Vol. 79: 31–48, 2017
https://doi.org/10.3354/ame01817

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online April 7

Aerobic and anaerobic ammonium oxidizers in the Cariaco Basin: distributions of major taxa and nitrogen species across the redoxcline

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ABSTRACT: Depth distributions of cells and functional gene copies from anaerobic ammonium-oxidizing (anammox) bacteria, aerobic ammonium-oxidizing bacteria (AOB) and archaea (AOA) in the Cariaco Basin, Venezuela were obtained using FISH and q-PCR assays. These distributions were compared to concentrations of dissolved ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), hydrogen sulfide (H_2S) and oxygen (O_2) along the redoxcline during 3 cruises. Cell counts of anammox bacteria and copies of their nitrite reductase gene (Scalindua-*nirS*) were consistently observed in 2 distinct layers: the suboxic zone (≤1.1 × 10^6 cells l^{-1}) and the upper euxinic zone (≤4.7 × 10^6 cells l^{-1}). We hypothesize that anammox bacteria vertically organize in response to NO_2^- sources, with NO_2^- for the shallower assemblage supplied by nitrification and for the deeper assemblage by denitrification. Peaks in AOA cell abundances (up to 14.8 × 10^6 cells l^{-1}) consistently coincided with copy numbers of archaeal ammonia monooxygenase subunit A gene (archaeal *amoA*) in the lower oxic zone. Peak abundances of beta- and gammaproteobacterial AOB cells (up to 24.0 × 10^6 cells l^{-1}) and one of their ammonia monooxygenase genes (β-*amoA*) overlapped above the shallow anammox peak. Our results suggest that anammox bacteria AOB, AOA and denitrifiers are metabolically interdependent and ultimately controlled by vertical fluxes of O_2, NO_3^-, NO_2^-, and NH_4^+ in the Cariaco Basin.

KEY WORDS: Anammox · Ammonium oxidation · Cariaco · q-PCR · FISH · Nutrient flux

INTRODUCTION

Transitional waters between oxic and anoxic conditions, known as redoxclines, can include a suboxic zone, where dissolved O_2 and hydrogen sulfide (H_2S) concentrations are below routine detection limits (1 to 2 µM and 1 µM, respectively). Suboxic zones are favorable habitats for chemoautotrophic bacteria dependent upon reduced inorganic substrates for energy (Sorokin 1972, Taylor et al. 2001). The Cariaco Basin off Venezuela's north coast is permanently stratified, with a pronounced redoxcline, a dynamic suboxic zone and permanently euxinic (anoxic and sulfidic) conditions below depths historically varying between 250 and 350 m (Scranton et al. 2014). Early studies of the Cariaco Basin detected chemoautotrophic bacterial activity peaks within the redoxcline, attributed to sulfur oxidizers (Tuttle & Jannasch 1973, 1979). However, geochemical profiles also suggest oxidation of NH_4^+ occurs in the suboxic zone (Taylor et al. 2001, 2006). This process has received far less attention than sulfur cycling in the Cariaco Basin.

Similar to other O_2-deficient areas, such as oxygen minimum zones (OMZs) extending out from ocean

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basin margins, N:P ratios in Cariaco Basin's redoxcline are very low due to fixed nitrogen loss (Richards & Vaccaro 1956, Cline & Richards 1972). For decades, only 2 bacterial lineages in the *Beta-* and *Gammaproteobacteria* were thought to be the major organisms converting NH_4^+ to oxidized nitrogen species in the ocean, and therefore crucial to the oceanic nitrogen budget (Head et al. 1993, Teske et al. 1994, Kowalchuk & Stephen 2001). More recently, members of the *Thaumarchaeota* clade were shown to aerobically oxidize NH_4^+ to NO_2^- (Könneke et al. 2005). This clade is ubiquitous throughout the world's oceans and its role in the oceanic nitrogen cycle is likely extensive (Venter et al. 2004, Treusch et al. 2005).

Somewhat surprisingly in the Cariaco Basin, the depths of N_2 depletion and measured N_2/Ar excesses strongly suggest fixed nitrogen is being lost in anoxic water, making aerobic NH_4^+ oxidation unlikely (Richards & Benson 1961, Montes et al. 2013). An anaerobic NH_4^+ oxidation pathway involving sulfate as the oxidant was hypothesized long ago for the Cariaco Basin by Richards & Benson (1961). Broda (1977) demonstrated that anaerobic NH_4^+ oxidation to N_2, i.e. anammox, was thermodynamically feasible using NO_2^- as the oxidant. However, bacteria carrying out the anaerobic oxidation of ammonium with NO_2^- eluded isolation attempts for some years until Strous et al. (1999) obtained an enrichment culture of *Planctomycetes* from a sewage treatment plant and verified the existence of a biological anammox pathway.

Extensive field research has subsequently demonstrated that anammox is a common process in many O_2-depleted aquatic habitats, contributing substantially to fixed nitrogen losses and N_2 production in marine environments. Anammox bacteria have been reported from a range of O_2-depleted environments, including arctic sediments and fjords (Rysgaard et al. 2004, Schmid et al. 2007, Zhang et al. 2007), the eastern tropical Pacific OMZs (Stevens & Ulloa 2008, Galán et al. 2009, Lam et al. 2009), the Arabian, Black, Barent, and Baltic seas (Kuypers et al. 2003, Hannig et al. 2007, Lam et al. 2007, Schmid et al. 2007, Jensen et al. 2008, 2011), the Benguela upwelling system (Kuypers et al. 2005), the Gulf of Dolce (Dalsgaard et al. 2003) and Mid-Atlantic Ridge hydrothermal vents (Byrne et al. 2009). The presence of anammox bacteria was recently confirmed within the Cariaco Basin by ladderane lipid biomarker signatures and fluorescent *in situ* hybridization (FISH) analyses (Wakeham et al. 2012), suggesting that nitrogen stoichiometric imbalances originally reported by Richards & Vaccaro (1956) for this system may be explained, at least in part, by activities of this unique group of *Bacteria*. Aerobic NH_4^+ oxidizers typically thrive at low O_2 concentrations (Kowalchuk & Stephen 2001, Kalvelage et al. 2011) while anammox activity has been observed at micromolar concentrations of oxygen (Kalvelage et al. 2011). Therefore, the precise distributions of anammox organisms, aerobic ammonium-oxidizing bacteria (AOB), and aerobic ammonium-oxidizing archaea (AOA) relative to geochemical profiles are not well understood.

Furthermore, many studies have suggested that nitrification, anammox, and denitrification are coupled. One pathway for NO_2^- supply to anammox populations is NO_3^- reduction during the denitrification process (Dalsgaard et al. 2003, 2012). In OMZs, this is likely the rate-limiting step for anammox (Ward 2013). Additionally, NO_2^- can also be supplied by nitrification, where NH_4^+ is oxidized to NO_2^- by either AOA or AOB. Investigations in the Black Sea, an euxinic end-member system similar to the Cariaco Basin, have suggested that the source of NO_2^- for anammox is NH_4^+ oxidation by autotrophic nitrifiers rather than heterotrophic denitrifiers (Lam et al. 2007).

The main objective of the present study is to understand anammox bacterial dynamics in the Cariaco Basin and explore their possible relationships with AOB, AOA and nutrient distributions. Using class and genus-specific FISH probes, we describe vertical distributions of anammox bacteria, AOB in the *Beta-* and *Gammaproteobacteria* (hereafter 'β-AOB' and 'γ-AOB', respectively) and AOA (*Thaumarchaeota*) populations during 3 cruises spaced 6 mo apart. In addition, functional genes involved in NH_4^+ oxidation by *Bacteria* (*amoA*) and *Thaumarchaeota* (archaeal *amoA*), as well as the nitrite reductase (Scalindua-*nirS*) gene specific for anammox bacteria from the genus '*Candidatus* Scalindua' were profiled using q-PCR. Depth distributions of anammox, AOB and AOA cells and functional genes were compared to those of dissolved nitrogen species and other redox-sensitive chemicals. Temporal variations in vertical flux estimates of NH_4^+ and NO_3^- and NO_2^- inventories in the suboxic zone were then related to those in anammox, AOB and AOA inventories.

MATERIALS AND METHODS

Site description

The Cariaco Basin is a semi-enclosed, tectonically-formed depression located on the northern continental shelf of Venezuela. The basin is divided into 2

sub-basins by a saddle rising up to 900 m above the 1400 m deep seabed. The basin links with the Caribbean Sea through the 146 m deep Centinela Channel to the west (Febres-Ortega & Herrera 1975) and the 135 m deep Tortuga Channel to the north (Richards et al. 1965). Waters above about 100 m depth exchange freely with the Caribbean Sea. The Basin's geomorphologically limited lateral exchange, thermal stratification, and high productivity, supported by seasonal upwelling result in permanent O_2 depletion at depths below 100 m and complete permanent anoxia below 250 to 350 m resulting in an euxinic volume of approximately 5200 km^3 (Richards 1975, Zhang & Millero 1993, Scranton et al. 2001).

Sample collection and storage

Water samples were collected at the Cariaco Ocean Time Series station onboard the R/V 'Hermano Ginés' (operated by Estación de Investigaciones Marinas (EDIMAR), Fundación la Salle de Ciencias Naturales on Isla Margarita, Venezuela) during 3 cruises on 6 December 2010, 6 May 2011 and 10 November 2011 (CAR-DEC2010, CAR-MAY2011 and CAR-NOV 2011, respectively). Samples from 18 depths were collected with a SeaBird SBE-25 rosette equipped with 12 polytetrafluoroethylene (PTFE) coated 8 l Niskin bottles. The Seabird rosette included a CTD with conductivity, temperature and pressure probes, a SBE-43 O_2 probe and a Chelsea Sea Tec beam transmissometer (660 nm). Discrete dissolved oxygen samples were analyzed by Winkler titrations (Aminot 1983). Beam attenuation peaks indicated particle maxima close to the euxinic boundary and were used as a basis for adaptive sampling. Water was collected at the particle maximum, then at 10 to 20 m intervals above and below it, and at wider intervals outside the redoxcline (Taylor et al. 2001). When drawing samples for chemical analyses, Niskin bottles were slightly pressurized with argon gas to minimize chances of sample oxygenation (Taylor et al. 2001). Nutrient profiles for NH_4^+, NO_2^- and NO_3^- were provided by Dr. K. A. Fanning from the University of South Florida following standard protocols (Gordon et al. 1993). H_2S profiles were obtained following Cline (1969) with modifications detailed in Percy et al. (2008) that yielded a detection limit of 0.6 µM. Chemoautotrophic assimilation of dissolved inorganic carbon (DIC) (hereafter also referred to as 'dark DIC assimilation') was measured by ^{14}C-bicarbonate incorporation into particles (Taylor et al. 2001).

For FISH analyses, 45 ml water samples were taken in duplicate for each depth, fixed with filtered borate-buffered formaldehyde (2% final concentration) on board and stored at −20°C. In the laboratory, samples were thawed and filtered onto white polycarbonate membranes (47 mm diameter, 0.2 µm pore size, EMD© Millipore) and stored at −80°C until further analysis (Pernthaler et al. 2001).

For the DNA samples, 2 to 2.5 l samples from 12 depths were directly collected from Niskin bottles into low-O_2 permeable sterile Secure™ ethylene vinyl acetate (EVA) compounder bags (Capitol Medical). Compounder bags were attached to in-line filter holders containing GTTP type membranes (as above) and attached to a vacuum filtration manifold. The enclosed system minimized atmospheric exposure and permitted filtration of 7 samples simultaneously. Filters were stored in 5 ml PowerWater™ Bead Tubes (MO BIO Laboratories®), submerged in 4 ml of LifeGuard™ Soil Preservation Solution (MO BIO Laboratories®), frozen in the field and stored at −80°C until further processing.

FISH and CARD-FISH

Previous FISH studies in the Cariaco Basin demonstrated that simple FISH protocols for the bacterial clades surveyed yielded results indistinguishable from those obtained by parallel Catalyzed Reporter Deposition FISH (CARD-FISH) assays, but led to underestimation of archaeal populations (Lin et al. 2007). Therefore, FISH was applied for anammox bacteria and AOB following Pernthaler et al. (2001) and CARD-FISH analyses were performed for AOA following Pernthaler et al. (2002). For FISH, briefly, small wedges were cut from the filters and incubated in hybridization buffer containing the oligonucleotide probes (Table 1) labeled with the fluorescent dye, cyanine 3 (Cy3). The probes Nso1225 and Nso190 were used for detection of betaproteobacterial NH_4^+ oxidizers by successive hybridizations (Wagner et al. 1994). For CARD-FISH, filter sections were embedded in 0.2% low-gelling-point agarose, permeabilized and incubated overnight at 46°C submerged in hybridization buffer containing horseradish peroxidase (HRP) labeled Cren679 probe, which appears to be specific for *Nitrosopumilus maritimus,* an NH_4^+-oxidizing member of *Thaumarchaeota* (Labrenz et al. 2010) (Table 1). After hybridization, filters were incubated with Tyramide-Alexa Fluor™ 555 for signal amplification using the Tyramide Signal Amplification Kit (Molecular Probes™, Invitrogen Detection Technologies®). After

Table 1. Probes used for FISH analyses

Organism targeted	Probe name	Sequence 5′–3′	SILVA (% specificity)	Source
All anammox bacteria	Amx368[a]	CCT-TTC-GGG-CAT-TGC-GAA	75.2	Schmid et al. (2003)
Genus '*Candidatus* Scalindua'	Sca1309[a]	TGG-AGG-CGA-ATT-TCA-GCC-TCC	41.3	Schmid et al. (2003)
Betaproteobacteria	Bet42a[a]	GCC-TTC-CCA-CTT-CGT-TT	N/A[d]	Manz et al. (1992)
Betaproteobacterial ammonium-oxidizing bacteria	Nso190[ac] Nso1225[ac]	CGA-TCC-CCT-GCT-TTT-CTC-C CGC-GAT-TGT-ATT-ACG-TGT-GA	95.6 85.3	Mobarry et al. (1996)
Gammaproteobacteria	Gam42a[a]	GCC-TTC-CCA-CAT-CGT-TT	N/A[d]	Manz et al. (1992)
Gammaproteobacteria Nitrosococcus oceani, N. halophilus	Nscoc128[a]	CCC-CTC-TAG-AGG-CCA-GAT	100	Juretschko (2000)
Thaumarchaeota, *Nitros-opumilus maritimus*, MGI c.	Cren679[b]	TTT-TAC-CCC-TTC-CTT-CCG	78.6	Labrenz et al. (2010)

[a]Probe labeled with Cy3; [b]Probe used in Catalyzed Reporter Deposition FISH (CARD-FISH) analysis. Labeled with horse-radish peroxidase (HRP); [c]Probes used in successive hybridization; [d]Database not available for 23s probes

hybridization, all filters were washed and counter-stained with 4,6 diamidino-2-phenylindole (DAPI), a general DNA stain. All filters were mounted on glass slides using Citifluor™ anti-fading oil (Electron Microscopy Sciences®) and visualized with a Zeiss® Axioskop™ epifluorescent microscope. A minimum of 500 DAPI-stained cells were counted. Detection limits were calculated using a conservative estimate of one probe-positive cell per field of vision, or 3.6×10^5 cells l^{-1}. Probes were checked *in silico* against SILVA's ribosomal RNA database (SSU 128). The number of hits and proportions of targeted hits are listed in Table 1. Non-targeted organisms that were hit by probes included some which were close relatives of the targeted organisms and likely had similar functions, some that were unlikely to be found in seawater, and some that were not closely related but should be expected in seawater. Therefore, it is not possible to determine the precise proportion of targeted organisms that were actually hit by the probes *in situ*; however, the probes were generally found to be highly specific for the target organisms. Results were interpreted cautiously with this in mind.

DNA extraction and quantitative PCR

Before DNA extraction, the LifeGuard™ Soil Preservation Solution was removed from the samples by centrifuging the bead tubes containing the filters at 2500 × *g* for 5 min and removing the supernatant according to the manufacturer's instructions. DNA was captured in silica spin columns and eluted in 100 µl of PCR grade sterile water using the PowerWater™ DNA Isolation Kit (MO BIO Laboratories®) following the manufacturer instructions. Samples were stored at –80°C until further analysis.

q-PCR protocols were developed for amplification of functional genes related to the nitrogen cycle which were unique to AOB, AOA and anammox bacteria. Details of the genes targeted, primer sequences, and melting temperatures are presented in Table 2. The PCR reaction mixes were as follows: 500 nM of forward and reverse primers, 100 to 500 ng of DNA, 0.5 µl of enzyme blend (Failsafe™ PCR System, Epicentre Biotechnologies®), 25 µl of PreMix 2X D/F (Failsafe™ PCR System, Epicentre Biotechnologies®), SYBR Green (Lonza Group®) as a nucleic acid stain

Table 2. Primer sets used for the PCR and q-PCR reactions. T_m = melting temperature

Target organism	Functional gene	Primer name	Sequence (5′–3′)	T_m (°C)	Source
Anammox bacteria '*Candidatus* Scalindua'	*nirS*	Scnir372F Scnir845R	TGT-AGC-CAG-CAT-TGT-AGC-GT TCA-AGC-CAG-ACC-CAT-TTG-CT	56.8 57.2	Lam et al. (2009)
β-ammonium-oxidizing bacteria	*amoA*	*amoA*1F *amoA*2R	GGG-GTT-TCT-ACT-GGT-GGT CCC-CTC-KGS-AAA-GCC-TTC-TTC	54.1 59.2	Rotthauwe et al. (1997)
Archaeal ammonium oxidizers	Archaeal *amoA*	Arch-*amoA*F Arch-*amoA*R	STA-ATG-GTC-TGG-CTT-AGA-CG GCG-GCC-ATC-CAT-CTG-TAT-GT	52.6 57.5	Francis et al. (2005)

(0.2 to 0.25×) and ROX (Stratagene-Agilent Technologies®) as a reference dye (0.32 nM). PCR products and specificity of the reactions were checked with gel electrophoresis (1% agarose) stained with ethidium bromide (EtBr) and observed under UV light.

All q-PCR samples and no template controls (NTC) were run in triplicate on a Stratagene Mx3000P (Agilent Technologies) q-PCR system and data were analyzed with MxPro QPCR software. NTC were used to check for reagent contamination and to avoid false positives (Nolan et al. 2006, Bustin et al. 2009). Standard curves ranging from 1 to 10^7 gene copies per reaction were run in triplicate with the samples and demonstrated amplification efficiencies of 90 to 130% and linear responses (r^2 = 0.95 to 0.99). After q-PCR amplification all samples were evaluated for reaction specificity by examining their dissociation curves for possible primer-dimer formation and by gel electrophoresis (1% agarose) stained with EtBr.

Flux model

Vertical fluxes of NH_4^+ and NO_3^- were estimated to examine their relationship to AOB, AOA and anammox bacteria within the suboxic zone. This flux model assumes that transport occurs exclusively by vertical eddy diffusion and necessarily ignores possible vertical or horizontal advection because they are poorly understood in this system (Scranton 1988, Li et al. 2012). The vertical fluxes (J) were obtained using Fick's first law:

$$J = -Kz\frac{\Delta C}{\Delta z} \quad (1)$$

where Kz is the vertical eddy diffusion coefficient and $\Delta C/\Delta z$ is the concentration gradient.

Kz was estimated from the density gradient equations (Gargett 1984, Li et al. 2012):

$$Kz = a_0\sqrt{\left(\frac{1}{N^2}\right)} \quad (2)$$

where:

$$N = \sqrt{\left(-\frac{g}{\rho}\frac{\delta\rho}{\delta z}\right)} \quad (3)$$

The constant a_0 is related to the input of energy to the basin via internal waves and other processes that cannot be directly measured, and has previously been estimated to be 0.0004 $cm^2\ s^{-2}$ for the Cariaco Basin (Li et al. 2012). The g term is the gravitational acceleration constant and ρ is the density at a given depth. Water density gradients, $\delta\rho/\delta z$, were determined from σ_θ (density using *in situ* salinity, potential temperature, and pressure = 0, −1000 kg m^{-3}) profiles provided by the CTD at 1 m resolution and were well approximated by linear regressions ($r^2 > 0.98$) through the finite depth intervals of interest (Li et al. 2012).

RESULTS

Variations in redox conditions

Vertical profiles of O_2, H_2S and cellular dark DIC assimilation for each cruise are presented in Fig. 1 to illustrate temporal variations in redox conditions and biogeochemical responses associated with them.

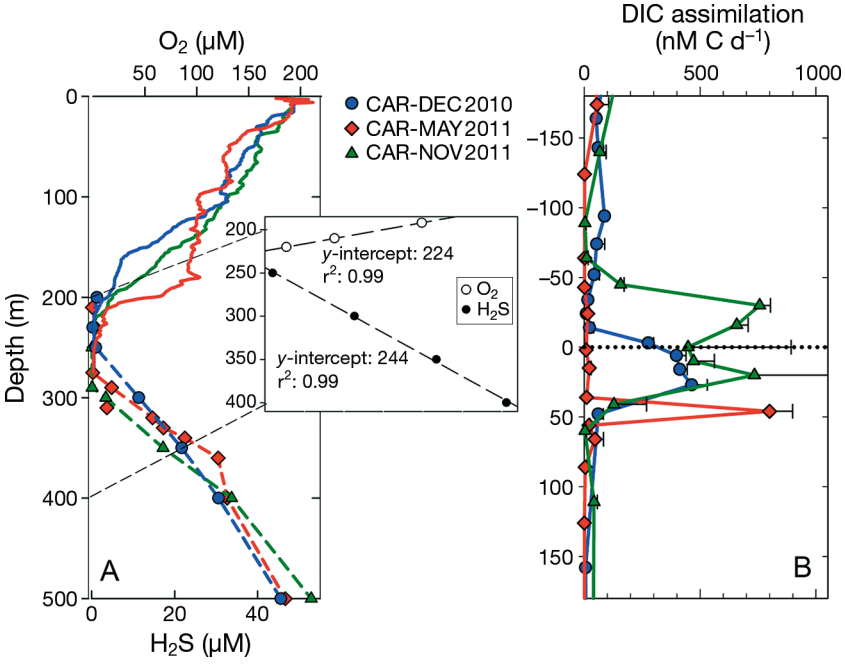

Fig. 1. Vertical distributions of (A) dissolved O_2 (solid lines) and H_2S (dashed lines) , and (B) dark dissolved inorganic carbon (DIC) assimilation observed in samples obtained from the Cariaco Basin, Venezuela, on 6 December 2010 (CAR-DEC2010), 6 May 2011 (CAR-MAY2011) and 10 November 2011 (CAR-NOV2011). The inset in Panel (A) shows the estimated first appearance of H_2S and O_2 for CAR-DEC2010. Profiles for DIC assimilation are plotted relative to the euxinic boundary (horizontal dotted line) which is defined as the depth of the first appearance of H_2S. Boundary position was derived from the intercept of the regression of the first 3 to 4 positive H_2S concentrations (Panel A, inset) against depth. Error bars are SE

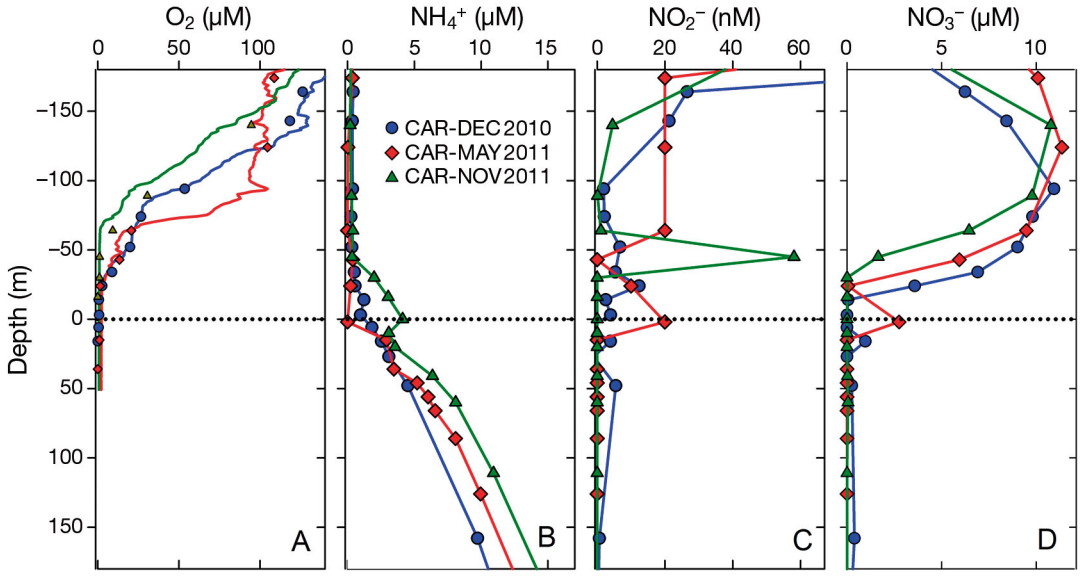

Fig. 2. Vertical distributions of (A) O_2, (B) NH_4^+, (C) NO_2^-, and (D) NO_3^- observed in samples obtained from the Cariaco Basin, Venezuela, during CAR-DEC2010, CAR-MAY2011 and CAR-NOV2011 cruises. All depth profiles are plotted relative to the euxinic boundary (horizontal dotted line), defined as depth of the first appearance of H_2S (see Fig. 1A)

Density profiles in the depth range of interest are homogeneous (not shown) and heterogeneous features in geochemistry are not considered to be significantly controlled by density gradients (Taylor et al. 2001). Based on the *z*-intercept from regressing the first 4 positive H_2S concentrations with depth (Fig. 1A, inset), we estimate that H_2S first appeared below 244 ± 3 m (mean ± SE), during CAR-DEC2010, 274 ± 1 m during CAR-MAY2011, and 290 ± 2 m (r^2 = 0.99) during CAR-NOV2011 (Fig. 1A). Consequently, redox-sensitive processes are expected to occur at different depths among the 3 cruises. To compare such processes and the responsible organisms among cruises, sample depths were referenced relative to the euxinic boundary, i.e. estimated first depth of H_2S appearance.

Distributions of DIC assimilation rates into cells, presumably attributable to chemoautotrophy fueled by oxidation of reduced sulfur species or ammonium, also varied distinctly among these cruises (Fig. 1B). The deepest peak relative to the euxinic boundary (CAR-MAY2011) also had the lowest integrated value of DIC assimilation (10 ± 1.2 mmol C m^{-2} d^{-1}), while the CAR-DEC2010 peak was intermediate in depth and strength (17.6 ± 1.0 mmol C m^{-2} d^{-1}), and the CAR-NOV2011 peak was broadest, shallowest and strongest (48 ± 8 mmol C m^{-2} d^{-1}). Evidently, biogeochemical conditions supporting chemoautotrophic production also differed markedly among these 3 cruises ($p < 0.002$, ANOVA among the 3 chemoautotrophy datasets).

Oxygen disappearance, computed by regressing the deepest 3 Winkler O_2 titrations values above the detection limit against depth (Fig. 1A, inset), occurred at 224 ± 3 m during CAR-DEC2010, indicating a 20 m thick suboxic zone (Fig. 2A). For CAR-MAY 2011, O_2 penetrated to 254 ± 5 m, again resulting in a 20 m suboxic zone. During CAR-NOV2011, however, O_2 penetration shoaled to 243 ± 5 m, resulting in a 47 m suboxic layer. This broader, shallower suboxic layer is consistent with the broader, shallower DIC assimilation peak observed for CAR-NOV2011 (Fig. 1B).

Next, we examined whether distributions of nitrogen species were similar when referenced to the euxinic boundary. Below the euxinic boundary, NH_4^+ increased to concentrations of 20 to 28 µM at 1300 m depth (http://imars.marine.usf.edu/CAR/). Above the euxinic boundary, NH_4^+ concentrations were at or near detection limits at all depths during CAR-DEC2010 and CAR-MAY2011, but were significantly above detection in the broad suboxic zone of CAR-NOV2011 (Fig. 2B). NO_2^- concentrations were low throughout the water column, with narrow peaks reaching no more than 60 nM within the redoxcline region (Fig. 2C). The dominant NO_2^- peak consistently appeared at the depth immediately above the first detectable NH_4^+ in all cruises. A NO_3^- maximum in the oxic disphotic zone (>100 m) was found during all cruises, consistently reaching maxima of 10 to 11 µM and disappearing within the suboxic zone

(Fig. 2D). CAR-MAY2011 was the exception in which a small secondary NO_3^- peak (2.8 µM) appeared at the euxinic boundary, coinciding with a NO_2^- peak.

Aerobic ammonium-oxidizing archaea and bacteria

Vertical profiles of AOA, AOB and anammox bacterial cells and their ammonia-oxidizing (AOA and AOB) or nitrite-reduction (anammox bacteria) enzyme encoding genes were examined in relation to distributions of nitrogen species and redox conditions.

Profiles of Cren679 probe-positive AOA revealed maximal cell abundances in the lower oxic zone during all 3 cruises, 20 to 40 m above the suboxic zone, with CAR-DEC2010 also exhibiting a secondary peak in the suboxic zone (Fig. 3). AOA cell abundances attained maxima of between 4.4 × 10^6 and 14.8 × 10^6 cells l^{-1} among cruises and approached detection limits in deeper waters. During CAR-DEC2010, unlike the other 2 cruises, depth distributions of AOA and total cell counts were similar (Fig. 3A,B). AOA cells accounted for 2.3 ± 0.27% of all prokaryotes enumerated by acridine orange throughout the redoxcline and no more than 14% at their peak abundance during any cruise.

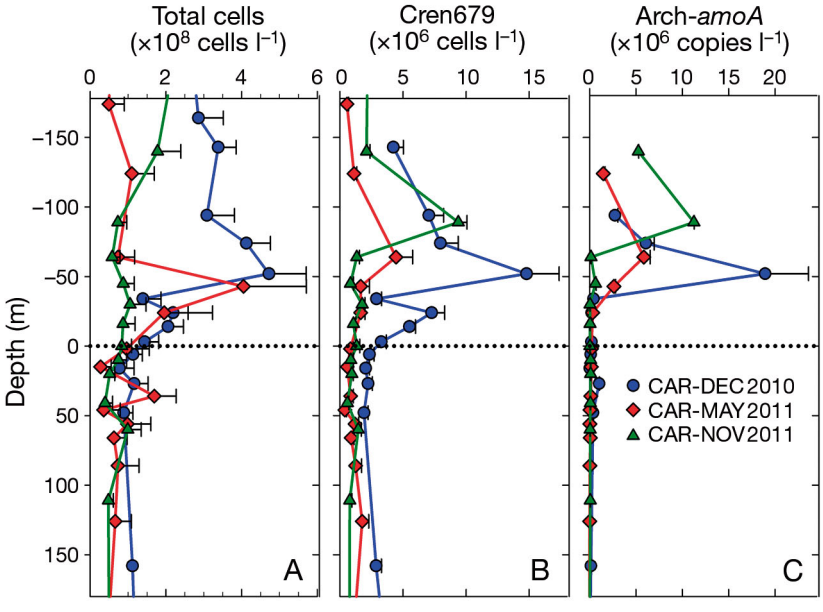

Fig. 3. Vertical distributions of total prokaryotic cells determined by epifluorescence microscopy of (A) acridine orange-stained samples, (B) Cren679 CARD-FISH probe-positive cell abundances and (C) archaeal ammonia mono-oxygenase (archaeal *amoA*) gene copy numbers using q-PCR and primers specific for thaumarchaeotal archaeal *amoA* observed in samples obtained from the Cariaco Basin, Venezuela, during CAR-DEC2010, CAR-MAY2011 and CAR-NOV2011 cruises. All depth profiles are plotted relative to the euxinic boundary (dotted line). Error bars are SE

Vertical distributions of archaeal ammonia monooxygenase subunit A (archaeal *amoA*) gene copies exhibited large peaks at the same depths as AOA cell abundances (Fig. 3C,B). However, the secondary peak evident in the CAR-DEC2010 FISH profile was absent from the archaeal *amoA* gene profile. Archaeal *amoA* gene abundances were highest in the lower oxic zone, varying between 5.8 × 10^6 and 18.9 × 10^6 gene copies l^{-1}, and declined to detection limits in deeper waters. Gene copy distributions in shallower waters are unknown, because q-PCR samples were not collected above 150 m.

The 2 AOB FISH probes produced similar vertical profiles from cruise to cruise. For example, peak abundances for γ-AOB and β-AOB were coincident in the lower oxic zone during CAR-DEC2010 and CAR-NOV2011 or in the upper suboxic zone during CAR-MAY2011. Primary peaks for γ-AOB and β-AOB varied from 3.4 to 24.0 × 10^6 and from 8.1 to 13.7 × 10^6 cells l^{-1}, respectively (Fig. 4A,B). Secondary peaks in γ-AOB and β-AOB abundance were also detected in the suboxic and upper euxinic zones during CAR-DEC2010 (3.8 and 9.4 × 10^6 cells l^{-1} for β-AOB and 1.2 and 8.1 × 10^6 cells l^{-1} for γ-AOB) and in the upper euxinic zone during CAR-MAY2011 (1.7 and 3.1 × 10^6 cells l^{-1} for β-AOB and γ-AOB, respectively). AOB represented small proportions of the total prokaryotic community; γ-AOB and β-AOB cells on average accounted for 2.4 ± 0.28% and 2.8 ± 0.33% of all acridine orange stained prokaryotes among all redoxcline depths and cruises and at most 11 and 16% of the total within their maxima, respectively. Although β-AOB appears on average to be the more abundant group of aerobic ammonia oxidizers, differences between AOA, β-AOB and γ-AOB were not significant ($p > 0.05$, ANOVA and Tukey and Student-Newman-Keuls pairwise comparisons).

Depth profiles of the β-AOB-specific ammonia monooxygenase gene (β-*amoA*) exhibited abundance maxima in the lower oxic zone (CAR-DEC2010 and CAR-NOV2011) or upper suboxic zone (CAR-MAY2011) varying from 0.96 to 10.1 × 10^5 gene copies l^{-1} (Fig. 4C). Smaller secondary peaks were observed in the upper euxinic zone in CAR-MAY2011 and CAR-NOV2011 varying from 0.4 to 1.5 × 10^5 gene copies l^{-1}, but comparable peaks

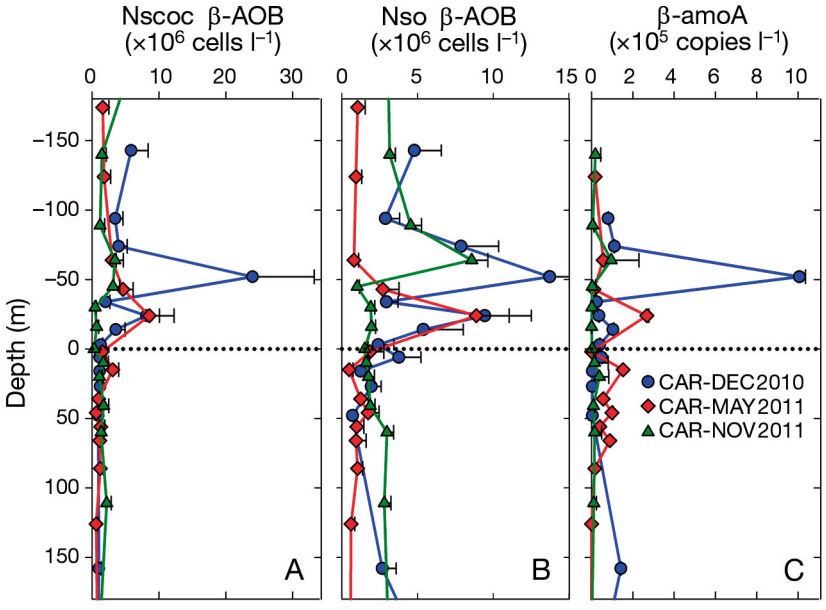

Fig. 4. Vertical distributions of (A) γ-AOB cell abundances determined by FISH using a Nscoc128 probe, (B) β-AOB cell abundances using a Nso1225-190 FISH probe, and (C) betaproteobacterial ammonia mono-oxygenase (β-*amoA*) gene copy concentrations observed in samples obtained from the Cariaco Basin, Venezuela, during CAR-DEC2010, CAR-MAY2011 and CAR-NOV2011 cruises. All depth profiles are plotted relative to the euxinic boundary (dotted line). Error bars are SE

were not evident in the β-AOB FISH profiles (Fig. 4B,C). A small secondary peak (1.0×10^5 gene copies l^{-1}) was evident during CAR-DEC2010 that overlapped with β-AOB (and γ-AOB) FISH peaks. No q-PCR profiles for γ-AOB ammonia monooxygenase were produced because of failed attempts at developing a standard for this gene.

Anammox bacteria

Distributions of anammox bacteria are compared to NO_2^- profiles in Fig. 5 because they are expected to be responsive to availability of this electron acceptor. A FISH probe targeting all known anammox *Planctomycetes* (Amx368) and another reputedly specific for the anammox genus '*Candidatus* Scalindua' (Sca1309) produced almost identical depth profiles of cell abundance. During all 3 cruises, depth distributions of Sca1309 (Fig. 5B) and Amx368 (Fig. 5C) and probe-positive cells exhibited at least 2 peaks. The larger one varied from 7.0 to 11.0×10^6 cells l^{-1} located at 245 to 250 m depth, within the upper suboxic zone, and the secondary peak varied between 3.0 and 4.7×10^6 cells l^{-1} and was located below the euxinic boundary. In the context of the total microbial community, Amx368 and Sca1309 probe-positive cells accounted for an average of ~1.9 % (SE ±0.29) of

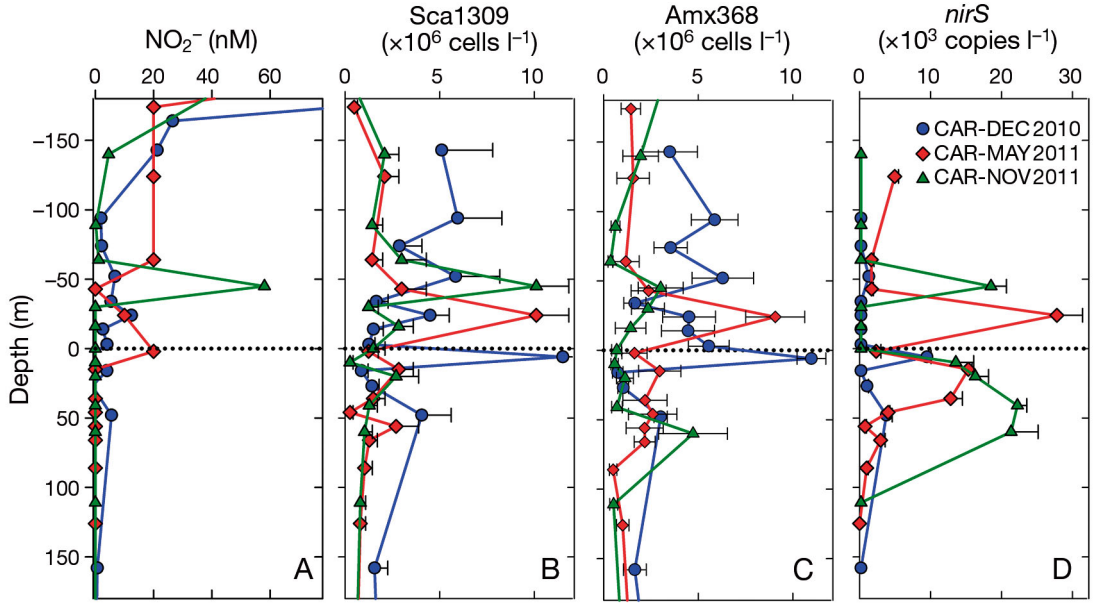

Fig. 5. Vertical distributions of (A) NO_2^-, (B) Sca1309 FISH probe-positive cell abundances, (C) Amx368 FISH probe-positive cell abundances and (D) nitrite reductase (Scalindua-*nirS*) gene copy numbers, using q-PCR and primers specific for anammox bacteria of the genus '*Candidatus* Scalindua', observed in samples obtained from the Cariaco Basin, Venezuela, during CAR-DEC2010, CAR-MAY2011 and CAR-NOV2011 cruises. All depth profiles are plotted relative to the euxinic boundary (dotted line). Error bars are SE

all prokaryotes enumerated by general acridine orange staining throughout the redoxcline and no more than 10% at their peak abundances.

Peaks in copy number of the functional gene, nitrite reductase from the genus '*Candidatus* Scalindua' (Scalindua-*nirS*), coincided with those of '*Candidatus* Scalindua' cell abundance profiles for all 3 cruises (Fig. 5B–D). Within the suboxic and euxinic zones, peak abundances varied between 1.0 and 22.0×10^3 gene copies l^{-1}. At depths above and below these peaks, Scalindua-*nirS* gene copy numbers were at or near detection limits (0.2×10^3 gene copies l^{-1}).

Nutrient and cell distribution comparisons

Nutrient profiles (Fig. 2) were compared to cell abundance of AOA (Fig. 3), AOB (Fig. 4) and anammox bacteria (Fig. 5) by Spearman rank order correlation (Table 3), since variables are not normally distributed (Shapiro-Wilks analysis). Abundances of AOA and cells hybridizing to the general β-AOB and the specific γ-AOB probe varied inversely with NH_4^+ concentrations (Cren679: $r = -0.4$, $p < 0.01$; Bet42a: $r = -0.6$, $p < 0.001$; Nscoc128: $r = -0.5$, $p < 0.01$; $n = 35$) and were positively correlated with NO_2^- (Cren679: $r = 0.5$, $p < 0.05$; Bet42a: $r = 0.4$, $p < 0.05$; Nscoc128: $r = 0.4$, $p < 0.05$; $n = 35$) and NO_3^- concentrations (Cren679: $r = 0.5$, $p < 0.001$; Bet42a: $r = 0.6$, $p < 0.001$; Nscoc128: $r = 0.6$, $p < 0.001$; $n = 35$) over the 150 to 400 m depth interval. Abundances of cells hybridizing to the specific β-AOB probe were only positively correlated with NO_3^- concentrations (Nso1225-190: $r = 0.4$, $p < 0.05$, $n = 35$) from depths between 150 and 400 m, whereas cells hybridizing to the general *Gammaproteobacteria* FISH probe (Gam42a) did not significantly correlate with NH_4^+, NO_2^- or NO_3^- concentrations. Anammox bacteria were significantly correlated with NO_2^- concentrations over the 150 to 400 m depth interval (Sca1309: $r = 0.5$, $p < 0.05$; Amx368: $r = 0.4$, $p < 0.025$; $n = 35$). In contrast, anammox cell distributions were not correlated ($p > 0.05$) with NH_4^+ or NO_3^- concentrations.

Nitrogen species supply

Based on the results of the correlations between nutrients and AOA, AOB and anammox bacteria, we examined the potential for covariance between vertical fluxes of those nutrients and cellular inventories in order to more clearly understand processes controlling distributions of these functional groups. Vertical fluxes for NH_4^+ and NO_3^- were computed for all 3 cruises using a 1-D eddy diffusion model dependent on gradients in N-species concentration and water density. The values of *Kz* computed for the depths above the euxinic boundary for the CAR-DEC2010, CAR-MAY2011 and CAR-NOV2011 cruises were 1.1, 1.1, and 0.80 $m^2\ d^{-1}$, respectively. Depth ranges considered for NO_3^- gradient calculations were: 180–230 m (CAR-DEC2010), 165–250 m (CAR-MAY2011), and 155–216 m (CAR-NOV2011). Among these cruises, computed downward NO_3^- fluxes to the suboxic zone varied between 80 ± 46 and 138 ± 77 μmol N $m^{-2}\ d^{-1}$ (Table 4). The calculated *Kz* values for depth below the euxinic boundary for cruises CAR-DEC2010, CAR-MAY2011 and CAR-NOV2011 were 1.8, 2.0, and 1.8 $m^2\ d^{-1}$, respectively. Depth ranges considered for the NH_4^+ gradient estimations were 220–321 m (CAR-DEC2010), 270–351 m (CAR-MAY2011), and 250–350 m (CAR-NOV2011). The computed upward flux of NH_4^+ varied between 83 ± 11 and 117 ± 32 μmol N $m^{-2}\ d^{-1}$ (Table 4).

Nitrite distributions typically exhibit 2 narrow and coarsely resolved peaks one at the nitracline's base and the other at top of the sulfide gradient and were thus not amenable to reliable flux estimations. Therefore, integrated NO_2^- inventories were calculated instead to assess supply. These inventories varied from 364 ± 36 to 1020 ± 60 μmol N m^{-2} (Table 4). NO_3^- and NH_4^+ fluxes and NO_2^- invento-

Table 3. Spearman rank order correlation (n = 35). Correlation coefficient and p-values (in *italics*) are shown with significant values in **bold**. See Table 1 for organisms targeted by the probes

	Cren679	Bet42a	Nso1225-190	Gam42a	Nscoc128	Amx368	Sca1309
NH_4^+	**−0.422** ***0.0119***	**−0.616** ***<0.0001***	−0.301 *0.0790*	−0.329 *0.0539*	**−0.528** ***0.0012***	−0.239 *0.165*	−0.292 *0.0884*
NO_3^-	**0.536** ***<0.001***	**0.640** ***<0.0001***	**0.351** ***0.0389***	0.311 *0.0685*	**0.559** ***0.0005***	0.124 *0.476*	0.269 *0.117*
NO_2^-	**0.446** ***0.0075***	**0.387** ***0.0217***	0.0666 *0.701*	0.165 *0.341*	**0.389** ***0.0210***	**0.381** ***0.0241***	**0.475** ***0.0041***

Table 4. Inventories of anammox and total bacterial aerobic NH_4^+ oxidizers (AAO), computed upward fluxes of NH_4^+ from the euxinic zone, downward NO_3^- fluxes, and integrated NO_2^- inventories within the lower oxic and suboxic zones. All values are mean ± SE. Microbial and NO_2^- inventories were estimated from peak integrations

Cruise	Anammox (10^{11} cells m^{-2})	AAO (10^{11} cells m^{-2})	NH_4^+ (μmol N m^{-2} d^{-1})	NO_3^- (μmol N m^{-2} d^{-1})	NO_2^- (μmol N m^{-2})
CAR-DEC2010	7.4 ± 1.6	21.0 ± 5.3	83 ± 11	138 ± 77	364 ± 36
CAR-MAY2011	4.5 ± 1.4	11.3 ± 3.7	113 ± 23	98 ± 55	600 ± 58
CAR-NOV2011	3.3 ± 1.3	11.1 ± 1.9	117 ± 32	80 ± 46	1020 ± 60

ries were then compared to inventories of anammox bacteria, β-AOB, γ-AOB and AOA within the 150 to 400 m layer. Trends between supply of dissolved nitrogen species and all aerobic NH_4^+ oxidizers were not significantly different. Therefore, inventories of β-AOB, γ-AOB and AOA were summed (Table 4). Upward fluxes of remineralized NH_4^+ from deep waters tended to be lower when inventories of anammox bacteria and all aerobic NH_4^+ oxidizers were higher. Higher downward NO_3^- fluxes tended to correspond to larger inventories of aerobic NH_4^+ oxidizers and anammox bacteria, roughly a doubling in estimated NO_3^- fluxes corresponded to a doubling in inventories of these functional groups. Lower NO_2^- inventories corresponded to larger inventories of anammox bacteria and all aerobic NH_4^+ oxidizers.

Comparison of cell and gene abundances

Reliable FISH quantification depends on probe selection/design that balances specificity with depth of coverage for a particular lineage. Data from all 3 cruises were compiled to compare q-PCR results for functional genes (Scalindua-*nirS*, archaeal *amoA*, β-*amoA*) with those obtained by FISH. Further, we assessed how well the specific probes (Sca1309, Cren679, Nscoc128 and Nso1225-190) captured each NH_4^+-oxidizing functional group, i.e. anammox bacteria, AOA, γ-AOB and β-AOB, respectively. These results were then compared to yields from general probes (Amx368, Gam42a and Bet42a) to assess the proportion of a given clade represented by a specific lineage.

Variance in Cren679 probe-positive cells explained 75% of the variance in the archaeal *amoA* gene abundances (Fig. 6B). The highly significant regression slope (m) ($p < 0.001$) indicates that 97 to 119% (m = 1.08 ± 0.11) of the archaeal *amoA* genes detected by q-PCR can be attributed to cells hybridizing against the Cren679 probe. Distributions of cells hybridizing with the probe specific for γ-AOB (Nscoc128) were significantly correlated ($p < 0.001$) with those of the general *Gammaproteobacteria* (Gam42a) probe, but only 29% of their variance was shared (Fig. 6C). Between 150 and 400 m, about 71% of bacteria recognized with the general *Gammaproteobacteria* probe (m = 0.71 ± 0.18) are also recognized by the specific probe for γ-AOB. Thus, the majority of probe-recognized *Gammaproteobacteria* in Cariaco's redoxcline during these cruises appear to be AOB lineages closely affiliated with the *Nitrosococcus* genus.

To assess the proportions of *Betaproteobacteria* that are *Nitrosomonas* and *Nitrosospira*-related NH_4^+-oxidizers, distributions of Bet42a and Nso1225-190 probe-positive cells were compared (Fig. 6D). Abundances obtained from the 2 probes covaried significantly ($p < 0.001$), where 75% of the variance in Nso1225-190 probe-positive cells is explained by that of the Bet42a probe. Between 150 and 400 m, on the order of 81% (m = 0.81 ± 0.12) of the *Bacteria* recognized by the general *Betaproteobacteria* probe were also recognized by the β-AOB specific probe. However, within the major abundance peaks and within analytical error, Nso1225-190 probe-positive cells appear to account for nearly 100% of the *Betaproteobacteria*, i.e. at 52, 24 and 64 m above the H_2S boundary during CAR-DEC2010, CAR-MAY2011 and CAR-NOV2011, respectively (Fig. 4B).

Significant, but weak linear relationships ($p < 0.05$) were found between *β-amoA* gene abundances and both the general *Betaproteobacteria* probe (Bet42a) and the β-AOB-specific probes (Nso1225-190). Variance in β-*amoA* gene copy numbers explained slightly more of the variance in Nso1225-190 probe-positive cells than that of Bet42a, 22% and 14%, respectively (data not shown). In both cases, most of the variance in these probes remains unexplained by β-*amoA* gene copy numbers.

Variance in the Sca1309 was significantly ($p < 0.005$) correlated with that of Scalindua-*nirS*, but only ex-

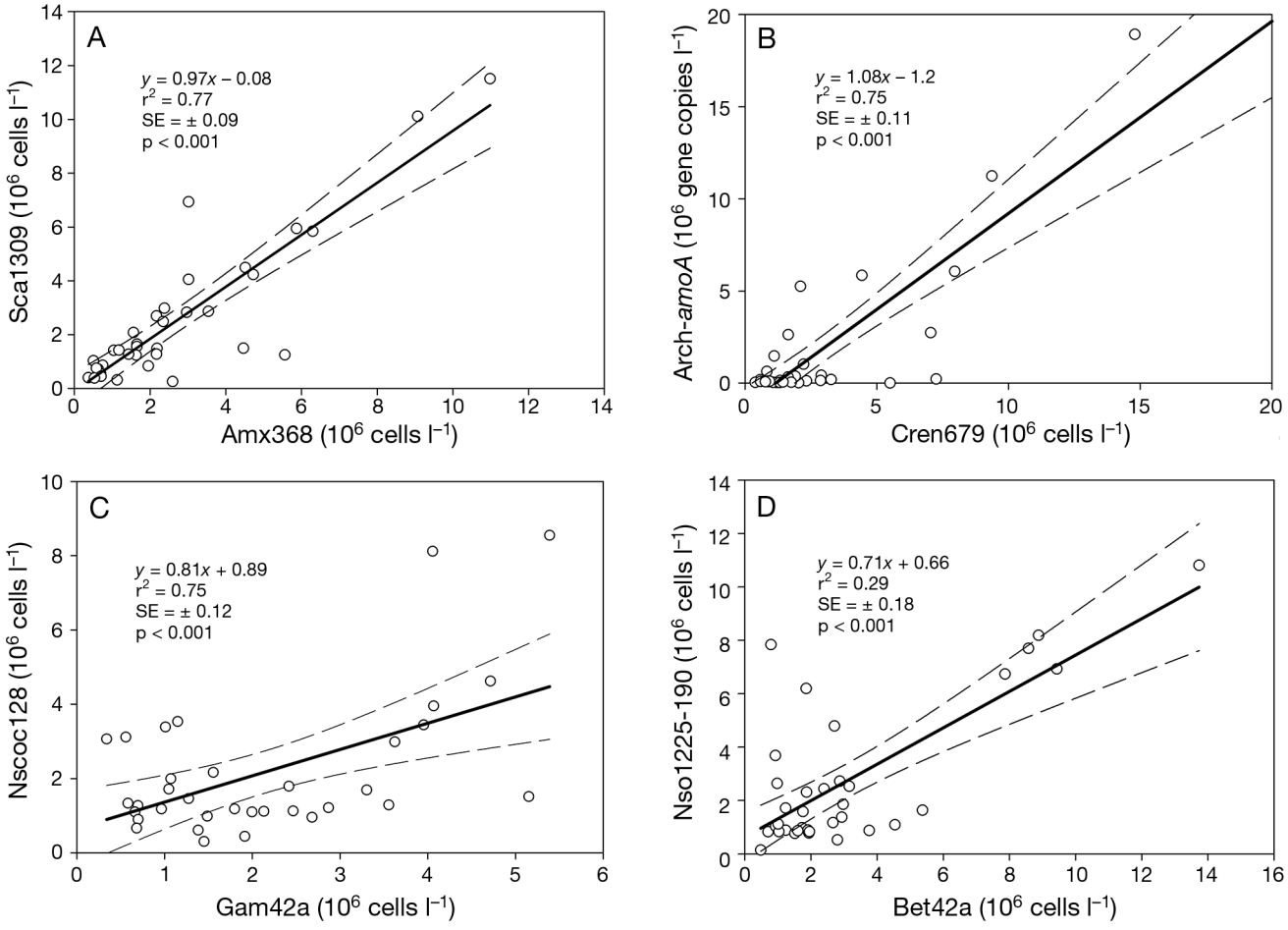

Fig. 6. Comparison of FISH probes and q-PCR results for all cruises and depths: (A) FISH results for anammox bacteria from the genus '*Candidatus* Scalindua' (Sca1309) vs. all known anammox bacteria (Amx368); (B) q-PCR archaeal *amoA* gene copies vs. Cren679 probe-positive cell abundances; (C) Nscoc128 (γ-NH_4^+-oxidizing bacteria) vs. Gam42a (most *Gammaproteobacteria*) probe-positive cell abundances; (D) Nso1225-190 (β-AOB) vs. Bet42a (most *Betaproteobacteria*) probe-positive cell abundances. 95% confidence intervals are shown

plained 15% of the variance (not shown). Nonetheless, peak abundances in the Scalindua-*nirS* gene and probe-positive cells mirrored one another across the redoxcline, corroborating the functional role of *Planctomycetes* enumerated by FISH.

Comparison of the 2 anammox probes yielded a highly significant ($p < 0.001$) linear relationship, where 77% of the variance in the Sca1309 probe-positive cells is explained by those hybridizing with the Amx368 probe (Fig. 6A). The regression slope was 0.97 ± 0.09, which means that within our analytical error 100% of the cells recognized by the general anammox Amx368 probe were also recognized by the Sca1309 probe specific for the genus '*Candidatus* Scalindua'. This suggests a very low diversity of anammox bacteria within the Cariaco Basin, an assemblage most likely composed of a single genus, if not single species.

DISCUSSION

Distributions of aerobic and anaerobic ammonium-oxidizers

Temporal and vertical variability in redox conditions, chemical distributions, and abundance markers of aerobic ammonia oxidizers was apparent within transitional waters during this 1-yr period. AOBs and AOAs exhibited peaks in the lower oxic zone and occasionally secondary peaks in the lower suboxic and euxinic layers, and total inventories of AOB and AOA were not significantly different during each cruise. Similarly, Lam et al. (2007, 2009) showed that total gene and transcript copies of *amoA* from AOA and AOB were comparable in both the Black Sea and eastern tropical South Pacific (ETSP). In contrast, gene abundances and NH_4^+ oxidation rates by

AOA were shown to be greater than for AOB in the eastern tropical North Pacific (ETNP) (Beman et al. 2012, Peng et al. 2015) and ETSP (Peng et al. 2016), the central California Current (Santoro et al. 2010), the Gulf of California (Beman et al. 2012), Puget Sound (Horak et al. 2013) and estuarine bottom waters and sediments (Caffrey et al. 2007). Environmental factors are presumed to control the relative abundances of AOB to AOA and, in particular, the presence of sulfide has been shown to negatively impact abundances of AOA (Caffrey et al. 2007). Therefore, the presence of sulfide in the Cariaco Basin and Black Sea (Lam et al. 2007) may explain why AOA are relatively less abundant than in other oxygen-depleted water columns.

The effects of upwelling on O_2 and NO_3^- distributions are evident by comparing the CAR-MAY2011 profile, collected during the upwelling season, to those from CAR-DEC2010 and CAR-NOV2011, collected during the non-upwelling season (Fig. 2A,D). The suboxic zone may contain trace quantities of oxygen, because the detection limit for Winkler titrations is about 1 to 2 µM. Such oxygen intrusions may promote episodic localized aerobic NH_4^+ oxidation while biogeochemical conditions are favorable, especially considering that NH_4^+ oxidation has been detected at very low concentrations (<1 µM) (Kalvelage et al. 2011, Bristow et al. 2016). The inactive cells may remain behind for some finite period after O_2 is depleted.

Variations in dissolved oxygen penetration may also explain varying distributions of DIC assimilation relative to the euxinic boundary. Because nitrification is an obligately aerobic process (Zehr & Ward 2002), the intermittent secondary AOB and β-*amoA* peaks in euxinic waters may be inactive relics of water masses previously influenced by lateral intrusion of oxygenated water from outside the basin (Scranton et al. 2001), rather than indicative of anaerobic NH_4^+ oxidation.

The 2 peaks in anammox cell and Scalindua-*nirS* gene abundances suggests trophic coupling with nitrifiers and denitrifiers. Nitrifiers plausibly reside at micro-oxic depths and release NO_2^- through aerobic oxidation of NH_4^+. Denitrifiers are more likely to reside in the lower suboxic and upper euxinic layers and release NO_2^- through anaerobic reduction of NO_3^-. While there is usually no detectable NO_3^- in the lower suboxic zone, the detection limit of the method used by the Cariaco Time Series is 0.13 µM, suggesting there could be nanomolar concentrations at these depths. Furthermore, the small positive NO_2^- and NO_3^- values reported for CAR-DEC2010 below the euxinic boundary could reflect relics of a very recent lateral intrusion of oxygenated water, previously described in the Cariaco Basin (Astor et al. 2003), and similar to those described in the Black Sea (Konovalov et al. 2003, 2008). Denitrification is possible in slightly sulfidic waters, and specifically autotrophic denitrification (oxidation of sulfide with NO_3^-) is likely occurring, similar to the Black Sea (Fuchsman et al. 2012). Trophic coupling such as this has been previously suggested from incubation experiments in the OMZ off Peru (Lam et al. 2009), off northern Chile (Galán et al. 2009), and in the Black Sea (Lam et al. 2007) and demonstrated in a laboratory based model system (Yan et al. 2012). Our hypothesis is also supported by similar observations of 2 anammox bacterial abundance maxima, belonging to 2 different cluster of '*Candidatus* Scalindua', in the upper and lower suboxic zone in the Black Sea (Fuchsman et al. 2012, Kirkpatrick et al. 2012). Furthermore, these results suggest a high sulfide tolerance for anammox organisms. Confirmation of this hypothesis would require direct evidence of activity through ^{15}N tracer experimental manipulations.

Oxygen sensitivity of anammox bacteria should also strongly influence their depth distributions. The shallower anammox bacterial peak in Cariaco's redoxcline corresponds to O_2 concentrations as high as 20 µM. Previous studies have reported abundance peaks of anammox bacteria within the upper OMZs off Peru and Costa Rica, where O_2 reached concentrations of up to 15 µM (Kalvelage et al. 2011, Podlaska et al. 2012). O_2 tolerance of the anammox reaction has been shown to be reversibly inhibited by O_2 concentrations of 1 µM and irreversibly inhibited by the O_2 concentration of 18 µM in enrichment cultures (Egli et al. 2001). In field populations, the anammox reaction exhibited 50% inhibition at 886 nM O_2 exposures and complete inhibition at 2 µM O_2, but rapidly recovered when O_2 was removed, suggesting regulation at the enzymatic, rather than genetic level (Dalsgaard et al. 2014). Microorganisms living within Cariaco's upper suboxic zone would thus be living close to their O_2 tolerance limit, but not necessarily inactivated.

Vertical profiles of genes from anammox bacteria (Scalindua-*nirS*), β-AOB (β-*amo*A), and AOA closely mirrored those produced for FISH cell abundances. However, abundances of those for anammox bacteria and β-AOB genes were one or 2 orders of magnitude lower than those of probe-positive cells, while those for AOA were closer to a 1:1 relationship with the Cren679 probe. While it is reassuring that vertical profiles of functional gene copies reproduce those derived from FISH, the quantitative disagreement between the 2 approaches is problematic. DNA

stocks were shared among all q-PCR assays and extracted by the same protocol from all samples. Thus, it is possible that our protocol more efficiently recovered DNA from *Thaumarcheota* than from *Planctomycetes* or *Betaproteobacteria*. More likely though, low q-PCR yields may have resulted from non-optimal q-PCR reaction chemistries, PCR inhibition or primer mismatches for Scalindua-*nirS* and β-*amo*A. Low taxon richness based on 16S rRNA within the anammox community in the Cariaco Basin was suggested by the 1:1 ratio of the probe Amx368 to the probe Sca1309. Furthermore, exclusive phylogenetic membership of the genus '*Candidatus* Scalindua' to the Cariaco's marine anammox bacterial assemblage has previously been shown in a wide variety of marine systems (Schmid et al. 2007). However, Scalindua-*nirS* gene sequences amplified from the Black Sea have been shown to differ from those found in the Peruvian OMZ (Kirkpatrick et al. 2012), suggesting that the Cariaco Basin possibly has unique and endemic Scalindua-*nirS* gene sequences. Therefore, the Scalindua-*nirS* primers, originally designed for organisms in the Peruvian OMZ, may not have amplified a significant fraction of those in the Cariaco Basin.

The apparent low Scalindua-*nirS* and β-*amoA* yields also may have resulted from a calibration bias caused by differential amplification efficiencies between genomic DNA from samples and the plasmid DNA from standards, or due to a lower efficiency of *nirS* primers. Available data do not permit resolution of our FISH versus q-PCR discrepancies. Nonetheless, we are confident that our FISH results accurately reflect actual abundances of NH_4^+-oxidizing functional groups. Furthermore, functional gene q-PCR profiles corroborate features within FISH depth profiles and confirm the presence of organisms capable of NH_4^+ oxidation through the presence of the β-*amoA* and archaeal *amoA* genes and NO_2^- reduction by abundant Scalindua-*nirS* gene copies.

The Cren679 probe is specific to the genus *Nitrosopumilus.* Without further phylogenetic resolution, it is not possible to determine if all *Thaumarchaeota* in the Cariaco Basin are related to this genus and it is possible that total AOA quantities were underestimated. However, low diversity of AOA has been observed previously in the Baltic Sea (Labrenz et al. 2010). The 1:1 correspondence between FISH and gene copy number is consistent with the interpretation of Hallam et al. (2006) of genomic data for NH_4^+-oxidizing *Thaumarchaeota*, i.e. each cell possesses a single archaeal *amoA* gene copy. The 1:1 correspondence is also indicative of the high efficiency of the qPCR primer pair selected for this group.

Geochemical control of ammonia oxidizers

The observed covariance of AOA and AOB distributions with NH_4^+, NO_2^- and NO_3^- concentrations and anammox bacteria with NO_2^- is consistent with the metabolic requirements of these microorganisms. Higher downward NO_3^- fluxes tended to correspond to larger inventories of aerobic NH_4^+ oxidizers and anammox bacteria, with roughly a doubling in estimated NO_3^- fluxes equivalent to a doubling in inventories of these functional groups. AOA and AOB should be the primary aerobic oxidizers of NH_4^+ in the nitrification zone. Higher inventories of these 2 functional groups suggest active nitrification, which would decrease the concentrations of NH_4^+, thus increasing concentrations of oxidized nitrogen.

Lower NO_2^- inventories corresponded to larger anammox bacteria inventories, while the response of aerobic NH_4^+ oxidizers to NO_2^- supply was ambiguous. Our conservatively estimated upward fluxes of remineralized NH_4^+ from deep waters tended to be lower when inventories of the deeper anammox bacteria population were higher. This is consistent with intensive NH_4^+ drawdown by the deeper anammox populations at the base of the suboxic zone. Rigorous statistical analyses were not possible due to the small number of cruises.

Sources of ammonium for shallow and deep ammonium oxidizers

Assessing sources of NH_4^+ for aerobic AOA and AOB and shallow anammox populations is challenging because NH_4^+ concentrations were at or near detection limits at most depths where cells and functional gene copies (archaeal *amoA*, β-*amoA*) were abundant and O_2 was detected during our 3 cruises. The most plausible explanation is that demands for this energy substrate by 'shallow' ammonium oxidizers are instantaneously balanced by NH_4^+ regenerated from local sources within this layer, thereby preventing accumulation. Globally, surface export production of sinking organic matter is known to supply NH_4^+ to depth as organic nitrogen, which is remineralized through ammonification by bacterial hydrolysis during transit through the water column (Wheeler & Kirchman 1986).

Potential ammonification rates in this layer can be estimated from differences in bi-weekly particulate nitrogen (PN) fluxes to the 225 and 410 m sediment traps maintained by the CARIACO Ocean Time Series. Observed depth-dependent flux decreases

suggested that up to 1.8 mmol NH_4^+ m^{-2} d^{-1} could be released daily from sinking organic matter with an average of ~0.4 mmol NH_4^+ m^{-2} d^{-1} between November 1995 and June 2012 (n = 195). Among the 3 cruises in this study, integrated inventories for all NH_4^+ oxidizers (AOA + AOB + anammox) within the lower oxic and suboxic zone averaged 1.95 × 10^{12} cells m^{-2}. If all cells were equally active, individual NH_4^+ oxidizers could account for oxidation of ~0.20 fmol NH_4^+ $cell^{-1}$ d^{-1} on average. This per capita rate is significantly lower than potential NH_4^+ oxidation rates reported from *in vitro* incubation studies, which vary from 2–4 fmol NH_4^+ $cell^{-1}$ d^{-1} for AOA and anammox bacteria to 7.2–1270 fmol NH_4^+ $cell^{-1}$ d^{-1} for AOB (Laanbroek & Gerards 1993, Wuchter et al. 2006, Kartal et al. 2007, Magalhães et al. 2009). Our per capita rate estimates assume that the entire NH_4^+ oxidizing membership actively participates to the same degree in the process, which likely underestimates the per cell activity for those active cells. These calculations suggest *in situ* ammonification is a plausible explanation for high inventories of NH_4^+ oxidizers.

While remineralization of sinking PN is a likely source of NH_4^+ to the shallower ammonia-oxidizing populations, alternative sources also merit consideration. Remineralization of suspended PN (sus-PN) within this layer could also supply NH_4^+, but is difficult to evaluate. Monthly sampling is too coarse to derive meaningful time-dependent release rates. Excretion by migrating mesozooplankton and nekton as well as by resident phagotrophic protists is another potential local NH_4^+ source within the lower oxic and suboxic zones (Bianchi et al. 2014). Select members of the nekton and mesozooplankton community are known to migrate well into the euxinic zone on a diel basis (Baird et al. 1974, Love et al. 2004) and may excrete NH_4^+ as well as defecate labile PN during transit. Thus, they actively translate reduced nitrogen pools from surface waters down to the transitional zone. However, their contribution to NH_4^+ flux escapes detection by the techniques routinely employed by the CARIACO Ocean Time Series program. The redoxcline is also known to harbor abundant and diverse phagotrophic protists (Taylor et al. 2006, Edgcomb et al. 2011). These organisms are likely to be important agents for nutrient regeneration in this and many other systems because of their nitrogen-rich diet (primarily prokaryoplankton and other protists), high reproductive potentials, and agile metabolic responsiveness (Taylor 1982, Berman et al. 1987, Caron 1994). Nonetheless, the NH_4^+ contribution of vertical migrators and resident protists to nitrifiers and anammox bacteria cannot be adequately assessed with available information. Furthermore, as was shown in the ETSP OMZ (Lam et al. 2009), DNRA by heterotrophic bacteria is also a potential, but small, source of ammonium within suboxic zones.

Shallow and deep '*Candidatus* Scalindua' populations are likely to have distinct sources of NH_4^+. The largest reservoir of NH_4^+ to support ammonium oxidation resides in Cariaco's deep waters where remineralized NH_4^+ accumulates from organic nitrogen diagenesis and is presumably transported upward by eddy diffusion and vertical advection to the suboxic zone (Fig. 2B). Presumably, deep anammox populations receive essentially all their NH_4^+ from deep waters. Stoichiometrically, the anammox reaction oxidizes 1 mole of NH_4^+ with 1 mole of NO_2^- to produce 1 mole of N_2 ($NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$). In CAR-DEC2010, downward flux of NO_3^- exceeded the amount needed to completely oxidize the upward diffusive flux of NH_4^+ into the suboxic zone. In CAR-MAY2011 and CAR-NOV2011, downward fluxes estimated for NO_3^- nearly balanced upward diffusive flux of NH_4^+. These results are consistent with direct coupling of NO_3^- reduction to NO_2^- from denitrification with NH_4^+ oxidation by anammox leading to nitrogen loss as previously observed in the Black Sea (Kuypers et al. 2003). We note that these mass balance computations are based on a limited set of instantaneous measurements and consequently do not adequately reflect physical and chemical dynamics of the system, such as advective processes. We also note that denitrifying thioautotrophs may also be another significant sink for NO_3^- arriving at the euxinic boundary (Bruckner et al. 2013).

CONCLUSIONS

Evidence presented in this study suggests that NH_4^+ oxidation by AOB, AOA and anammox are co-occurring in the suboxic zone of the Cariaco Basin. In the upper suboxic zone, peaks of AOB and AOA suggest aerobic NH_4^+ oxidation occurs at micro-oxic conditions. The source of NH_4^+ in these layers is likely from remineralization. On the other hand, anammox occurs in both the upper and lower regions of the suboxic zone, coupled to aerobic NH_4^+ oxidation and denitrification, respectively. The relative distributions of these organisms suggests close trophic coupling among members of the nitrogen cycling community and some sulfide tolerance of anammox bacteria in the Cariaco Basin.

Subsequent research should combine molecular analyses with rate measurements for a more detailed verification of activity in this environment. For anammox bacterial dynamics in particular, genes encoding for hydrazine synthase subunits (*hzsA, hzsB*) or hydrazine oxidoreductase (*hzo*), or primers specific to Cariaco Basin bacterial assemblages should be designed. Of special interest would be those genes involved in the denitrification process, such as genes encoding for the NO_3^- reductase (*narG, napA*) or NO_2^- reductase (*nirS, nirK, nrfA*) enzymes, which mediate reduction of NO_3^- to NO_2^- and reduction of NO_2^- to NO, respectively (Braker et al. 1998, Michotey et al. 2000, Philippot et al. 2002). Results from this study suggested that denitrification could be a source of NO_2^- for anammox in shallow sulfidic layers. q-PCR assays of genes such as *nirK* would be particularly interesting, as this gene is used by autotrophic denitrifiers (Walsh et al. 2009). All of these analyses should be accompanied by ^{15}N isotopic pairing experiments to assess the relative roles of nitrification, denitrification, anammox, and DNRA. This information will be essential for comparing nitrogen cycling in the Cariaco Basin with other oxygen-depleted marine ecosystems globally and for identifying unifying themes, as well as unique features.

Acknowledgements. This work was supported by the National Science Foundation (grant number OCE- 0752014 to M.I.S. and G.T.T.) and the Fondo Nacional de Ciencia, Tecnologia e Innovacion (FONACIT), Venezuela (Grant number 2011000353). We thank Dr. Kent A. Fanning for supplying nutrient data, and Dr. Martin Könneke and Dr. Mike S.M. Jetten for kindly sharing their *Nitrosopumilus maritimus* cell stocks and anammox DNA, respectively. The authors also thank the crew of the R/V 'Hermano Ginés', staff of EDIMAR for their invaluable help in the field and V.P. Edgcomb and M.G. Pachiadaki for insightful comments on an earlier draft of this paper. This is the Stony Brook University's School of Marine & Atmospheric Sciences contribution no. 1435.

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Editorial responsibility: Klaus Jürgens, Rostock, Germany

Submitted: May 2, 2016; Accepted: January 24, 2017
Proofs received from author(s): March 22, 2017