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Assimilatory nitrate utilization by bacteria on the West Florida Shelf as determined by stable isotope probing and functional microarray analysis

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stable isotope probing; marine bacteria; nitrogen cycling; nitrate.

Abstract

Dissolved inorganic nitrogen (DIN) uptake by marine heterotrophic bacteria has important implications for the global nitrogen (N) and carbon (C) cycles. Bacterial nitrate utilization is more prevalent in the marine environment than traditionally thought, but the taxonomic identity of bacteria that utilize nitrate is difficult to determine using traditional methodologies. ¹⁵N-based DNA stable isotope probing was applied to document direct use of nitrate by heterotrophic bacteria on the West Florida Shelf. Seawater was incubated in the presence of 2 μM ¹⁵N ammonium or ¹⁵N nitrate. DNA was extracted, fractionated via CsCl ultracentrifugation, and each fraction was analyzed by terminal restriction fragment length polymorphism (TRFLP) analysis. TRFs that exhibited density shifts when compared to controls that had not received ¹⁵N amendments were identified by comparison with 16S rRNA gene sequence libraries. Relevant marine proteobacterial lineages, notably *Thalassobacter* and *Alteromonadales*, displayed evidence of ¹⁵N incorporation. RT-PCR and functional gene microarray analysis could not demonstrate the expression of the assimilatory nitrate reductase gene, *nasA*, but mRNA for dissimilatory pathways, i.e. *nirS*, *nirK*, *narG*, *nosZ*, *napA*, and *nrfA* was detected. These data directly implicate several bacterial populations in nitrate uptake, but suggest a more complex pattern for N flow than traditionally implied.

Introduction

Uptake of dissolved inorganic nitrogen (DIN) by marine heterotrophic bacteria has important implications for the global nitrogen (N) and carbon (C) balances (Kirchman, 1994; Allen *et al.*, 2005). This is particularly true in environments where bacteria actively compete for nitrate with phytoplankton, as this can significantly affect estimates of new vs. recycled productivity (Kirchman, 2000). Traditionally, it has often been suggested that heterotrophic bacteria in marine systems satisfy their N demand primarily via the uptake of organic N, while being responsible for remineralization of N in the form of ammonium (Allen *et al.*, 2001). It is now known that nitrate-utilizing bacteria are far more prevalent in marine systems than

was previously recognized (Allen *et al.*, 2001, 2005; Cai & Jiao, 2008), and that, nitrate uptake by heterotrophic bacteria can account for a sizable proportion of total inorganic N utilization in natural populations (Allen *et al.*, 2002); for example, in a study of bacterial DIN uptake in the Barents Sea, it was observed that bacterial nitrate uptake accounted for as much as 14–40% of total nitrate uptake and a greater proportion of total nitrate utilization near the marginal ice zone (as opposed to the open Atlantic Ocean) and with depth (Allen *et al.*, 2002). Bacteria can, therefore, contribute to nitrate utilization in marine systems and should not be ignored in estimates of new production (Allen *et al.*, 2002). Molecular tools have shed some light on the diversity of bacteria containing assimilatory nitrate reduction genes in marine

environments (Allen *et al.*, 2001; Cai & Jiao, 2008), suggesting that a large diversity of such organisms exists. It has further been observed that their distribution and community structure are correlated with nitrate availability and utilization patterns (Allen *et al.*, 2005).

Despite these observations, unresolved questions remain with respect to nitrate utilization by heterotrophic bacteria in marine systems; for example, the presence of genes does not necessarily imply activity. Another important limitation of prior studies has been the inability to differentiate N uptake by individual microbial populations. Distinguishing between bacterial and phytoplankton uptake is challenging, because N uptake measurements traditionally employ glass fiber filters (e.g. Whatman GF/F), with a nominal pore size of 0.7 μm . The rates calculated from such experiments are typically attributed to phytoplankton, but a sizable and variable bacterial component is retained on such filters (Gasol & Moran, 1999; Berg *et al.*, 2001). Furthermore, measurements are taken on bulk communities, in which hundreds of different bacterial and phytoplankton populations can exist simultaneously. As a result, it is not always clear which phytoplankton or bacteria utilize different forms of N and how these populations interact. Culture-independent approaches have provided some insight into the distribution, diversity, and abundance of microbial populations, but they cannot demonstrate uptake of N directly.

^{15}N -based DNA stable isotope probing (SIP) allows tracking of N into individual microbial taxa via the separation of DNA of differing isotopic composition through a cesium chloride gradient (Buckley *et al.*, 2007a, b; Wawrik *et al.*, 2009). The method was first applied to study bacterial communities in soils, demonstrating the presence of uncultivated diazotrophic lineages (Buckley *et al.*, 2007a, b). More recently, the approach has been applied in marine environments to investigate N nutrition of *Synechococcus* and diatoms on the West Florida Shelf (Wawrik *et al.*, 2009), demonstrating that N nutrition of these two phytoplankton is more complex than previously thought. Together, these studies illustrate that ^{15}N -based DNA SIP is a powerful tool for investigating the N nutrition of specific microbial populations in the environment. This study relates to our previous work on phytoplankton N uptake (Wawrik *et al.*, 2009) by exploring nitrate utilization by heterotrophic bacterioplankton in the same community. SIP experiments presented here demonstrate that N from ^{15}N nitrate is readily incorporated into DNA of several bacterial populations. This suggests that both phytoplankton (Wawrik *et al.*, 2009) and bacterioplankton are simultaneously utilizing the available nitrate pool on the West Florida Shelf.

Materials and methods

Work presented here is a companion study to a previously described SIP experiment describing phytoplankton N uptake (Wawrik *et al.*, 2009). All environmental samples, incubations, and SIP fractions correspond to those previously described. A detailed description of the site, water chemistry, as well as N concentrations [ammonium, nitrate, urea, dissolved primary amines (DPA), dissolved organic nitrogen (DON)], uptake (ammonium, nitrate, urea and DPA), and regeneration rates (ammonium and nitrate) can also be found in the preceding study. Also included there are results from SIP control experiments comparing labeled and unlabeled DNA from cultures, ambient community DNA, and no treatment control (NTC) samples (Wawrik *et al.*, 2009). No artifacts or unexpected density shifts were observed in any of the SIP controls, and control experiments are, therefore, not reiterated here.

Field site and incubation experiments

All field samples were collected during an ECoGY Of Harmful Algal Blooms (ECOHAB) cruise on the R/V Pelican on October 10, 2008. Samples were collected in the morning from 2-m depth using 20-L Niskin bottles at a station c. 2 km west of Narrows Key, outside of Charlotte Harbor (Lat. 26.6183 N, Long. -82.2455 W). Ambient seawater controls (T0) were filtered immediately, and filters were stored under liquid nitrogen. The following incubations of 400 mL of seawater were conducted in duplicate: no N source (NTC) and 2 $\mu\text{mol N L}^{-1}$ addition of $^{15}\text{NH}_4\text{Cl}$ or $\text{Na}^{15}\text{NO}_3$. All incubations were for 24 h to ensure that sufficient time had passed to allow DNA replication by some portion of the bacterial population. Using rate measurements, we estimated that within 24 h, about 15% of the label was recycled (see Discussion). Longer incubations were, therefore, deemed undesirable, as they would have likely prevented any detection of differences among treatments because of significant cross-feeding. As a result, any detected differences probably originated from major, fast growing populations. It should be noted that seawater was filtered by 0.45- μm filters, which may not have captured some small cells. In our experience, however, this tends to be a minor problem, and somewhere between 90% and 95% of DAPI countable cells are typically collected on these filters (B. Wawrik, unpublished data). Comparisons of DNA from cultures, the ambient community (T0), NTC bottles, and N treatments are found in the companion study and demonstrated that this was sufficient time to allow substantial labeling of *Synechococcus* and diatom populations at our study site (Wawrik *et al.*, 2009). All incubations

were conducted in 500-mL acid-cleaned polycarbonate bottles that were placed under two layers of neutral density screening under ambient light to simulate light intensity at *c.* 2-m depth. Continuous flow of seawater through the incubator was used to maintain temperatures near those of the sampled seawater.

DNA extraction

Seawater (400 mL) was filtered onto 0.45 µm Supor filters (PALL Life Sciences). Filters were stored in 0.75 mL of STE buffer (1 M NaCl, 100 mM Tris·HCl pH 8.0, 10 mM EDTA pH 8.0) and frozen in liquid N₂ in the field. The filters were stored at -80 °C until DNA extraction. For extraction, 75 µL of 5% SDS and 20 µL of 20 mg mL⁻¹ proteinase K were added, and samples were incubated for 30 min at 37 °C. Muffled glass beads (50–100 mg of 0.1 mm beads) were added, and samples were homogenized by bead-beating for two minutes before extraction with phenol/chloroform/isoamyl alcohol (25 : 24 : 1). DNA was precipitated with 0.1 volumes of 3 M sodium acetate (pH 5.3) and 0.6 volumes of isopropanol and then re-suspended in 50 µL of TE buffer (1 mM Tris pH 9.0, 10 mM EDTA pH 8.0).

CsCl gradient ultracentrifugation

Centrifugation and fractionation were conducted as previously described (Buckley *et al.*, 2007a, b; Wawrik *et al.*, 2009). Five to ten micrograms of DNA was fractionated, and each fraction's density was determined by measuring the refractive index. Density calculations were recorded as described by Birnie (1978), applying a small empirically determined correction factor for the effect of ethidium bromide (0.0038) to the measured refractive index (Wawrik *et al.*, 2009).

Terminal restriction fragment length polymorphism (TRFLP) analysis

DNA from each fraction was amplified using universal 16S primers 27F 5'-AGAGTTTGATCMTGGCTC-3' and 1525R 5'-AAGGAGGTGTCCARCC-3' (Nakatsu & Marsh, 2007). The forward primer was labeled with carboxyfluorescein (FAM). PCR amplifications (30 µL volumes) were set up using PCR Supermix (Invitrogen, Carlsbad, CA) containing 0.5 µM of each primer and 1 µL of template DNA. An initial denaturation step of 95 °C for 3 min was followed by 35 cycles of: 96 °C for 30 s, 45 s at 55 °C, and 72 °C for 1.2 min. A final extension of 10 min at 72 °C was applied. PCR products were quantified by agarose gel electrophoresis and comparison of band brightness to duplicate lanes of 0.5 µg of Lambda HindIII

DNA using a Kodak Gel Logic 100 Imaging System (Kodak). A 2.5-µL aliquot of each PCR was digested at 37 °C for 4 h using 2 units (10 U µL⁻¹) of AluI restriction enzyme (Promega) in 2 µL of 10× buffer and 14.5 µL of sterile water. After digestion, samples were precipitated with 1.9 µL of 0.75 M sodium acetate (pH 5.2), 0.31 µL of 20 mg mL⁻¹ glycogen, 0.09 µL ABI loading dye, and 37 µL of 100% ethanol. Pellets were re-suspended into HiDi formamide (Applied Biosystems) containing size standard LIZ 600 (Applied Biosystems) and run on ABI 3730 capillary sequencer for analysis. TRFLP data were then imported into MATLAB (MathWorks) to identify fragments that exhibited density shifts as a result of incubation with ¹⁵N-labeled ammonium or nitrate. This is performed visually by generating heat maps of the data for each of the treatments (see Supporting Information, Fig. S2). Only fragments with discernable distribution maxima were included in the analysis. Fragments that produced saturating signals on the ABI sequencer were not considered, because their distribution maxima cannot be clearly identified. To estimate sizing error, run-to-run variability was analyzed by considering individual peaks in the data set; for example, the peak corresponding to the 248-bp fragment was a major signal (> 200 fluorescence units) in 14 fractions of the ¹⁵N nitrate treatment. The standard deviation of the associated peak size in these fractions was 0.67 bp. This means that > 87% of the respective peak maxima were within ±1 bp (1.5 SD). All peak maxima were within < 1% of the predicted size.

Community analysis

Two approaches were used to analyze the ambient bacterial community composition (T0). For longer sequence reads (needed for *in silico* comparison to terminal restriction fragments), PCR products were generated using 27F and 1525R bacterial rRNA gene primers as indicated earlier. PCR products were gel-purified using a Gel Purification Kit (Qiagen) and ligated into pCR4.0-TOPO (Invitrogen) according to the manufacturer's instructions. Four hundred colonies were picked and inserts were sequenced using both the M13F and M13R priming regions on the vector. Reads were then assembled into operational taxonomic units (OTUs) at the 95% identity level and trimmed to remove vector. Primer sequences were included in the analysis to allow correct calling of terminal restriction fragment lengths. All sequences, for which the lengths of the terminal AluI fragment could not be unambiguously determined, were removed from the analysis.

For a more in-depth survey of bacterial community composition, 16S rRNA gene primers 27F 5'-AGAGT

TTGATCMTGGCTC-3' and 338R 5'-GCTGCCTCCCG-TAGGAGT-3' were used to amplify community DNA using the following cycling conditions: 95 °C for 3 min, followed by 35 cycles 96 °C for 15 s, 20 s at 55 °C, and 30 s at 72 °C. The forward primer was tagged for pyrosequencing and also included a unique identifying hexamer sequence (Hamady *et al.*, 2008). Sequencing was conducted via FLX 454 chemistry at the University of South Carolina's Environmental Genome Sequencing Core.

Classification of sequences or OTUs was achieved using MOTHUR (v.1.21.1) (Schloss *et al.*, 2009) by alignment with the SILVA reference alignment using the NAST algorithm (Pruesse *et al.*, 2007). Sequences containing ambiguous bases, homopolymers ($N > 8$), or a quality of < 25 were removed. Only sequences longer than 100 bp were included in the analysis. Potentially chimeric sequences were removed using CHIMERASLAYER (www.broadinstitute.org). Each sequence was then classified using the RDP's naive Bayesian rRNA classifier (Wang *et al.*, 2007).

RNA extraction

Ambient seawater (T0) was filtered onto 0.45 µm Supor filters and stored in 2-mL screw-cap tubes containing 0.75 mL of RLT buffer (Qiagen RNeasy Kit) and 50 mg of muffled glass beads (0.1 mm). Tubes were frozen in liquid N₂ in the field and maintained at -80 °C until extraction. Extraction proceeded as previously described (Wawrik *et al.*, 2002, 2003), by performing DNase digestion on the RNeasy column as recommended by the manufacturer. PCR with 16S rRNA gene primers was used to assay for the complete removal of contaminating DNA from RNA extracts before proceeding to reverse transcription (data not shown).

RT-PCR of nitrogen cycling genes

Reverse transcription was performed on RNA extracted from ambient seawater (T0) using a Superscript Vilo cDNA Synthesis Kit (Invitrogen), as recommended by the manufacturer. All PCR (cDNA template) were performed with PCR Supermix (Invitrogen) using PCR conditions described in respective references. PCR for the nitrite reductase genes *nirS* and *nirK* were conducted as described by Braker *et al.* (1998, 2000) using the following primers: *nirS1F* 5'-CCTAYTGCCGCCRCART-3', *nirS6R* 5'-CGTTGAACTTRCCGGT-3', *nirK1F* 5'-GGMATGGTKCCSTGGCA-3', and *nirK5R* 5'-GCCTCGATCAGRTTTRTGG-3'. Nested RT-PCR for the nitrate assimilation gene *nasA* was performed as previously described by Allen *et al.* (2001) using the following nested PCR primer set: *nas22* 5'-TGYCCNTAYTGYGGNGT-3', *nas964* 5'-CARC CNAAYGCNATGGG-3', *nasA1735* 5'-ATNGTRTGCCAY

TGRTC-3', and *nas1933* 5'-CARTGCATNGGNAYRAA-3'. As a positive control and to assay for the quality of the extracted RNA, RT-PCR for *rbcL* genes was conducted using primers for the Form IA/B of RubisCO as previously described (Wawrik & Paul, 2004) (forward: 5'-TCI GCITGRAACTAYGGTCG-3'; reverse: 5'-GGCATRTGCC AIACRTGRAT-3').

GeoChip analysis

The GeoChip 3.0 is a functional gene array that contains c. 28 000 probes and includes more than 57 000 gene sequences in 292 gene families (He *et al.*, 2010). GeoChip analyses for both DNA and RNA were performed by amplification of template as described previously (Wu *et al.*, 2006; Gao *et al.*, 2007; Van Nostrand *et al.*, 2009). Hybridizations were performed with a MAUI Hybridization System (BioMicro systems, UT) at 42 °C for 10 h according to the manufacturer's recommended method. The microarray was scanned by ScanArray Express Microarray Scanner (Perkin Elmer, Boston, MA) at 633 nm. IMAGEGENE v. 6.0 (Biodiscovery, El Segundo, CA) was used to estimate spot intensity and identify poor-quality spots. Spots with a signal-to-noise ratio [$((\text{signal intensity} - \text{background}) / \text{standard deviation of background}) < 2.0$] were removed as poor quality (He & Zhou, 2008). Mean Cy3 intensity of the universal standards in each subgrid was used to normalize the Cy5 intensity for samples in the same subgrid. The Cy5 intensity after the first normalization was normalized again by the mean value of three technical replicates. In addition, the data were normalized by the mean intensity of universal standards on the Cy3 channel in all slides for Cy5 intensity of the samples. If any of the replicates exceeded twice the standard deviation (signal-mean), the replicate was removed as an outlier, repeating this process iteratively until no such replicates were identified. A minimum of two positive spots were required for each gene to be considered for data analysis. Only spots for which both DNA and RNA signals were observed were considered positive. Combining RNA and DNA data was performed to gain confidence that detected signals originated from sizable and active populations. We acknowledge that rare community members can sometimes be highly active, but given the quantitative limitations of the microarray approach; this was deemed a more conservative approach than analyzing RNA alone.

Results

Samples were taken near Charlotte Harbor, FL, as part of an Ecology of Harmful Algae Blooms (EcoHAB) sampling cruise. A detailed site description, chemical characteristics,

nitrogen uptake rates, nitrogen recycling rates, and phytoplankton species composition for the sampling time and location are described elsewhere (Wawrik *et al.*, 2009).

Ambient bacterial species composition was surveyed using 454 pyrosequencing of 16S rRNA gene PCR products, yielding 4114 quality 16S sequences across the V1 and V2 regions of the 16S gene. Binning of sequences at the 97% identify level indicated the presence of 720 bacterial taxa. Keeping in mind that the choice of primers and the number of PCR cycles can influence the final composition of clone libraries (Polz & Cavanaugh, 1998; Acinas *et al.*, 2005), it was observed that dominant among the classifiable sequences were *Alpha*-, and *Gamma*-*proteobacteria*, as well as *Bacteroidetes*, *Actinomycetales*, and cyanobacteria (Fig. 1). Most of the alphaproteobacterial OTUs were classified within the *Roseobacter* as well as within the SAR11 groups. Almost all of the gammaproteobacterial lineages were classified as *Oceanospirillales* and *Alteromonadales*. Classifiable cyanobacterial sequences were all associated with the *Prochlorococcus* and *Synechococcus* lineages, while those associated with the *Bacteroidetes* clustered within the *Sphingobacteria*. The detected *Deltaproteobacteria* belonged to lineages that could not be identified with confidence beyond the phylum level. Most of the detected Actinobacterial sequences were classified within the *Acidimicrobiales*. Sequences originated from chloroplasts accounted for 3.6% of the library. Approximately 3% of all quality reads could not be classified at any taxonomic level.

DNA was extracted from ambient seawater, NTC samples, as well as incubations with 2 μ M of 15 N ammonium

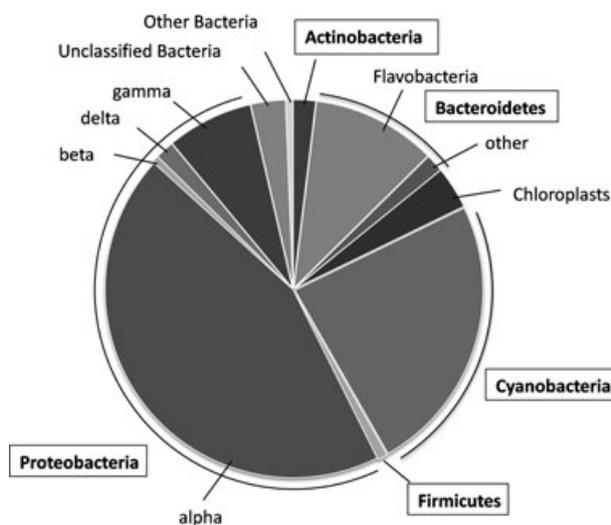


Fig. 1. Phylogenetic analysis of bacterial 16S rRNA gene sequences ($N = 4114$) obtained by 454-based pyrosequencing of 16S PCR products. Class-level phylogenetic distribution is shown.

or 2 μ M of 15 N nitrate, respectively. After ultracentrifugation and fractionation of this DNA, each fraction was examined via TRFLP analysis. The distribution of DNA in CsCl gradients is dependent on G+C content, as well as the degree of isotopic labeling (Buckley *et al.*, 2007a, b; Wawrik *et al.*, 2009). A comparison of the distribution of TRFs between control DNA and ammonium and nitrate treatments was, therefore, conducted to identify TRFs that exhibited DNA density shifts in response to the presence of 15 N label (Fig. 2). TRF analysis implicated four bacterial populations in nitrate uptake, producing the terminal fragments with length of *c.* 128 nt (Fig. 2a,b), 151 nt (Fig. 2c,d), 194 nt (Fig. 2e,f), and 248 nt (Fig. 2g,h). TRFs 128 and 151 also exhibited notable density shifts in 15 N ammonium incubations, suggesting mixed N nutrition of these bacteria. TRFs 194 and 248 only displayed minor density shifts in the presence of 15 N ammonium, suggesting that nitrate, but not ammonium, was utilized as an N source by these bacteria.

A clone library of full-length 16S PCR products (27F to 1525R) was generated from ambient microbial population (T0) for comparison with TRFLP data, and four hundred clones were sequenced bidirectionally using traditional Sanger sequencing. The resulting data were assembled and analyzed *in silico* to identify sequences that could produce specific TRFs (Table 1). Sequences corresponding to the 128, 151, and 248 nt fragments were identified. Sequences that corresponded to the TRF at 128 nt fell within poorly classified alpha- and gammaproteobacterial lineages. One of the four OTUs that matched the TRF at 128 nt was classified within the genus *Roseovarius*. Allowing for a < 1% sizing error during TRFLP analysis, the fragment at 151nt was ascribed to the sequences in clone libraries producing a 152 nt fragment, which fell within unclassified gammaproteobacterial lineages belonging to the orders *Alteromonadales* and *Oceanospirillales*. More prominently, 61 sequence reads assembled into four OTUs that can produce a terminal fragment of 248 nt. These sequences were all classified within the alphaproteobacterial lineage *Thalassobacter*. Sequences corresponding to the 194 nt fragment were not detected in the library. The 194-bp fragment was, however, a relatively weak signal, compared with other fragments for which density shifts were observed (see Fig. S2). The absence of a corresponding sequence in the clone library was assumed to result from low abundance.

A secondary aim of this study was to assay for mRNA of assimilatory nitrate reduction genes to corroborate SIP data. It was hypothesized that nitrate-assimilating bacteria would express the gene for the catalytic subunit of assimilatory nitrate reductase, *nasA*, which has been used in prior studies as the biomarker gene for the detection of nitrate-assimilating bacteria in marine systems (Allen

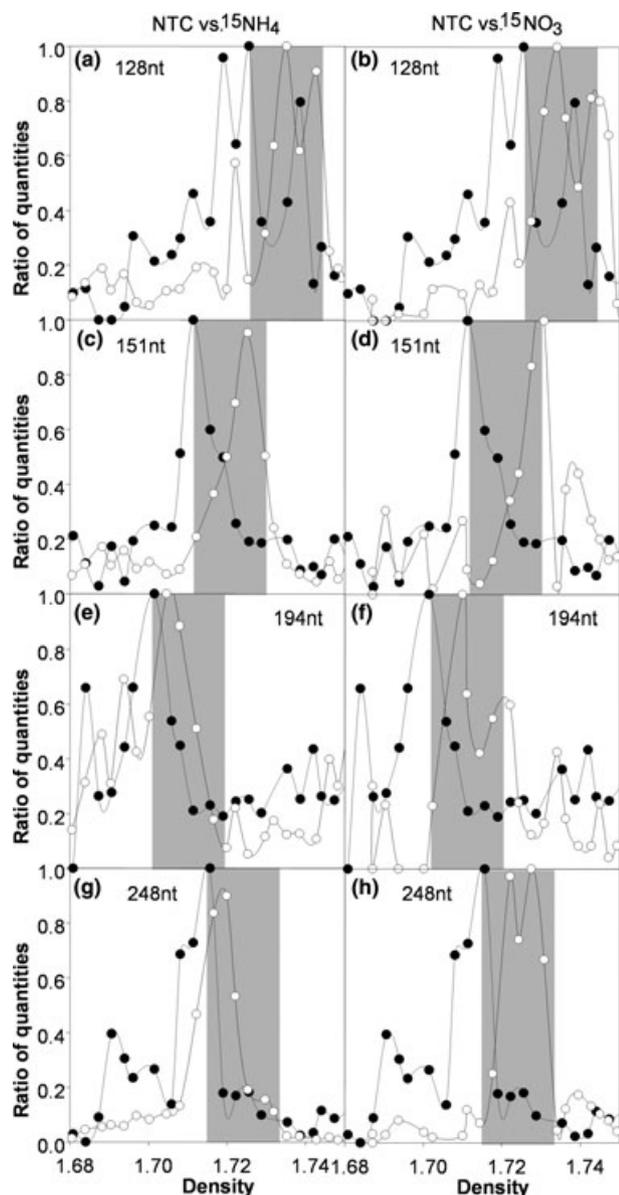


Fig. 2. Analysis of TRFs detected in each density fraction of (●) ambient community DNA (NTC = no treatment control) and DNA extracted from bottle incubations with (○) ^{15}N ammonium or ^{15}N nitrate. Fragments for which a density shift was observed were plotted as a function of density (x-axis) and relative peak height (y-axis). Peak height was averaged across ± 1 nt to account for small sizing errors and then normalized to the highest detected signal. The width of gray boxes indicates the expected density shift for 100% labeling of DNA with ^{15}N . The gray boxes are aligned on the left with the maximum peak height of ambient control DNA (NTC). Shown are ratios of quantities, which are calculated by dividing the peak height in each fraction by the highest value measured in any of the fractions obtained in a given CsCl column.

et al., 2001; Adhitya *et al.*, 2007; Cai & Jiao, 2008). These studies demonstrated the presence of *nasA* genes, but did not provide information regarding expression. We, there-

fore, analyzed community mRNA via RT-PCR and functional gene microarray analysis. RT-PCR of *nasA* genes was conducted using previously published primers (Allen *et al.*, 2001). *nasA* mRNA was not detected, despite repeated amplification attempts and several rounds of PCR optimization (including testing of different primer concentrations, annealing temperatures, template concentrations, and adding excess TAQ polymerase; data not shown). RNA was considered to be of good quality and free of inhibitors, as other genes, such as the large subunit gene of RubisC/O (*rbcL*), nitrite reductase (*nirS*), and 16S rRNA gene could be easily amplified by RT-PCR (see Fig. S1). Primers and PCR conditions appeared to be adequate, as *nasA* genes could readily be PCR amplified and cloned from community DNA (see Fig. S1).

The unexpected lack of *nasA* mRNA in RT-PCR experiments prompted us to seek confirmation of these data. A secondary approach for mRNA was applied by utilizing the GeoChip 3.0 for mRNA hybridization. The GeoChip functional gene microarray contains probes for many of the key N-cycling genes (Fig. 3), including *nasA*. DNA and mRNA hybridization were detected for *ureC*, *nirS*, *nirK*, *narG*, *nosZ*, *napA*, *nrfA*, *amoA*, and *nifH* (Fig. 3, Table 2), representing key N-transformation genes for urea cycling, denitrification, dissimilatory nitrate and nitrite reduction, as well as N fixation. There was no evidence for the expression of assimilatory nitrate reduction genes, *nasA* (bacterial) and *narB* (cyanobacterial). Among the positive N-cycling gene probes (DNA + RNA hybridization signal), 35% originated from the *Alpha*- and *Gammaproteobacteria*, corroborating the importance of these two lineages at the site. Other bacterial lineages (Table 2) implicated in N cycling by the GeoChip belonged to the *Beta*- and *deltaproteobacteria*, *Clostridia*, *Aquificales*, *Halobacteria*, and *Chloroflexi*. Bacterial and archaeal *amoA* mRNA were detected.

Discussion

^{15}N -based DNA SIP is an emerging methodology for testing hypotheses with respect to microbial N cycling in the environment. The technique allows for the interrogation of N nutrition of specific microbial populations and has been applied to identify novel diazotrophic bacteria in soil (Buckley *et al.*, 2007a, b), to study N uptake by *Synechococcus* and diatoms in marine systems (Wawrik *et al.*, 2009), and to study N utilization by bacteria in petroleum-contaminated arctic soils (Bell *et al.*, 2011). The aim of the current study was to apply a community-based ^{15}N SIP approach to identify bacterial populations in a coastal, subtropical, marine environment that were actively utilizing N in the form of nitrate.

An important limitation of SIP studies is the potential for 'cross-feeding', where N in one form is transformed,

Table 1. *In silico* prediction of terminal restriction fragments and phylogenetic classification of cloned 16S rRNA gene fragments

Class	Order	Family	Genus	Predicted AluI TRF	# of clones
<i>Alphaproteobacteria</i>	Unclassified	Unclassified	Unclassified	66	2
<i>Alphaproteobacteria</i>	Unclassified	Unclassified	Unclassified	72	2
<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	Unclassified	Unclassified	72	1
<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	<i>Rhodobacteraceae</i>	<i>Thalassobacter</i>	73	1
<i>Bacteroidetes</i>	Uncultured	Unclassified	Unclassified	73	2
<i>Gammaproteobacteria</i>	<i>Legionellales</i>	<i>Coxiellaceae</i>	<i>Coxiella</i>	74	125
<i>Gammaproteobacteria</i>	Unclassified	Unclassified	Unclassified	74	16
<i>Gammaproteobacteria</i>	Unclassified	Unclassified	Unclassified	74	1
<i>Bacteroidetes</i>	Unclassified	Unclassified	Unclassified	74	1
<i>Gammaproteobacteria</i>	<i>Oceanospirillales</i>	<i>Oleiphilaceae</i>	Uncultured	74	1
<i>Gammaproteobacteria</i>	<i>Oceanospirillales</i>	<i>Oleiphilaceae</i>	Uncultured	82	2
<i>Gammaproteobacteria</i>	<i>Pseudomonadales</i>	<i>Moraxellaceae</i>	<i>Acinetobacter</i>	85	1
<i>Gammaproteobacteria</i>	Unclassified	Unclassified	Unclassified	127	1
<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	<i>Rhodobacteraceae</i>	<i>Roseovarius</i>	127	1
<i>Alphaproteobacteria</i>	Unclassified	Unclassified	Unclassified	128	2
<i>Gammaproteobacteria</i>	Unclassified	Unclassified	Unclassified	128	1
<i>Gammaproteobacteria</i>	<i>Oceanospirillales</i>	<i>Oleiphilaceae</i>	Uncultured	138	2
<i>Alphaproteobacteria</i>	<i>Rhodobacterales</i>	<i>Rhodobacteraceae</i>	<i>Thalassobacter</i>	139	2
<i>Betaproteobacteria</i>	<i>Burkholderiales</i>	Unclassified	Unclassified	139	1
<i>Gammaproteobacteria</i>	<i>Oceanospirillales</i>	<i>Oleiphilaceae</i>	Uncultured	147	1
<i>Gammaproteobacteria</i>	<i>Alteromonadales</i>	Unclassified	Unclassified	152	14
<i>Gammaproteobacteria</i>	<i>Alteromonadales</i>	Unclassified	Unclassified	152	3
<i>Gammaproteobacteria</i>	<i>Alteromonadales</i>	Uncultured	Unclassified	152	2
<i>Gammaproteobacteria</i>	Unclassified	Unclassified	Unclassified	152	1
<i>Gammaproteobacteria</i>	<i>Oceanospirillales</i>	<i>Oleiphilaceae</i>	Uncultured	152	1
<i>Gammaproteobacteria</i>	Unclassified	Unclassified	Unclassified	152	1
<i>Alphaproteobacteria</i>	<i>Rhodobacterales</i>	<i>Rhodobacteraceae</i>	<i>Thalassobacter</i>	201	3
<i>Alphaproteobacteria</i>	Unclassified	Unclassified	Unclassified	201	2
<i>Verrucomicrobia</i>	Unclassified	Unclassified	Unclassified	201	1
<i>Gammaproteobacteria</i>	<i>Oceanospirillales</i>	<i>Oleiphilaceae</i>	Uncultured	201	1
<i>Gammaproteobacteria</i>	Unclassified	Unclassified	Unclassified	204	2
<i>Bacteroidetes</i>	<i>Flavobacteriaceae</i>	Unclassified	Unclassified	206	1
<i>Bacteroidetes</i>	Unclassified	Unclassified	Unclassified	207	2
<i>Cyanobacteria</i>	<i>Chroococcales</i>	Unclassified	Unclassified	217	1
<i>Alphaproteobacteria</i>	<i>Rhodospirillaceae</i>	Unclassified	Unclassified	221	1
<i>Deltaproteobacteria</i>	Unclassified	Unclassified	Unclassified	232	1
<i>Gammaproteobacteria</i>	<i>Oceanospirillales</i>	Uncultured	Uncultured	234	25
<i>Gammaproteobacteria</i>	<i>Oceanospirillales</i>	<i>Oleiphilaceae</i>	Uncultured	234	1
<i>Alphaproteobacteria</i>	<i>Rhodobacterales</i>	<i>Rhodobacteraceae</i>	<i>Thalassobacter</i>	248	56
<i>Alphaproteobacteria</i>	<i>Rhodobacterales</i>	<i>Rhodobacteraceae</i>	<i>Thalassobacter</i>	248	2
<i>Alphaproteobacteria</i>	<i>Rhodobacterales</i>	<i>Rhodobacteraceae</i>	<i>Thalassobacter</i>	248	2
<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	<i>Rhodobacteraceae</i>	<i>Thalassobacter</i>	248	1
<i>Gammaproteobacteria</i>	Unclassified	Unclassified	Unclassified	253	2
<i>Gammaproteobacteria</i>	<i>Alteromonadales</i>	<i>Endobugula</i>	Uncultured	273	1
<i>Alphaproteobacteria</i>	<i>Rhodobacterales</i>	<i>Rhodobacteraceae</i>	<i>Thalassobacter</i>	307	2
<i>Alphaproteobacteria</i>	<i>Rickettsiales</i>	Uncultured	Unclassified	352	1
<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	<i>Rhodobacteraceae</i>	<i>Thalassobacter</i>	391	1

through any number of processes, into another form. It is possible, for example, that some of the ^{15}N label in ^{15}N nitrate amendments could have been converted to ^{15}N ammonium via remineralization of particulate organic nitrogen (PON). The degree to which cross-feeding is a problem, however, is dependent on the question being addressed. Generally, if only the final fate of N substrates (i.e. ammonium or nitrate) is in question, then cross-

feeding is not an issue. The approach used here traces the transfer of N into bacterial DNA, irrespective of the actual form of the N when it was taken up. More specifically, if one seeks to define which N substrates are utilized, then the results can be more ambiguous and additional measurements are helpful. With respect to the bacterial community studied here, the fate of the added ^{15}N nitrate is clear – uptake into DNA. To address the

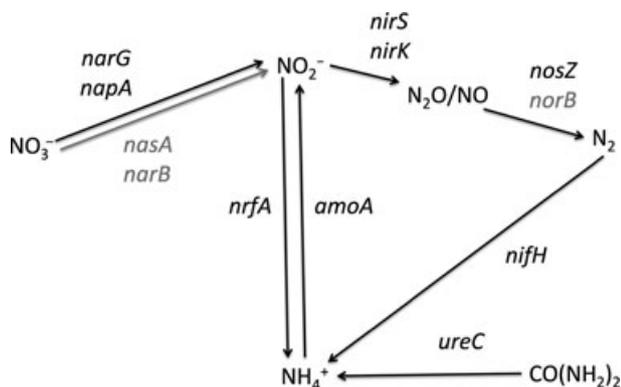


Fig. 3. Schematic representation of a partial N cycle and the genes corresponding to pathways represented on the GeoChip 3.0. Black arrows and gene symbols indicate that DNA and mRNA hybridization signals were detected for these genes/pathways. Gray arrows and gray gene names indicate that mRNA was not detected.

more specific concern, the following case can be made that the effect of ‘cross-feeding’ on SIP data was likely minor and that direct nitrate uptake by bacteria was indeed occurring. The concentration of PON was $5.78 \mu\text{mol N L}^{-1}$, while the nitrate uptake rate was $0.065 \mu\text{mol N L}^{-1} \text{h}^{-1}$. After 24 h of incubation, ^{15}N could, therefore, account for at most 27% of total PON. Given a 0% labeled PON pool at T0 and 27% labeling at the end, it follows that *c.* $0.27 \mu\text{moles}$ of ^{15}N ammonium could have been produced, given the measured ammonium regeneration rate of $0.082 \mu\text{M N h}^{-1}$ (Wawrik *et al.*, 2009). The result would be that about 15% of total ammonium uptake could originate from recycled ^{15}N , which is less than the observed density shifts observed in ^{15}N nitrate treatments. In addition, at least two of the observed fragments (TRFs 194 and 248) increased in density in the presence of ^{15}N nitrate, but not in the presence of ^{15}N ammonium. While replicates would be helpful for statistical comparison of nitrate and ammonium treatments, the data indicate that ^{15}N nitrate conversion to ammonium was likely a minor contributor to density shifts. If nitrate was first converted to ammonium, TRFs 194 and 248 would be equally labeled in ^{15}N ammonium and ^{15}N nitrate treatments, as the actual microbial activity observed would be ammonium uptake.

SIP analysis implicates several poorly classified marine gammaproteobacterial lineages, as well as the alphaproteobacterium *Thalassobacter* in nitrate uptake. *Thalassobacter* belongs to the Alphaproteobacteria and is closely related to the marine *Roseobacter* species. Alphaproteobacteria are well known to be important components of marine bacterioplankton, and sequences belonging to this lineage often make up a sizable percentages of 16S clone libraries; for example, in two libraries from the coastal Pacific (coast

of California), 55% of sequences was classified as Alphaproteobacteria, and FISH analysis indicated that this group accounted for 3–34% of DAPI countable cells (Cottrell & Kirchman, 2000). Within the Alphaproteobacteria, distinct lineages have been demonstrated to be particularly abundant. Sar 11 (*Pelagibacter ubique*), for example, can account for 50% of the bacterioplankton in the open ocean (Morris *et al.*, 2002). Similarly, the *Roseobacter* group can account for 10–20% of bacterial populations, particularly in coastal marine environments (Gonzalez & Moran, 1997; Moran *et al.*, 2007). Nitrate utilization genes can be identified in the genomes of species belonging to the *Roseobacter* group, including *Sulfitobacter* sp., *Roseovarius* sp., *Roseobacter* sp., and *Octadecabacter* sp. They are also absent from the genomes of other *Roseobacter* species, notably *Silicibacter* (Moran *et al.*, 2007). *Thalassobacter* sp. was first isolated from a site in the Mediterranean Sea (Macian *et al.*, 2005) and is strictly aerobic, bacteriochlorophyll *a*-containing, and heterotrophic, with a wide salinity tolerance range (0.85–7%). It utilizes pyruvate and acetate, but is unable to utilize simple sugars as growth substrates. The ability to reduce nitrate to nitrite is absent from the type strain, and denitrification has not been observed (Macian *et al.*, 2005). Genomic information is not available for bacteria within this genus, and confirmation of the presence of a nitrate-reducing complex can, therefore, not be obtained. Data described here, however, suggest that N from nitrate was incorporated into *Thalassobacter* DNA in the environment, suggesting that different ecotypes of *Thalassobacter* may exist.

Prior studies describing the diversity of assimilatory nitrate reductase genes (*nasA/narB*) in marine environments revealed the presence of cytophagal, cyanobacterial, clostridial, and proteobacterial lineages (Allen *et al.*, 2001; Adhitya *et al.*, 2007; Cai & Jiao, 2008). Alphaproteobacterial lineages were among those detected (Adhitya *et al.*, 2007; Cai & Jiao, 2008), but Gammaproteobacteria typically represent the bulk of the *nasA* gene diversity. Within the Gammaproteobacteria, the Alteromonadales appear to be a particularly dominant group (Adhitya *et al.*, 2007; Cai & Jiao, 2008). In line with prior reports for this lineage, SIP data described here (TRF at 151 nt) also suggest that Alteromonadales are relevant in nitrate uptake. Alteromonadales are primarily associated with marine environments where they can be facultatively anaerobic and occur across a wide range of oceanic conditions, including sea ice, surface water, and deep sea sediments (Bowman & McMeekin, 2005). The type strain, *Alteromonas macleodii*, does not appear to utilize nitrate (Bowman & McMeekin, 2005), but a search of the two available genomes (*A. macleodii* ATCC 27126, and ‘Deep Ecotype’) indicates the presence of a nitrate

Table 2. DNA and mRNA GeoChip 3.1 analysis of N-cycling genes

Genbank ID	Gene	Category	Class	Order	Organism	RNA*	DNA*	RNA/DNA
150029098	<i>ureC</i>	Ammonification	Alphaproteobacteria	Rhizobiales	<i>Sinorhizobium medicae</i>	4899	4139	1.18
113941017	<i>ureC</i>	Ammonification	Chloroflexi	Herpetosiphonaceae	<i>Herpetosiphon aurantiacus</i>	6357	22227	0.29
151280243	<i>ureC</i>	Ammonification	Betaproteobacteria	Burkholderiales	<i>Janthinobacterium</i> sp.	7030	23908	0.29
34494851	<i>ureC</i>	Ammonification	Halobacteria	Halobacteriales	<i>Haloarcula aidinensis</i>	22409	31262	0.72
67985209	<i>ureC</i>	Ammonification	Actinobacteria	Actinomycetales	<i>Kineococcus radiotolerans</i>	66929	19056	3.51
57335474	<i>nirS</i>	Denitrification	Gammaproteobacteria	Alteromonadales	Uncultured sp.	4775	11493	0.42
149783097	<i>nirK</i>	Denitrification	Alphaproteobacteria	Rhodobacterales	Uncultured sp.	5138	10923	0.47
26278828	<i>narG</i>	Denitrification	Actinobacteria	Actinomycetales	Uncultured sp.	5864	27759	0.21
41349577	<i>narG</i>	Denitrification	Halobacteria	Halobacteriales	<i>Haloferax mediterranei</i>	6549	6441	1.02
116634745	<i>narG</i>	Denitrification	Deltaproteobacteria	Desulfuromonadales	Uncultured sp.	7589	9401	0.81
29125972	<i>nosZ</i>	Denitrification	Gammaproteobacteria	Pseudomonadales	Uncultured sp.	8061	7608	1.06
63081994	<i>nosZ</i>	Denitrification	Alphaproteobacteria	Rhodospirillales	Uncultured sp.	10543	14274	0.74
26278770	<i>narG</i>	Denitrification	Betaproteobacteria	Burkholderiales	Uncultured sp.	20703	51300	0.4
124488145	<i>napA</i>	Dissimilatory N reduction	Aquificales	Hydrogenothermaceae	Uncultured sp.	10912	7703	1.42
127511439	<i>nrfA</i>	Dissimilatory N reduction	Gammaproteobacteria	Enterobacteriaceae	<i>Shewanella loihica</i>	12646	5930	2.13
124488141	<i>napA</i>	Dissimilatory N reduction	Gammaproteobacteria	Enterobacteriaceae	Uncultured sp.	16142	14382	1.12
109645564	<i>nrfA</i>	Dissimilatory N reduction	Clostridia	Clostridiales	<i>Desulfitobacterium hafniense</i>	20143	28867	0.7
19481356	<i>amoA</i>	Nitrification	Betaproteobacteria	Nitrosomonadale	Uncultured sp.	4674	8923	0.52
82570859	<i>amoA</i>	Nitrification	Unclassified Crenarcheota	Unclassified	Uncultured sp.	5295	10711	0.49
95116083	<i>amoA</i>	Nitrification	Unclassified Crenarcheota	Unclassified	Uncultured sp.	9364	10400	0.9
44829089	<i>nifH</i>	Nitrogen fixation	Clostridia	Clostridiales	Uncultured sp.	7026	35833	0.2
148568718	<i>nifH</i>	Nitrogen fixation	Chloroflexi	Chloroflexales	<i>Roseiflexus</i> sp.	11549	9975	1.16
3157608	<i>nifH</i>	Nitrogen fixation	Alphaproteobacteria	Rhizobiales	Uncultured sp.	20649	5284	3.91

*Fluorescence signal detected during GeoChip 3.1 analysis. Only those spots for which both DNA and RNA signal were detected were considered positive.

reduction operon that includes nitrate reductase, nitrite reductase, as well as an ABC-dependent nitrate/nitrite transporter.

Despite observations from SIP experiments, which suggest that N from ^{15}N nitrate is readily incorporated into the DNA of several bacterial populations, no evidence for the expression of *nasA* genes was observed using the two different approaches. Several factors may have contributed to this observation. First, it is possible that *nasA* PCR primers used for RT-PCR contain sequence mismatches to relevant bacteria at our study site. The primers were originally designed on the basis of sequences from *Gammaproteobacteria*, *Bacteroidetes*, and *Cytophaga* (Allen et al., 2001). Marine alphaproteobacterial *nasA* sequences were not available at the time, but a comparison of the nested primer pairs to *nasA* sequences of *Roseobacter* sp.

MED193, *Roseovarius* sp. 217, and *Octadecabacter antarcticus* 307 indicates that only a single mismatch exists in one of the four primers (*nas1933*). Furthermore, the primers have been successfully employed to amplify *nasA* from a wide range of environmental bacteria and locations, including a Norwegian fjord, the Barents Sea, the Skidaway River, the South Atlantic Bight, the North Pacific Gyre, and the South China Sea (Allen et al., 2001; Cai & Jiao, 2008). We, therefore, expect that primer biases were of minor importance. Support for this assumption is provided by the observation that *nasA* mRNA was also not observed using the GeoChip. Detection by hybridization is typically less sensitive to biases introduced by single base-pair mismatches. Although the GeoChip cannot be considered to be a comprehensive array with respect to marine environments, it does contain a sizable number

of relevant N-cycling gene probes from a diversity of important marine species. The array contains 106 assimilatory nitrate reductase gene probes (See Table S2). Of these, 82 are of marine origin, including *nasA* genes from alpha-, beta-, and gammaproteobacteria (53 probes), as well as several marine cyanobacterial *narB* gene sequences (29 probes). Included are probes representing several *Roseobacter* strains as well as sequences detected in several environmental surveys (Allen *et al.*, 2001; Adhitya *et al.*, 2007; Cai & Jiao, 2008). The array, therefore, represents a relatively comprehensive representation of known marine *nasA/narB* sequences. Parallel analysis via the GeoChip and RT-PCR amplification should, therefore, be sufficiently sensitive to detect *nasA* mRNA from relevant marine *Alpha*- and *Gammaproteobacteria*.

Disparate observations between SIP and mRNA experiments might be explained by active denitrification as evidenced by mRNA signals for denitrification genes (*nirS*, *nirK*, and *nosZ*), which were readily detected by RT-PCR and GeoChip analysis. Denitrification proceeds via nitrite as a shared intermediate in both assimilatory and dissimilatory nitrate reduction pathways. mRNA for both the periplasmic and membrane-bound nitrate reductase genes (*napA* and *narG*, respectively) was detected. Dissimilatory nitrate reduction could, therefore, provide a continuous supply of nitrite for both denitrification and further assimilatory N reduction to ammonium. Under such circumstances, bacterial populations capable of regulating assimilatory nitrate vs. nitrite reduction genes would gain an energetic advantage. Dissimilatory nitrate reduction (denitrification), as a metabolic trait, is widely distributed among facultatively anaerobic bacterial and archaeal species. Historically, denitrification is, therefore, understood as a process that primarily occurs across aerobic/anaerobic interfaces (Zehr & Ward, 2002). It has, however, been reported that denitrification can occur in the presence of atmospheric levels of O₂ in the alphaproteobacterium, *Paracoccus pantotrophulus* (Robertson *et al.*, 1995). Similar observations have been made in a strain of *Pseudomonas stutzeri* (Su *et al.*, 2001), suggesting that this trait may be more widely distributed. The environmental significance of aerobic denitrification remains unclear (Zehr & Ward, 2002), but GeoChip analysis in this study indicated mRNA transcription of several denitrification genes (Table 2) in *Alpha*- and *Gammaproteobacteria*.

Nitrogen reduction via dissimilatory nitrate reduction to ammonium (DNRA) has also been reported for some marine systems. The importance of DNRA as a nitrate sink is traditionally only recognized for sediments with high organic loading (Christensen *et al.*, 2000). The process is, therefore, typically considered to be most relevant in coastal sediment (Bonin *et al.*, 1998), and it has

been suggested that it is unlikely to occur in the water column (Zehr & Ward, 2002). More recently, however, DNRA has been observed in the Peruvian oxygen minimum zone (Lam *et al.*, 2009). ¹⁵N ammonium was produced during ¹⁵N-nitrogen oxide incubations, and high levels of *nrfA* mRNA were detected. *nrfA* encodes a cytochrome *c* nitrite reductase (Einsle *et al.*, 1999) and is considered to be the functional biomarker gene for DNRA (Lam *et al.*, 2009). We also detected *nrfA* mRNA in the current study (Table 2). Another potential pathway for N from nitrate to ammonium is anammox (Kartal *et al.*, 2007). *Kuenia stuttgartiensis* reduces nitrate to nitrite and ammonium, and then subsequently utilizes both of these substrates in the anammox process. Neither DNRA nor anammox, however, are likely to be the key mechanisms behind ¹⁵N incorporation from nitrate into DNA observed in SIP analysis. Ammonium generated by anammox bacteria is converted to N₂ gas, and is therefore not available for assimilation by other community members. Further, any DNRA or ammonium release by anammox would be detected as part of the measured ammonium regeneration rates (see Discussion of N recycling and cross-feeding earlier).

Overall, these observations speak to the complexity of N flow in marine systems and the fact that individual N transformation processes are often tightly linked. The present study applies ¹⁵N-based DNA SIP to pelagic marine bacterial communities and provides evidence for the uptake of N from nitrate into several proteobacterial lineages, notably *Thalassobacter* and *Alteromonadales*. mRNA data presented here, however, highlight the importance of multiple experimental approaches to understand N flow dynamics. ¹⁵N-based DNA SIP measurements are well suited to addressing questions regarding the N cycle and the fate of different N substrates, but are greatly informed by an integrated approach that includes targeted ¹⁵N tracer studies and other molecular techniques conducted in parallel.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. RT-PCR of N-cycling genes.

Fig. S2. Example of heat map used to identify DNA from bacterial populations that exhibits density shifts in the presence of ^{15}N label.

Table S1. Complete list of probes that displayed positive hybridization signals for DNA and RNA during GeoChip 3.1 analysis.

Table S2. Probes for assimilatory nitrate reductase genes (*nasA/narB*) on the GeoChip 3.1.

Table S3. Comparison of 16S rRNA gene sequence library data obtained by 454 and Sanger sequencing.

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