Primer selection influences abundance estimates of ammonia oxidizing archaea in coastal marine sediments

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A B S T R A C T

Quantification of the α-subunit of ammonia monooxygenase (*amoA*) through PCR is an established technique for estimating the abundance of ammonia oxidizing archaea (AOA) in environmental samples. This study quantified AOA with two established primer sets in 1 cm increments from the sediment surface (0–1 cm) to a depth of 10 cm at two locations within Port Phillip Bay (PPB), Australia. Primer choice had a significant effect on within sample estimates of AOA with copy numbers ranging from 10^2 to 10^4 copies per ng DNA. Variation in AOA abundance patterns with increasing sediment depth were site and primer specific. Sequence mismatches between the primer binding region of the isolated *amoA* sequences from PPB and *Nitrosopumilus maritimus* SCM1 were identified and may explain the high variation identified between primer estimates. Our results highlight the need for testing multiple primer pairs that target different regions of the AOA *amoA* sequence prior to large-scale marine sediment environmental studies.

1. Introduction

Marine sediments harbour the largest diversity of microorganisms on Earth (Whitman et al., 1998) and support a diverse community of ammonia oxidizing archaea (AOA) (Biller et al., 2012). Despite the dominant presence of AOA in sediments we only have a preliminary understanding of their contribution to ammonia oxidation and global nitrification (Beman et al., 2012; Labrenz et al., 2010; Mosier and Francis, 2008; Santoro et al., 2008; Smith et al., 2014, 2015). Amplification of the α-subunit of the ammonia monooxygenase gene (*amoA*) through quantitative PCR (qPCR) is an established technique for estimating the environmental abundance of AOA (Francis et al., 2005; Pester et al., 2012; Treusch et al., 2005; Wuchter et al., 2006). Yet the technique is limited by the choice of primer as archaeal *amoA* sequence diversity is high within the conserved primer binding regions, and increasing primer degeneracy to target more diverse sequences limits accuracy by decreasing PCR amplification efficiency (Meinhardt et al., 2015; Tolar et al., 2017). Due to this limitation, qPCR analysis of AOA *amoA* is considered a "closed-format" (Zhou et al., 2015) molecular technique as it can select for discrete AOA clades and potentially

underrepresent the total environmental abundance of AOA *amoA* gene sequences (Agogué et al., 2008; Church et al., 2010; De Corte et al., 2008; Kalanetra et al., 2009; Konstantinidis et al., 2009; Meinhardt et al., 2015; Santoro et al., 2008). Ideally, identifying the environmental sequence diversity of AOA *amoA* through more "open-format" approaches (Zhou et al., 2015), such as metagenomics, may reduce the risk of underestimating AOA abundance with qPCR due to inappropriate primer selections. However, these pre-selection tools are not always available or appropriate. In lieu of next generation sequencing, the recommended approach is to test multiple primers that target different conserved regions of the AOA *amoA* sequence to avoid underestimating their abundance in environmental studies (Meinhardt et al., 2015). Yet this approach rarely occurs, and many sediment studies have estimated and compared both the archaeal and bacterial *amoA* sequence abundances without understanding the complexity of the sediment archaeal *amoA* sequence pool.

This study compared two commonly used archaeal *amoA* primers: Arch-*amoA*-for/Arch-*amoA*-rev (Wuchter et al., 2006) and Arch-*amoA*F/Arch-*amoA*R (Francis et al., 2005) (hereafter referred to as AOA_Wuchter and AOA_Francis) to determine if primer based

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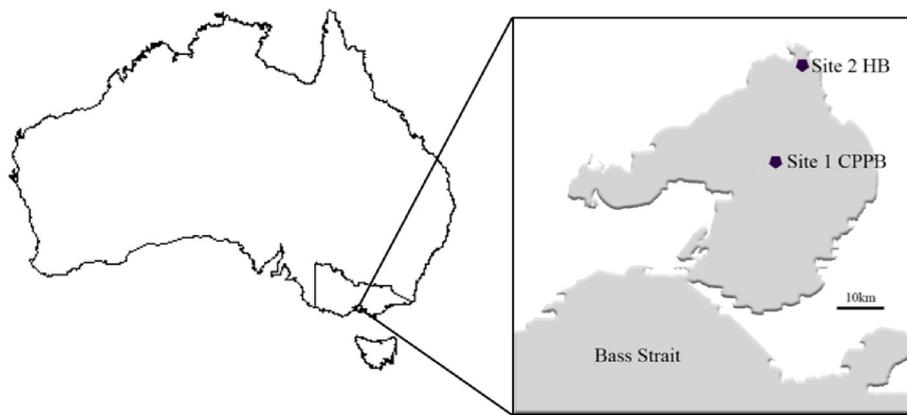
Fig. 1. Map of sample locations within Port Phillip Bay (PPB), Victoria, Australia. Central PPB (Site 1 CPPB) and Hobsons Bay (Site 2 HB).

differences occur in the quantification of archaeal *amoA* gene estimates in sediments collected from two locations within Port Phillip Bay (PPB), Australia (38.1732° S, 144.8731° E). AOA_Francis is a common choice for amplifying AOA *amoA* as the primer binding locations flank a large sequence region (635 bp) to avoid an area of high nucleotide diversity (Francis et al., 2005; Konstantinidis et al., 2009). We selected the AOA_Wuchter primers for comparison due to their shorter PCR amplified product size (256 bp), which is ideal for qPCR. These AOA_Wuchter primers have previously been used in soil and sediment environments, and recent phylogenetic findings have identified that AOA *amoA* sequences isolated from marine sediments cluster more closely with those from soil samples than with sequences isolated from ocean water column samples (Biller et al., 2012; Wuchter et al., 2006). We hypothesised that: (1) primer choice would affect final estimations of AOA due to known primer mismatches between the AOA_Wuchter primers and *amoA* sequences from marine metagenomes (Konstantinidis et al., 2009; Venter et al., 2004) and that (2) gene abundance patterns associated with increasing sediment depth would be maintained despite primer choice due to the influence of common environmental factors, such as high surface sediment mixing or the vertical zonation of major oxidants required by AOA (Froelich et al., 1979).

2. Materials and methods

Sediment cores were collected in the summer (January 2014) from two sites within PPB where nitrogen loss pathways have been studied through benthic chamber research (Berelson et al., 1998; Heggie et al., 1999). Site one (S38° 03.495′ E144°52.242′) is 24 m deep and is in the muddy sediment zone of central PPB (CPPB) away from point source inputs whereas site two, Hobsons Bay (HB) (S37° 52.065′ E144°55.654′) is 11 m deep, approximately 800 m from shore and is primarily influenced by the outflow of the Yarra River (Fig. 1). Five (95 mm × 300 mm) cores were diver-collected to a sediment depth of 20 cm at each site and kept on ice until they could be transferred to a −20 °C freezer. Cores were sectioned into 1 cm thick disks to a depth of 10 cm and frozen sediment was sampled from the centre of each disk. DNA was extracted in duplicate from 400 mg of the frozen sediment with the PowerSoil DNA extraction kit (Qiagen). Duplicate extractions were pooled, and DNA quality was assessed (average concentration in 30 μl (29 ± 9 ng/ul) (NanoDrop 2000 ThermoFisher Scientific)). TOPO One shot (ThermoFisher) cloned plasmid standards of representative AOA *amoA* sequences from PPB sediments were generated from each primer set and sequenced with an ABI 3730xl DNA analyser (Applied Biosystems, Mulgrave, Vic, Australia). The cloned sequences were confirmed as putative AOA *amoA* by Blast$_n$ (Altschul et al., 1990) analysis and have been deposited in GenBank (accession numbers MF176967-MF176980). Sequences were aligned at the primer binding

regions and manually checked with Sequencher 5.0 (Gene Codes Corporation, MI, USA). QPCR analyses were performed in duplicate in 384-well format (Biorad CFX384 Touch) using the primers and optimised amplification conditions listed in Table 1. Technical reproducibility was assessed for each sample in duplicate (AOA_Wuchter R^2 = 0.83; AOA_Francis R^2 = 0.98). Gene copy numbers were calculated from the average quantification cycle (C_q) of two replicates for each sample and compared against a standard curve (10-fold dilution with a linear range 10^{-3} to 10^{-7}), generated using the relevant plasmid for that assay and converted to copy number per ng of extracted DNA and copy number per gram of wet weight (g^{-1} WW) sediment. Abundance values were averaged for each sample and then square-root transformed. Correlation coefficients and Analysis of Variance (ANOVA) with Tukey's post hoc analysis was analysed in R with R Studio (R Core Team, 2014; RStudio Team, 2016).

3. Results

Quantification of AOA *amoA* within PPB sediments revealed primer specific biases in gene abundances with final estimations by AOA_Wuchter primers being an order of magnitude greater than those by AOA_Francis (Fig. 2). In CPPB (site one), which is furthest from external inputs into PPB, AOA_Wuchter primers indicated that AOA were homogenously distributed across the top 10 cm of sediment. In contrast, AOA_Francis primers indicated that AOA *amoA* abundance were homogeneous from the surface to a depth of 4 cm but then increased 6.6-fold from 4 cm to 10 cm in CPPB sediments (Fig. 2A and B). In comparison, in HB (site two), which is close to external inputs into PPB, AOA *amoA* abundances were homogeneous with increasing sediment depth regardless of primer choice (Fig. 2C and D). These results coincided with the measured concentration of extracted DNA. Central PPB displayed a depth specific decrease in the extracted concentration of DNA from 35 ng μl^{-1} at 0–1 cm to 18 ng μl^{-1} at the 9–10 cm depth. In comparison, there was no depth stratification in the concentration of extracted DNA in HB with DNA concentration ranging from 25 to 35 ng μl^{-1}. A positive correlation was identified between the concentration of extracted DNA g^{-1} WW and AOA *amoA* sequence abundances per gram wet sediment measured by AOA_Wuchter (R^2 = 0.69, p < 0.001) in CPPB, with no relationship at HB or at either site when AOA *amoA* abundances were determined with AOA_Francis primers.

The primer binding region of the *amoA* sequences isolated within this study were aligned to selected *amoA* reference sequences from GenBank (Fig. 3). AOA_Wuchter primers amplified *amoA* sequences (256 bp) that shared a 97.3% (1 bp mismatch) base pair sequence similarity with the primer binding region of *Nitrosopumilus maritimus* strain SCM1 and a 94.6% (2 bp mismatch) with *Nitrosopelagicus brevus*. The 2 bp mismatches with *Nitrosopelagicus brevus* occur at degenerate

Table 1
Primer details and qPCR amplification conditions.

Primer name	Forward sequence 5'- 3'	Reverse sequence 5'- 3'	Expected size (bp)	PCR amplification conditions	qPCR conditions	Master mix	qPCR Efficiencies	Reference
Arch-*amoA*F/ Arch-*amoA*R	STA ATG GTC TGG CTT AGA CG	GCG GCC ATC CAT CTG TAT GT	635	95 °C for 5 min; 35 cycles of 95 °C for 30 s, 55 °C for 45 s, 72 °C for 45 s; final extension of 72 °C for 10 min	Denaturation of 95 °C for 3 min; then 40 cycles of 95 °C for 5 s, 60 °C for 30 s, Melt curve 58 °C - 95 °C increments of 0.5 °C	Bioline SensiFAST	E-value (83.2%) R^2 = 0.998	(modified from Francis et al., 2005)
Arch-*amoA*-for/ Arch-*amoA*-rev	CTGAYTGGGCYTGACACATC	TTCTTCTTTGTTGCCCAGTA	256	95 °C for 5 min; 40 cycles of 95 °C for 30 s, 53 °C for 45 s, 72 °C for 45 s; final extension of 72 °C for 10 min		BIORAD SSO Advanced	E-value (89.6%) R^2 = 0.997	(modified from Wuchter et al., 2006)

locations within the AOA_Wuchter forward primer where a cytosine or guanine base can occur (Fig. 3). In contrast, AOA_Francis primers amplified sequences (635 bp) that shared 100% base pair sequence similarity at the primer binding region with *Nitrosopelagicus brevus* and 94.6% (2 bp mismatch) with *Nitrosotenius cloacae* (Fig. 3). A 92.3% (3 bp mismatch) base pair sequence similarity was identified between the alignment of the AOA_Francis forward (2 bp) and reverse (1 bp) primers and the binding location within the *Nitrosopumilus maritimus* reference alignment (Fig. 3).

4. Discussion

Two established AOA *amoA* primer sets were tested in this study on sediment collected from two locations in PPB. This approach identified primer specific biases in sediment AOA *amoA* gene abundances with final estimations by AOA_Wuchter primers being an order of magnitude greater than those by AOA_Francis. We hypothesised that primer choice would yield different final estimations of AOA abundance, yet we expected higher abundances with AOA_Francis primers than AOA_Wuchter primers. We expected higher abundances from the AOA_Francis primers due to three known base pair mismatches between the AOA_Wuchter reverse primer Arch-*amoA*-rev (Wuchter et al., 2006) and environmental sequences of AOA *amoA* (Konstantinidis et al., 2009; Venter et al., 2004). Our results from these coastal sediments support comparative primer studies in soil environments which have identified that primer choice affects AOA *amoA* abundances which can alter the final data interpretation (Meinhardt et al., 2015). Although Meinhardt et al. (2015) did not directly compare the primers used in this study they did report that in soil samples AOA_Francis primers comparatively amplified lower AOA *amoA* gene counts than other primers tested.

Altered estimates of AOA *amoA* abundance with these two different primer sets impacted our understanding of the ecological distribution of AOA *amoA* with increasing sediment depth in PPB. The patterns of AOA distribution with depth were both site and primer specific, which is a concern for large-scale sediment studies that look to describe the environmental distribution and role of archaeal nitrifiers, especially in relation to bacterial ammonia oxidizers. Primer mismatches between AOA_Wuchter and deep sea *amoA* sequences led initially to results that suggested some AOA may lack the *amoA* gene (Agogué et al., 2008; De Corte et al., 2008; Kalanetra et al., 2009). Due to this known primer mismatch, AOA_Francis primers were identified as the optimal choice for amplifying *amoA* in open ocean samples (Church et al., 2010; Konstantinidis et al., 2009; Santoro et al., 2010) as base pair mismatches can lead to reduced primer annealing during PCR (Bru et al., 2008). Marine sediment studies of AOA *amoA* have also preferentially quantified *amoA* with the AOA_Francis primers (Beman et al., 2012; Dang et al., 2013; Wang et al., 2017). Furthermore, ammonia monooxygenase belongs to a wider family of copper-containing membrane bound monooxygenases, which includes the membrane-bound particulate methane monooxygenase (encoded by *pmoA*) and differentiating between these two enzymes can be difficult at the sequence level (Tavormina et al., 2011). For example, it was only recently that complete ammonia-oxidation was considered in bacteria as the *amoA* gene within *Nitrospira* had been previously identified as a *pmoA* gene (Daims et al., 2015; Van Kessel et al., 2015). Although, we did not identify *pmoA*-like sequences in the Blast$_n$ analysis in this study, the increase in *amoA* gene abundance determined with the AOA_Francis primers and the maintenance of high *amoA* gene abundance with increasing sediment depth with the AOA_Wuchter primers implies that oxygen requiring processes could be occurring at depth in this system, in sediment environments previously considered anoxic (Burke, 1999).

5. Conclusion

This study supports the requirement for testing a suite of different degenerate primer sets when targeting Archaeal *amoA* through qPCR in
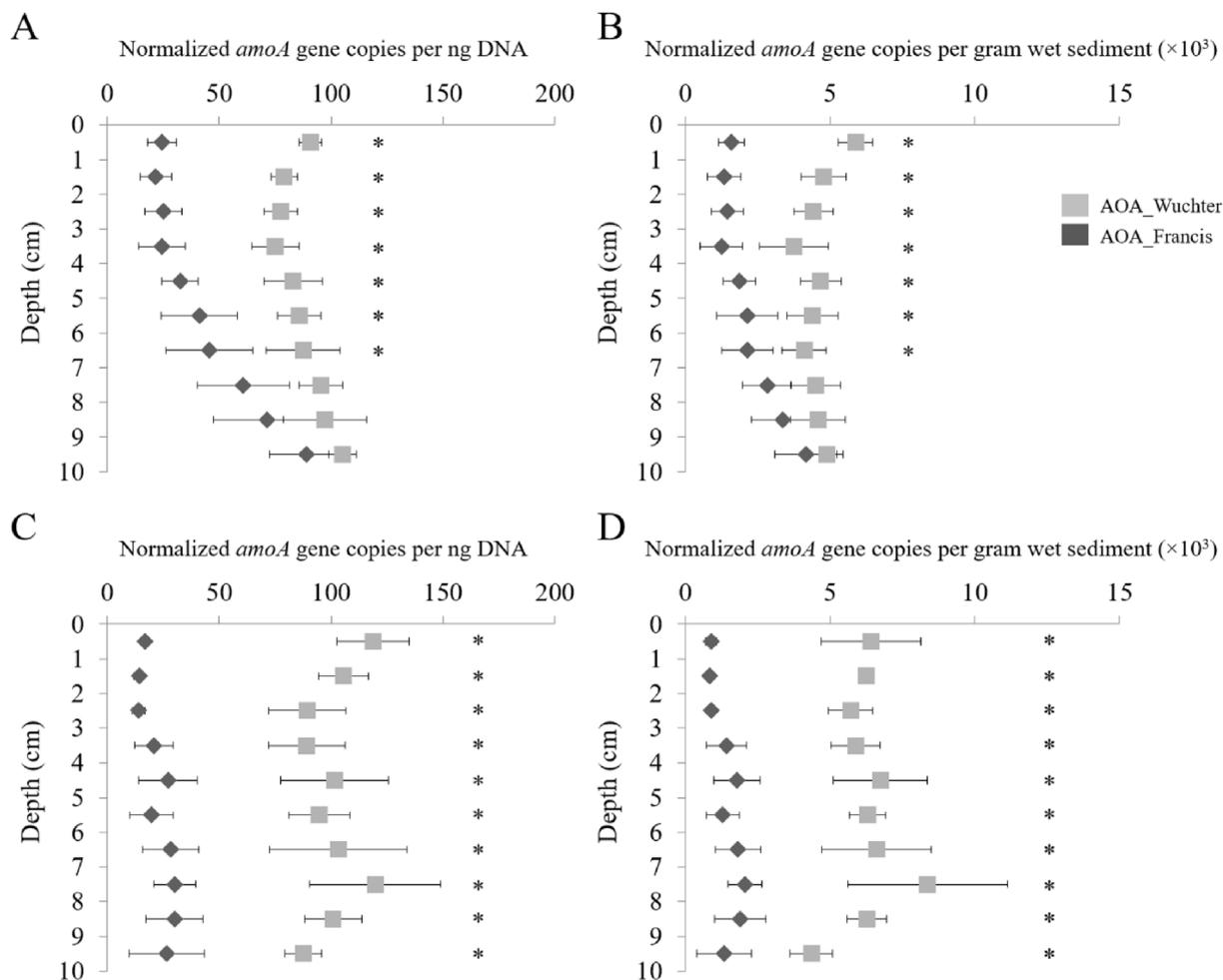
Fig. 2. Archaeal *amoA* gene copies per ng DNA (A and C) and per gram of extracted wet sediment (B and D) amplified from sediment extracts in 1 cm depth increments (0–10 cm) from two locations (Site 1 Central Port Phillip Bay: A and B; Site 2 Hobsons Bay: C and D) within Port Phillip Bay, Australia. Two primer choices targeting *amoA* are AOA_Wuchter (Arch-*amoA*-for/Arch-*amoA*-rev (Wuchter et al., 2006)) (light grey) and AOA_Francis (Arch-*amoA*F/Arch-*amoA*R (Francis et al., 2005)) (dark grey). Data are represented as the average of normalized (square root transformation) *amoA* gene copies per ng DNA or per gram of extracted wet weight sediment ± Standard Deviation. Significant differences (ANOVA $p < 0.05$) between AOA_Wuchter and AOA_Francis at each depth are noted by the asterisk symbol (*).

Fig. 3. Alignment of AOA_Francis (Arch-*amoA*F/Arch-*amoA*R (Francis et al., 2005)) and AOA_Wuchter (Arch-*amoA*-for/Arch-*amoA*-rev (Wuchter et al., 2006)) forward (left column) and reverse compliment (right column) primer sequences (5′- 3′) with primer binding locations within AOA genomes available from NCBI Refseq and sequences generated from this study. Locations where binding mismatches were identified between the primer sequence and the sequenced AOA *Nitrosopumilus maritimus* SCM1(CP000866.1) genome are indicated by the asterisk symbol (*).

environmental studies where the AOA community is uncharacterised. Primer choice was found to affect both the final abundance estimation of AOA *amoA* in sediment extracts in PPB and the associated sediment depth patterns in a site-specific manner. Whilst we did not compare the full available suite of AOA *amoA* primers, we found AOA_Wuchter primers provided higher abundance estimates of AOA *amoA* sequences in near shore marine sediment extracts from PPB than AOA_Francis primers. If *Nitrosopumilus*-like AOA are a dominant archaeal nitrifier within PPB sediments, mismatches at the AOA_Francis forward primer binding location may be a reason that estimated AOA *amoA* abundances were lower with this primer set. However, more "open format" molecular approaches such as 16S rRNA community profiling and metagenomics would be required and confirm this hypothesis. In future studies that look to compare the relationship between archaeal to bacterial *amoA* sequences in near shore sediment systems we recommend screening multiple primer pairs targeting archaeal *amoA* before embarking on quantitative assessments.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.marenvres.2018.06.001.

References

Agogué, H., Brink, M., Dinasquet, J., Herndl, G.J., 2008. Major gradients in putatively nitrifying and non-nitrifying Archaea in the deep North Atlantic. Nature 456, 788–791. https://doi.org/10.1038/nature07535.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Beman, J.M., Bertics, V.J., Braunschweiler, T., Wilson, J., 2012. Quantification of ammonia oxidation rates and the distribution of ammonia-oxidizing Archaea and Bacteria in marine sediment depth profiles from Catalina Island, California. Front. Microbiol. 3. https://doi.org/10.3389/fmicb.2012.00263.

Berelson, W.M., Heggie, D., Longmore, A., Kilgore, T., Nicholson, G., Skyring, G., 1998. Benthic nutrient recycling in Port Phillip Bay, Australia. Estuar. Coast Shelf Sci. 46, 917–934. https://doi.org/10.1006/ecss.1998.0328.

Biller, S.J., Mosier, A.C., Wells, G.F., Francis, C.A., 2012. Global biodiversity of aquatic ammonia-oxidizing archaea is partitioned by habitat. Front. Microbiol. 3. https://doi.org/10.3389/fmicb.2012.00252.

Bru, D., Martin-Laurent, F., Philippot, L., 2008. Quantification of the detrimental effect of a single primer-template mismatch by real-time PCR using the 16S rRNA gene as an example. Appl. Environ. Microbiol. 74, 1660–1663. https://doi.org/10.1128/AEM.02403-07.

Burke, C.M., 1999. Molecular diffusive fluxes of oxygen in sediments of Port Phillip Bay in south-eastern Australia, Molecular diffusive fluxes of oxygen in sediments of Port Phillip Bay in south-eastern Australia. Mar. Freshw. Res. 50, 557–566. https://doi.org/10.1071/MF98056.

Church, M.J., Wai, B., Karl, D.M., DeLong, E.F., 2010. Abundances of crenarchaeal *amoA* genes and transcripts in the Pacific Ocean. Environ. Microbiol. 12, 679–688. https://doi.org/10.1111/j.1462-2920.2009.02108.x.

Daims, H., Lebedeva, E.V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N., Palatinszky, M., Vierheilig, J., Bulaev, A., Kirkegaard, R.H., Bergen, M., von, Rattei, T., Bendinger, B., Nielsen, P.H., Wagner, M., 2015. Complete nitrification by Nitrospira bacteria. Nature 528, 504–509. https://doi.org/10.1038/nature16461.

Dang, H., Zhou, H., Yang, J., Ge, H., Jiao, N., Luan, X., Zhang, C., Klotz, M.G., 2013. Thaumarchaeotal signature gene distribution in sediments of the Northern South China Sea: an indicator of the metabolic intersection of the marine carbon, nitrogen, and phosphorus cycles? Appl. Environ. Microbiol. 79, 2137–2147. https://doi.org/10.1128/AEM.03204-12.

De Corte, D., Yokokawa, T., Varela, M.M., Agogué, H., Herndl, G.J., 2008. Spatial distribution of Bacteria and Archaea and *amoA* gene copy numbers throughout the water column of the Eastern Mediterranean Sea. ISME J. 3, 147–158. https://doi.org/10.1038/ismej.2008.94.

Francis, C.A., Roberts, K.J., Beman, J.M., Santoro, A.E., Oakley, B.B., 2005. Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. Proc. Natl. Acad. Sci. U. S. A 102, 14683–14688. https://doi.org/10.1073/pnas.0506625102.

Froelich, P.N., Klinkhammer, G.P., Bender, M.L., Luedtke, N.A., Heath, G.R., Cullen, D., Dauphin, P., Hammond, D., Hartman, B., Maynard, V., 1979. Early oxidation of organic matter in pelagic sediments of the eastern equatorial Atlantic: suboxic diagenesis. Geochem. Cosmochim. Acta 43, 1075–1090. https://doi.org/10.1016/0016-7037(79)90095-4.

Heggie, D.T., Skyring, G.W., Orchardo, J., Longmore, A.R., Nicholson, G.J., Berelson, W.M., 1999. Denitrification and denitrifying efficiencies in sediments of Port Phillip Bay: direct determinations of biogenic N_2 and N-metabolite fluxes with implications for water quality. Mar. Freshw. Res. 50, 589–596. https://doi.org/10.1071/MF98054.

Kalanetra, K.M., Bano, N., Hollibaugh, J.T., 2009. Ammonia-oxidizing archaea in the Arctic ocean and Antarctic coastal waters. Environ. Microbiol. 11, 2434–2445. https://doi.org/10.1111/j.1462-2920.2009.01974.x.

Konstantinidis, K.T., Braff, J., Karl, D.M., DeLong, E.F., 2009. Comparative metagenomic analysis of a microbial community residing at a depth of 4,000 meters at station ALOHA in the North Pacific Subtropical Gyre. Appl. Environ. Microbiol. 75, 5345–5355. https://doi.org/10.1128/AEM.00473-09.

Labrenz, M., Sintes, E., Toetzke, F., Zumsteg, A., Herndl, G.J., Seidler, M., Jürgens, K., 2010. Relevance of a crenarchaeotal subcluster related to Candidatus Nitrosopumilus maritimus to ammonia oxidation in the suboxic zone of the central Baltic Sea. ISME J. 4, 1496–1508. https://doi.org/10.1038/ismej.2010.78.

Meinhardt, K.A., Bertagnolli, A., Pannu, M.W., Strand, S.E., Brown, S.L., Stahl, D.A., 2015. Evaluation of revised polymerase chain reaction primers for more inclusive quantification of ammonia-oxidizing archaea and bacteria. Environ. Microbiol. Rep. 7, 354–363. https://doi.org/10.1111/1758-2229.12259.

Mosier, A.C., Francis, C.A., 2008. Relative abundance and diversity of ammonia-oxidizing archaea and bacteria in the San Francisco Bay estuary. Environ. Microbiol. 10, 3002–3016. https://doi.org/10.1111/j.1462-2920.2008.01764.x.

Pester, M., Rattei, T., Flechl, S., Gröngröft, A., Richter, A., Overmann, J., Reinhold-Hurek, B., Loy, A., Wagner, M., 2012. *amoA*-based consensus phylogeny of ammonia-oxidizing archaea and deep sequencing of *amoA* genes from soils of four different geographic regions. Environ. Microbiol. 14, 525–539. https://doi.org/10.1111/j.1462-2920.2011.02666.x.

R Core Team, 2014. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. [WWW Document]. GBIF.ORG. http://www.R-project.org/ (accessed 3.16.17).

RStudio Team, 2016. RStudio. Integrated Development for R. RStudio, Inc., Boston, MA.

Santoro, A.E., Casciotti, K.L., Francis, C.A., 2010. Activity, abundance and diversity of nitrifying archaea and bacteria in the central California Current. Environ. Microbiol. 12, 1989–2006. https://doi.org/10.1111/j.1462-2920.2010.02205.x.

Santoro, A.E., Francis, C.A., De Sieyes, N.R., Boehm, A.B., 2008. Shifts in the relative abundance of ammonia-oxidizing bacteria and archaea across physicochemical gradients in a subterranean estuary. Environ. Microbiol. 10, 1068–1079. https://doi.org/10.1111/j.1462-2920.2007.01547.x.

Smith, J.M., Casciotti, K.L., Chavez, F.P., Francis, C.A., 2014. Differential contributions of archaeal ammonia oxidizer ecotypes to nitrification in coastal surface waters. ISME J. 8, 1704–1714. https://doi.org/10.1038/ismej.2014.11.

Smith, J.M., Mosier, A.C., Francis, C.A., 2015. Spatiotemporal relationships between the abundance, distribution, and potential activities of ammonia-oxidizing and denitrifying microorganisms in intertidal sediments. Microb. Ecol. 69, 13–24. https://doi.org/10.1007/s00248-014-0450-1.

Tavormina, P.L., Orphan, V.J., Kalyuzhnaya, M.G., Jetten, M.S.M., Klotz, M.G., 2011. A novel family of functional operons encoding methane/ammonia monooxygenase-related proteins in gammaproteobacterial methanotrophs. Environ. Microbiol. Rep. 3, 91–100. https://doi.org/10.1111/j.1758-2229.2010.00192.x.

Tolar, B., Herrmann, J., Bargar, J., van den Bedem, H., Wakatsuki, S., Francis, C., 2017. Integrated structural biology and molecular ecology of N-cycling enzymes from ammonia-oxidizing archaea. Environ. Microbiol. Rep. 9, 484–491. https://doi.org/10.1111/1758-2229.12567.

Treusch, A.H., Leininger, S., Kletzin, A., Schuster, S.C., Klenk, H.-P., Schleper, C., 2005. Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. Environ. Microbiol. 7, 1985–1995. https://doi.org/10.1111/j.1462-2920.2005.00906.x.

Van Kessel, M.A.H.J., Speth, D.R., Albertsen, M., Nielsen, P.H., Op den Camp, H.J.M.,

Kartal, B., Jetten, M.S.M., Lücker, S., 2015. Complete nitrification by a single microorganism. Nature 528, 555–559. https://doi.org/10.1038/nature16459.

Venter, J.C., Remington, K., Heidelberg, J.F., Halpern, A.L., Rusch, D., Eisen, J.A., Wu, D., Paulsen, I., Nelson, K.E., Nelson, W., Fouts, D.E., Levy, S., Knap, A.H., Lomas, M.W., Nealson, K., White, O., Peterson, J., Hoffman, J., Parsons, R., Baden-Tillson, H., Pfannkoch, C., Rogers, Y.-H., Smith, H.O., 2004. Environmental genome shotgun sequencing of the Sargasso Sea. Science 304, 66–74. https://doi.org/10.1126/science.1093857.

Wang, J., Kan, J., Zhang, X., Xia, Z., Zhang, X., Qian, G., et al., 2017. Archaea dominate the ammonia-oxidizing community in deep-sea sediments of the eastern indian ocean—from the equator to the Bay of Bengal. Front. Microbiol. 8. http://dx.doi.org/10.3389/fmicb.2017.00415.

Whitman, W.B., Coleman, D.C., Wiebe, W.J., 1998. Prokaryotes: the unseen majority. Proc. Natl. Acad. Sci. Unit. States Am. 95, 6578–6583.

Wuchter, C., Abbas, B., Coolen, M.J.L., Herfort, L., Bleijswijk, J., van Timmers, P., Strous, M., Teira, E., Herndl, G.J., Middelburg, J.J., Schouten, S., Damsté, J.S.S., 2006. Archaeal nitrification in the ocean. Proc. Natl. Acad. Sci. Unit. States Am. 103, 12317–12322. https://doi.org/10.1073/pnas.0600756103.

Zhou, J., He, Z., Yang, Y., Deng, Y., Tringe, S.G., Alvarez-Cohen, L., 2015. High-throughput metagenomic technologies for complex microbial community analysis: open and closed formats. mBio 6, e02288–14. https://doi.org/10.1128/mBio.02288-14.