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

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ABSTRACT

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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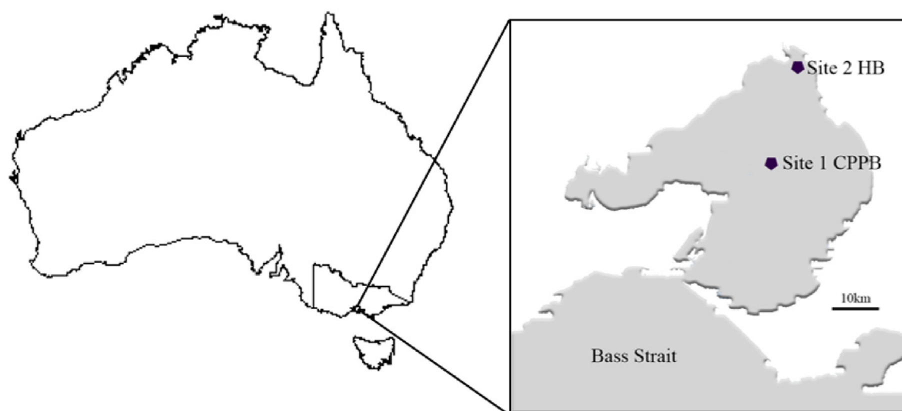

Fig. 1. Map of sample locations within Port Phillip Bay (PPB), Victoria, Australia. Central PPB (Site 1 CPPB) and Hobsons Bay (Site 2 HB).

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

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