mu\text{M}$  on day 9 followed by decreasing concentrations through the end of the incubation (Figure 5B). Nitrite concentrations in the remaining treatments remained constant with the exception of a transient increase in concentration in the  $\text{CH}_4$ -only and  $\text{CH}_4 + \text{NH}_4^+$  amended treatments on day 6. Nitrate accumulated throughout the experiment in the  $\text{NH}_4^+$ -only amended treatment and remained relatively unchanged in all other treatments (Figure 5C).

### Community Analysis and Sequencing

PCR reactions consistently yielded products of the correct size and position corresponding to positive controls. DGGE analysis of PCR products obtained from amplification with

the general eubacterial primers 341f/541r illustrated a change in community structure over time and between treatments (Figure 6A). Through day 2, all samples displayed 4–7 distinct bands. On day 4 and through the remainder of the experiment, all samples exhibited 2–3 distinct bands. The sequence generated from band gA had the most similarity (96%) with *Vogesella indigofera*, a *pseudomonas*-like member of the  $\beta$ -proteobacteria (Table 2). Band gB and gE sequences were most similar to a *Ralstonia* sp. (97%) and a *Pseudomonas* sp. (94%) from estuarine sediments. Bands gC and gD were most similar to *Methylomonas* spp. (Table 2).

Group specific primer products also revealed changes over time as well as differences between treatments. Amplicons from the nitAB primer pair yielded 4 to 6 DGGE bands (Figure 6B). The initial time point was dominated by bands that were most similar to *Vogesella indigofera* (bands nB and nD through nG; 88%–98% similarity). By day 6 and through the end of the enrichment, all samples exhibited 1–2 distinct bands. Sequences of



**FIGURE 6** DGGE analysis of PCR products from enrichment experiment DNA extractions using: Bacterial variable region 3 (A), nitAB (B), pmo (C), and MethT1/T2 (D) primer pairs. Treatments were control (c), methane (m), ammonium (a), and methane + ammonium (m + a).

**TABLE 2** Phylogenetic affiliations of sequences obtained from excised DGGE bands. GAP analyses (see Methods) were run on all sequences with the same closest relative. Identities were <90% except for mG and mF (99.0%) indicating that the different bands represented different microorganisms. Accession numbers AY152650–AY152670 correspond to excised DGGE bands in order of their appearance in the table

Band	Major taxa	Group	Closest relative (accession no.)	%16S rDNA similarity
gA	$\beta$ -Proteobacteria	<i>Neisseriaceae</i>	<i>Vogesella indigofera</i> (AB021385)	96
gB	$\beta$ -Proteobacteria	Ralstonia	<i>Ralstonia pickettii</i> (AF467977)	97
gC	$\gamma$ -Proteobacteria	<i>Methylococcaceae</i>	<i>Methylomonas</i> sp. LW21 (AF150800)	94
gD	$\gamma$ -Proteobacteria	<i>Methylococcaceae</i>	<i>Methylomonas methanica</i> (AF304196)	92
gE	$\gamma$ -Proteobacteria	<i>Pseudomonasaceae</i>	<i>Pseudomonas</i> sp. GOBB3-207 (AF321048)	94
nA	$\beta$ -Proteobacteria	Ammonia-oxidizing	<i>Nitrosomonas</i> sp. Band6a5 (AJ245755)	96
nB	$\beta$ -Proteobacteria	<i>Neisseriaceae</i>	<i>Vogesella indigofera</i> (AB021385)	90
nC	$\beta$ -Proteobacteria	Ammonia-oxidizing	<i>Nitrosomonas europaea</i> (AF353160)	90
nD	$\beta$ -Proteobacteria	<i>Neisseriaceae</i>	<i>Vogesella indigofera</i> (AB021385)	92
nE	$\beta$ -Proteobacteria	<i>Neisseriaceae</i>	<i>Vogesella indigofera</i> (AB021385)	88
nF	$\beta$ -Proteobacteria	<i>Neisseriaceae</i>	<i>Vogesella indigofera</i> (AB021385)	98
nG	$\beta$ -Proteobacteria	<i>Neisseriaceae</i>	<i>Vogesella indigofera</i> (AB021385)	91
mA	$\gamma$ -Proteobacteria	<i>Methylococcaceae</i>	<i>Methylomonas</i> LW21 (AF150800)	90
mB	$\gamma$ -Proteobacteria	<i>Methylococcaceae</i>	<i>Methylomonas rubra</i> (AF150807)	98
mC	$\gamma$ -Proteobacteria	<i>Methylococcaceae</i>	<i>Methylomonas rubra</i> (AF150807)	97
mD	$\gamma$ -Proteobacteria	<i>Methylococcaceae</i>	<i>Methylomonas methanica</i> (AF304196)	93
mE	$\gamma$ -Proteobacteria	<i>Methylococcaceae</i>	<i>Methylomonas rubra</i> (AF150807)	92
mF	$\gamma$ -Proteobacteria	<i>Methylococcaceae</i>	<i>Methylobacter</i> sp. LW14 (AY007296)	94
mG	$\gamma$ -Proteobacteria	<i>Methylococcaceae</i>	<i>Methylobacter</i> sp. LW14 (AY007296)	98
Band	Major taxa	Group	Closest relative (accession no.)	% <i>pmoA</i> similarity
pA	$\gamma$ -Proteobacteria	<i>Methylococcaceae</i>	<i>Methylomonas</i> LW19 (AF150799)	98
pB	$\gamma$ -Proteobacteria	<i>Methylococcaceae</i>	<i>Methylobacter</i> sp. LW14 (AY007286)	95

band nA and nC were most similar (96% and 90%) to *Nitrosomonas* spp. Band nA was prominent in the  $\text{NH}_4^+$ -only treatment while band nC was most prominent in the control,  $\text{CH}_4$ -only, and  $\text{CH}_4 + \text{NH}_4^+$  amendments.

Amplicons from the *pmoA* primer pairs consistently yielded two DGGE bands in all treatments (Figure 6C). Band pA was dominant, consistent, and most similar (98%) to a *Methylomonas* sp. from lake sediment. The intensity of band pB varied in strength between treatments and was consistently more prominent in the  $\text{CH}_4$ -only and  $\text{CH}_4 + \text{NH}_4^+$  treatments. The sequence of this band was most similar (95%) to a *Methylobacter* sp. from lake sediment. MethT1/MethT2 primer pairs revealed 8 distinct DGGE bands in all treatments which remained constant throughout the incubation period (Figure 6D). The sequences from bands mA through mE were most similar (90%–98%) to *Methylomonas* spp. from lake sediments while band mF and mG sequences were most similar (94% and 98%, respectively) to *Methylobacter* spp. from lake sediments (Table 2).

## Discussion

The laboratory enrichment experiments we performed identified unexpected interactions between methane oxidation and nitrification in Galveston Bay sediments. These data helped identify both microbiological and environmental controls on methane and ammonium cycling in this system. Specifically, we observed: (1) ammonium stimulation of nitrification, suggesting that nitrifiers are substrate limited; (2) stimulation of methane oxidation by ammonium, inferring nitrogen limitation of methanotrophs; (3) inhibition of nitrification by methanotroph activity, suggesting that methanotrophs out-compete nitrifiers for available ammonium; and, (4) community shifts in nitrifier populations, possibly resulting from competition with methanotrophs for available inorganic nitrogen.

### *Ammonium Enhancement of Ammonia and Methane Oxidation*

The  $\text{NH}_4^+$ -only amended slurry exhibited the highest potential ammonia oxidation rates (Figure 4A) suggesting that the activity of ammonia oxidizing bacteria (AOB) was limited by the ammonia supply. This result is not surprising since increased ammonia availability typically leads to an increased activity of nitrifiers in soils and sediments (Bruns et al. 1999; Mendum et al. 1999). Ammonia oxidation in the other treatments (including the control) increased by day 2 but no significant change in rates or differences between treatments was observed during the remainder of the experiment. Low initial ammonia oxidation rates may have been an artifact of the inherent perturbation imposed during slurry preparation. The observed initial increase in rates in all treatments may have reflected a recovery from that perturbation. No change in activity was observed throughout the remainder of the experiment in the control,  $\text{CH}_4$ -only, or  $\text{CH}_4 + \text{NH}_4^+$  amended treatments, suggesting little change or growth of the ammonia oxidizer population occurred in those treatments. Ammonia oxidation decreased in all treatments on day 15 possibly due to carbon ( $\text{CO}_2$ ) limitation caused by the low organic carbon content of the sediment (0.8%) although other possibilities such as trace metal limitation and bottle effects cannot be ruled out.

Potential methane oxidation rates increased in sediments amended with  $\text{CH}_4$ -only and with  $\text{CH}_4 + \text{NH}_4^+$  (Figure 4B). Soils exposed to elevated methane concentrations exhibit a high capacity for methane oxidation, presumably because of selection for methanotrophic bacteria (Whalen et al. 1990; Jones and Nedwell 1993). Rates of potential methane oxidation in the  $\text{NH}_4^+$ -only amended treatment were similar to rates of the control indicating that ammonia oxidizers, presumably selected for ammonium enrichment and clearly oxidizing ammonia, did not significantly contribute to methane oxidation. These results mirror

previous results that demonstrated that nitrifiers did not contribute to methane oxidation in rice planted microcosm soils (Bodelier and Frenzel 1999).

Potential methane oxidation rates were highest in the  $\text{CH}_4 + \text{NH}_4^+$  amended treatment. Since ammonia oxidizers were not contributing to methane oxidation, we suggest that increased ammonium availability enhanced methanotroph activity. Previous studies have pointed out that ammonium can inhibit methane oxidation in pure culture (King and Schnell 1994a), in forest soils (Megraw and Knowles 1987; Schnell and King 1994), and sediments (Bosse et al. 1993). However, predictable mechanisms of inhibition have failed to emerge (Gulledge and Schimel 1998). More recent results have illustrated stimulation of methane oxidation by ammonium-based fertilizers in mesocosms planted with rice, suggesting nitrogen limitation of methanotrophs (Bodelier et al. 2000; Eller and Frenzel 2001). Most inhibition studies focus on atmospheric methane oxidation, and thus utilize relatively low methane concentrations. Elevated methane concentrations (>500 ppm) decrease the inhibitory effect of ammonia on methane oxidation (Schnell and King 1994). Methane concentrations in our methane enrichments were much higher, presumably supporting a productive population of methanotrophs responsible for the increased potential methane oxidation rates we observed. Nitrogen depletion, illustrated by porewater ammonium concentrations in the  $\text{CH}_4 + \text{NH}_4^+$  amended treatment, likely limited methanotroph productivity in the  $\text{CH}_4$ -only amendment. Increased ammonium availability in the  $\text{CH}_4 + \text{NH}_4^+$  amended treatment alleviated nitrogen limitation and resulted in higher potential methane oxidation rates through increased methanotroph biomass.

### ***Inhibition of Nitrification by Methanotrophic Activity***

Ammonia oxidation rates in the  $\text{CH}_4$ -only amended slurry did not differ from the control rates (Figure 4A). If methanotrophs were selected for this enrichment and if they contributed to ammonia oxidation, this treatment should have exhibited higher ammonia oxidation rates than the control. Diluted freshwater sediment slurries supplemented with methane (1 to 24  $\mu\text{M}$ ) exhibited enhanced nitrification compared to unsupplemented slurries (Roy and Knowles 1994), suggesting that nitrification by methanotrophs was significant in those freshwater sediments. A major contribution of methanotrophs to nitrification in the rhizosphere of rice-planted microcosms has also been demonstrated (Bodelier and Frenzel 1999). However, there was no apparent contribution to nitrification by methanotrophs in Galveston Bay sediments. We hypothesize that methanotrophs in this system were N limited and that they assimilated available ammonia, thus limiting the ammonia oxidation potential in enrichments supplemented with methane.

This hypothesis is supported by the fact that ammonia oxidation rates in the  $\text{CH}_4 + \text{NH}_4^+$  amended slurry did not differ significantly from control rates (Figure 4A). The lack of stimulation of ammonia oxidation in the  $\text{CH}_4 + \text{NH}_4^+$  treatment suggests that the methane availability may have limited the development of the AOB population. Physiologically, methane can act as a competitive inhibitor of ammonia monooxygenase (Schimel et al. 1993) and active methanotrophs may suppress nitrification in humisol or polluted sediments (Megraw and Knowles 1987; Roy and Knowles 1994). Methanotrophs may also assimilate ammonium and thus compete with ammonia oxidizers directly for their primary substrate. Porewater ammonium concentrations in the  $\text{NH}_4^+$ -only amended treatment were relatively high, and corresponded with increased porewater nitrate and nitrite concentrations (Figure 5B). The  $\text{CH}_4 + \text{NH}_4^+$  amended treatment exhibited low porewater ammonium concentrations and nitrate or nitrite did not accumulate (with the exception of day 6 discussed later). This suggests that these methanotrophs efficiently assimilated ammonia and thus limited the activity of ammonia oxidizers in the  $\text{CH}_4 + \text{NH}_4$  amended treatments.

Potential methane oxidation rates in both methane amended treatments decreased at day 6. Several mechanisms have been reported for ammonia inhibition of methane oxidation including enzymatic substrate competition (Dunfield and Knowles 1995), nitrite toxicity (King and Schnell, 1994a; Schnell and King 1994), and salt effects (Gulledge and Schimmel 1998). Ammonia inhibition of methane oxidation via substrate competition is unlikely at the high methane concentrations used in our experiments (King and Schnell 1994b; Schnell and King 1994). Furthermore, chloride addition via ammonium enrichment (as  $\text{NH}_4\text{Cl}$ ) to these slurries was insignificant (0.16% increase of Cl per day for a total change of 2.4% over 15 days), so it is unlikely that variation in ionic strength led to the observed changes. The inhibition of potential methane oxidation we observed corresponded to an accumulation of nitrite in the slurry porewater (Figure 5B). Nitrite inhibition of methane oxidation has been documented (King and Schnell 1994a) and the suppression of methanotrophic activity has been attributed to the toxicity of nitrite produced by ammonia oxidation (Schnell and King 1994). Ammonia oxidation initially produces nitrite faster than it is oxidized to nitrate by nitrite oxidizers (Prosser 1989) resulting in an accumulation of nitrite in closed vessel incubations (as we observed, Figure 5B). Nitrite concentrations decreased by day 9 (Figure 5B) and at this time potential methane oxidation rates increased to their highest levels and maintained the initially observed difference between treatments (Figure 4B).

### Community Population Analysis

Changes in overall bacterial community composition were associated with changes in the rates of ammonia and methane oxidation. Sequences from individual DGGE bands indicated that *Vogesella*, *Ralstonia*, and *Pseudomonas* spp., which do not contribute to methane or ammonia oxidation, dominated the microbial population in all treatments during the initial days of the incubation. As substrate oxidation rates increased in amended slurries (Figure 4), microbial diversity decreased and methane-oxidizing bacterial abundance increased. DGGE analysis revealed 2–3 dominant bands on day 4 of the incubation (Figure 6A). *Ralstonia*-like and *Vogesella*-like organisms were still evident in the control, but a *Methylomonas*-like sp. (band gC) dominated the treatments amended with  $\text{CH}_4$ -only and  $\text{CH}_4 + \text{NH}_4^+$ . Methane-oxidizing bacteria became a more dominant component of the overall bacterial community in methane amended treatments concurrent with increased potential methane oxidation rates.

Ammonia oxidizers were not detected in any of the treatments utilizing the universal primer pair. Although ammonia-oxidizing bacteria are generally ubiquitous in freshwater and marine environments, they account for a very small proportion of the total bacterial population in natural environments (Voytek and Ward 1995; Phillips et al. 2000) and may have only been present in numbers that required a more sensitive, nested PCR approach for detection (Voytek and Ward 1995).

The nitAB primer pair is specific for nitrifying bacteria in the  $\beta$  subdivision of the Proteobacteria (Voytek and Ward 1995). With the exception of *Spirillum volutans*, DNA amplification of any closely related  $\beta$ -Proteobacteria that did not oxidize ammonia has not been reported. NitAB has also been used successfully to amplify DNA collected from a variety of environments including an Antarctic lake and the Southern California Bight (Voytek and Ward 1995), the Baltic coast of Germany, sewage treatment sludge, enrichment cultures, and several lakes in northern Germany (Ward et al. 1997), and the Arctic Ocean (Bano and Hollibaugh 2000). None of the sequences obtained in these cited studies had affinity for nonammonia-oxidizing bacteria. However, sequence data obtained in this study indicated that *Vogesella*-like organisms accounted for many of the bands present in our nitAB DGGE analysis (bands nB and nD-nG). *Vogesella*-like ammonia oxidizers

are *Pseudomonas*-like members of the  $\beta$  subdivision of the Proteobacteria and are found in freshwaters and lake sediments (Grimes et al. 1997). *Vogesella*-like bands were only present through day 2 of the enrichment. It is possible that, even with a nested approach, the initial ammonia oxidizer population was small and *Vogesella*-like organisms were present in sufficient numbers so that differential, low level, nonspecific amplification, possibly enhanced by the nesting procedure we used, led to detectable numbers of *Vogesella* nitAB amplicons. Overall, community changes, based on EUB primers, suggested that *Vogesella* became less dominant in all treatments over the course of the enrichment. This was also evident based the nitAB primer pair results.

By day 6 and through the end of the enrichment, the nitAB PCR/DGGE results showed 1–2 dominant bands (nA and nC) with sequences most similar (96% and 90%) to *Nitrosomonas* spp. (Figure 6B). GAP analysis using the Wisconsin Package (Version 10.2, Accelrys, Burlington, MA, USA) revealed an 84.4% identity between sequences from band nA and nC indicating two different microorganisms. Band nC was present in the control, CH<sub>4</sub>-only, and CH<sub>4</sub> + NH<sub>4</sub><sup>+</sup> treatments while band nA was dominant in the NH<sub>4</sub><sup>+</sup>-only treatment. Potential ammonia oxidation rates in the NH<sub>4</sub><sup>+</sup>-only treatment were 4 times greater than control rates and these elevated rates coincided with the appearance and dominance of *Nitrosomonas*-like band nA. Relatively low potential ammonia oxidation rates measured in the control, CH<sub>4</sub>-only, and CH<sub>4</sub> + NH<sub>4</sub><sup>+</sup> treatments were concurrent with *Nitrosomonas*-like band nC. These data suggest there was selection for different *Nitrosomonas*-type subpopulations coincident with the absence of methane in the enrichment and that this population supported higher potential ammonia oxidation rates.

Higher ammonia concentrations, such as those found in enrichment cultures, may favor the growth of *Nitrosomonas* sp. while low ammonia concentrations favor the growth of *Nitrospira* sp. (Hiorns et al. 1995; Phillips et al. 2000). It is possible that intragenus (*Nitrosomonas*) preferences for ammonia concentrations also exist. The NH<sub>4</sub><sup>+</sup>-only enrichment clearly had higher ammonia availability than all other treatments (Figure 5A). Band nA could represent a *Nitrosomonas*-like sp. that is better adapted to high ammonia concentrations and capable of higher potential ammonia oxidation rates. Different ammonia oxidizer populations in the NH<sub>4</sub><sup>+</sup>-only and CH<sub>4</sub> + NH<sub>4</sub><sup>+</sup> treatments (band nA and nC, respectively) suggest that the methane co-addition influenced species distribution (and activity) in the CH<sub>4</sub> + NH<sub>4</sub><sup>+</sup> enrichment. We suggest that methanotrophs effectively lowered ammonium availability and constrained the ammonia oxidizer population to resemble the population associated with the control and CH<sub>4</sub>-only amended treatments.

DGGE analysis of PCR products produced with *pmoA* primers suggested low methanotroph diversity (Figure 6C). Band pA was dominant (in all treatments), consistent, and most similar (98%) to a *Methylomonas* sp. from lake sediments. Band pB was most similar (95%) to a *Methylobacter* sp., varied in intensity between treatments, and was consistently more prominent in the CH<sub>4</sub>-only and CH<sub>4</sub> + NH<sub>4</sub><sup>+</sup> treatments. PCR/DGGE, as used in this study, is at best semiquantitative (Henckel et al. 1999; Bano and Hollibaugh 2000). Pitfalls of this approach include differential amplification based on gene copy number, template concentration, and genome size (Farrelly et al. 1995; Chandler et al. 1997; Von Wintzingerode et al. 1997). Nonetheless, by quantifying the intensities of the same bands obtained in independent PCR/DGGE analyses, Diez et al. (2001) demonstrated significant reproducibility (variability < 2%) of fingerprints of natural bacterial assemblages. Furthermore, changes in band intensity as a function of time or space along an environmental gradient may infer changes in population size (Ferris and Ward 1997). Although we did not quantify band intensity, multiple PCR/DGGE analyses were run and individual runs yielded bands with consistent relative visual differences suggesting that semiquantitative conclusions may be made. Methanotroph community diversity, based on the *pmoA* primer set, did

not change over the course of the incubation. However, visual assessment of band differences suggests that *Methylobacter* (band pB) were more abundant in treatments amended with methane (Figure 6C). Similar intensity of the dominant *Methylomonas* band (pA), between treatments with different relative methane oxidation rates, suggests that changes in physiological activity, as well as shifts in population size (illustrated by *Methylobacter* band pB), may have been responsible for the higher potential methane oxidation rates we observed in methane amended treatments.

Methanotroph specific 16S rDNA primers confirmed the dominant bands revealed by the *pmoA* primer pair but also illustrated a greater microdiversity of the methanotroph community. Several different *pmoA* primer pairs have been shown to have different specificities (Bourne et al. 2001) and a combination of different primers may have been useful for more accurately determining the total diversity of *pmoA* in these samples. MethT1/MethT2 primer pairs revealed 8 distinct DGGE bands which remained constant throughout the incubation (Figure 6D). Bands mA-mE were most similar (90%–98%) to a *Methylomonas* sp. from lake sediments. The range in percent similarity and percent identity (from GAP analysis) suggests that while closely related, these bands were distinct from *Methylomonas* LW 21 and from each other (Table 2). Bands mF and mG were most similar (94% and 98%, respectively) to a *Methylobacter* sp. from lake sediments. While the MethT1/MethT2 primer pairs provided greater resolution of the microdiversity of the methanotroph population (than did a single *pmoA* primer pair) they did not reveal additional community differences over time or between treatments.

Phylogenetic and functional molecular results reveal a conspicuous absence of type II methanotrophs in this study. Type II methanotrophs are known to fix molecular nitrogen and may be selected for nitrogen-limited environments (Graham et al. 1993). However, recent results have indicated both *nif* gene fragments and nitrogenase activity associated with type I *Methylomonas* and *Methylobacter* cultures (Auman et al. 2001). It is possible that the methanotrophs in Galveston Bay are nitrogen-fixing; however, our experimental design was not appropriate to evaluate that possibility.

## Summary

Our results expand the range of environments where methane oxidation is limited by the availability of fixed nitrogen. Stimulation of methane oxidation rates and depletion of dissolved inorganic nitrogen pools in CH<sub>4</sub> and CH<sub>4</sub> + NH<sub>4</sub><sup>+</sup> treatments showed clearly that methanotrophs rapidly and efficiently consumed bioavailable nitrogen. Phylogenetic results suggest that the activity of methanotrophs altered the community composition of other bacterial groups, such as AOB, by restricting their access to ammonia. Uptake of ammonia by methanotrophs may effectively sequester ammonia-nitrogen in microbial biomass, thereby decoupling nitrification and denitrification and limiting N-loss through coupled denitrification. Simply put, efficient methanotroph scavenging of ammonia creates a positive feedback loop that favors the nitrogen-limited methanotroph population by retaining bioavailable N in the sediments.

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