

SHORT COMMUNICATION

NC10 bacteria in marine oxygen minimum zones

Cory C Padilla^{1,8}, Laura A Bristow^{2,8}, Neha Sarode^{1,3}, Emilio Garcia-Robledo⁴, Eddy Gómez Ramírez⁵, Catherine R Benson⁶, Annie Bourbonnais⁷, Mark A Altabet⁷, Peter R Girguis³, Bo Thamdrup² and Frank J Stewart¹

¹School of Biology, Georgia Institute of Technology, Atlanta, GA, USA; ²Department of Biology and Nordic Center for Earth Evolution (NordCEE), University of Southern Denmark, Odense, Denmark; ³Biological Laboratories, Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA, USA; ⁴Department of Bioscience, Aarhus University, Aarhus, Denmark; ⁵Centro de Investigacion en Ciencias del Mar y Limnología (CIMAR), Universidad de Costa Rica, San Pedro de Montes de Oca, Costa Rica; ⁶Department of Molecular Biology and Biochemistry, Middlebury College, Middlebury, VT, USA and ⁷School for Marine Science and Technology, University of Massachusetts Dartmouth, New Bedford, MA, USA

Bacteria of the NC10 phylum link anaerobic methane oxidation to nitrite denitrification through a unique O₂-producing intra-aerobic methanotrophy pathway. A niche for NC10 in the pelagic ocean has not been confirmed. We show that NC10 bacteria are present and transcriptionally active in oceanic oxygen minimum zones (OMZs) off northern Mexico and Costa Rica. NC10 16S rRNA genes were detected at all sites, peaking in abundance in the anoxic zone with elevated nitrite and methane concentrations. Phylogenetic analysis of particulate methane monooxygenase genes further confirmed the presence of NC10. rRNA and mRNA transcripts assignable to NC10 peaked within the OMZ and included genes of the putative nitrite-dependent intra-aerobic pathway, with high representation of transcripts containing the unique motif structure of the nitric oxide (NO) reductase of NC10 bacteria, hypothesized to participate in O₂-producing NO dismutation. These findings confirm pelagic OMZs as a niche for NC10, suggesting a role for this group in OMZ nitrogen, methane and oxygen cycling.

The ISME Journal (2016) 10, 2067–2071; doi:10.1038/ismej.2015.262; published online 26 February 2016

Microbes have critical roles in methane (CH₄) consumption, with aerobic methane oxidation by methanotrophic bacteria and sulfate-dependent anaerobic oxidation of methane (AOM) by Archaea recognized as important sinks in the CH₄ cycle (Reeburgh, 2007). Studies of freshwater habitats have further linked AOM to the reduction of oxidized nitrogen compounds, including nitrate (NO₃) and nitrite (NO₂) (Raghoebarsing *et al.*, 2006; Haroon *et al.*, 2013). Recently discovered NO₂-reducing bacteria of the NC10 phylum couple CH₄ oxidation to N₂ production through a unique NO₂-dependent anaerobic methane oxidation (n-damo) pathway (Raghoebarsing *et al.*, 2006; Ettwig *et al.*, 2009). Characterized in the bacterium *Candidatus Methyloirabilis oxyfera* from freshwater sediments (Ettwig *et al.*, 2010), the n-damo pathway reduces NO₂ to nitric oxide (NO), which is then putatively dismutated into N₂ and O₂ gas, with O₂ serving as the oxidant for intra-aerobic methanotrophy.

The ecological significance of n-damo remains unclear, as does the environmental distribution of NC10 bacteria. Environmental evidence for NO₂ or NO₃-dependent AOM stems primarily from freshwater sediments and wetlands (for example, Deutzmann *et al.*, 2014; Norði and Thamdrup, 2014; Hu *et al.*, 2014), while NC10 genes have been detected in diverse anoxic freshwater habitats (Shen *et al.*, 2015), and recently in NO₂-rich sediments of the South China Sea (Chen *et al.*, 2015). However, the potential for NC10 bacteria and n-damo in the pelagic ocean remains unknown.

Oxygen minimum zones (OMZs) are a potential niche for NO₂-dependent n-damo. In the major upwelling-driven OMZs, including the world's largest OMZ in the Eastern Tropical North Pacific (ETNP), microbial respiration depletes [O₂] to anoxia (<10 nM) (Tiano *et al.*, 2014). The microbial reduction of NO₃ to NO₂ in these zones drives NO₂ to micromolar concentrations (Thamdrup *et al.*, 2012). OMZs are further characterized by elevated CH₄ concentrations (Naqvi *et al.*, 2010), and indeed represent the largest accumulation of open-ocean CH₄ in the world (Sansone *et al.*, 2001). Although sequence fragments related to NC10 bacteria (by BLAST) have been detected in OMZ meta-omic data

Correspondence: F Stewart, School of Biology, Georgia Institute of Technology, 311 Ferst Drive, Atlanta, 30332 GA, USA.

E-mail: frank.stewart@biology.gatech.edu

⁸These authors contributed equally to this work.

Received 9 September 2015; revised 25 November 2015; accepted 10 December 2015; published online 26 February 2016

sets (for example, Dalsgaard *et al.*, 2014), confirmation of a pelagic OMZ niche for NC10 bacteria is lacking.

We tested the hypothesis that NO_2^- -replete OMZs host *Methylomirabilis*-like NC10 bacteria, using samples from four sites spanning a nearshore to offshore transition through the ETNP OMZ off northern Mexico and at a site in the anoxic, coastal basin of Golfo Dulce (GD), Costa Rica (Supplementary Figure S1, Supplementary Table S1). At both locations, $[\text{O}_2]$ decreased from the surface, dropping below the detection limit (20 nM) at 70–130 m depth across sites (Figure 1). Characteristic to anoxic OMZs, $[\text{NO}_2^-]$ increased upon O_2 depletion to a prominent maximum (4–5 μM) in the upper anoxic zone in the ETNP (120–150 m), before declining with depth. In the GD, NO_2^- accumulated to 0.7 μM at 100 m in the anoxic zone. Nitrite here disappeared at 140 m near the interface to a bottom layer in which hydrogen sulfide and ammonium accumulated, reaching 6.6 and 3.4 μM , respectively, at 190 m.

Methane was present in the anoxic zone in the ETNP and GD. Challenges in methodology and calibrations prohibited us from determining exact concentrations in samples from the ETNP. Nevertheless, methane was detectable in samples from OMZ depths at ETNP sites, consistent with previous studies hypothesizing that the ETNP CH_4 maximum, along an isopycnal of 26.8 kg m^{-3} , reflects CH_4 advected from coastal sediments (Sansone *et al.*, 2001; Pack *et al.*, 2015). The CH_4 profile in the GD also suggested a sediment source, with $[\text{CH}_4]$ at trace levels above the oxycline, $\sim 6 \text{ nM}$ at the NO_2^- maximum, and $\sim 80 \text{ nM}$ in the sulfidic bottom water.

Quantitative PCR with NC10-specific 16S rRNA (16S) gene primers (Supplementary Table S2) detected NC10 16S genes at all OMZ sites, with maximum counts in the ETNP (25–174 copies ml^{-1} ; 0.02–0.05% of total 16S counts) at anoxic depths (~ 300 –400 m) below the NO_2^- maximum (Figure 1). This distribution suggests that in addition to anoxia, ETNP NC10 bacteria are linked to CH_4 rather than organic flux from above, in which case counts would be predicted to peak higher in the OMZ where organotrophic activity is highest (Babbin *et al.*, 2014). In the GD, counts peaked at 90 m (105 copies ml^{-1} ; 0.006% of total) at the top of the NO_2^- and CH_4 -enriched zone. NC10 16S rRNA genes were undetectable or near the detection limit (6 copies ml^{-1}) above and below the ETNP OMZ and above the GD OMZ.

Sequencing of the *pmoA* gene encoding particulate methane monooxygenase (pMMO), catalyzing the CH_4 oxidation step in methanotrophy, further confirmed the presence of NC10. Amplification with NC10-specific primers recovered *pmoA* from all screened OMZ samples, representing a subset from both sites (Supplementary Table S1). Phylogenetic analysis of cloned sequences (26 from ETNP and 2 from GD) revealed four closely related

phylotypes ($> 98.5\%$ shared amino-acid identity) related to *pmoA* from NC10 environmental clones and *Ca. M. oxyfera* (Figure 2), with highest similarity ($\sim 98\%$ amino-acid identity) to sequences from South China Sea sediments (Chen *et al.*, 2015). Recovery of these sequences suggests the potential for CH_4 oxidation by OMZ NC10 bacteria.

Metatranscriptomes confirmed that NC10-like bacteria are transcriptionally active within OMZs. Community cDNA from five 2014 ETNP samples and three GD samples from anoxic depths was shotgun sequenced, and analyzed with five existing data sets (from Ganesh *et al.*, 2015) from ETNP station 6 in June 2013. The relative abundance of NC10 16S rRNA transcripts increased with decreasing oxygen, peaking at the ETNP core (300 m) and at 90 m in the GD (Supplementary Tables S2 and S4), consistent with depths of maximum NC10 16S gene counts. Messenger RNA transcripts assigned to NC10 using a lowest common ancestor algorithm were detected in all data sets. NC10 mRNA transcripts varied in relative abundance and, with the exception of one sample (150 m, ETNP station 10), paralleled the distribution of NC10 rRNA, increasing with declining oxygen and peaking at $\sim 0.1\%$ of total mRNA transcripts in the ETNP OMZ core (Supplementary Table S2).

The OMZ NC10-like transcript pool contained genes with predicted roles in dissimilatory N transformations (Supplementary Figures S3 and S4, Supplementary Tables S5 and S6). Two *norZ* genes, encoding quinol-dependent NO reductases (qNor), were most abundant, together representing 51% of transcripts assignable to NC10 by the lowest common ancestor. For two samples (ETNP station 6, 300 m, 2013; station 10, 150 m, 2014), assemblies of qNor transcripts yielded full-length gene sequences that clustered phylogenetically with qNor variants (bootstrap: 100) encoded by loci DAMO_2434 and 2437 of the *Ca. M. oxyfera* genome (Figure 2). N_2O does not appear during NO_2^- reduction in *Ca. M. oxyfera* enrichments (Ettwig *et al.*, 2010) and it has been hypothesized that the qNor encoded by DAMO_2434 and 2437, instead of producing N_2O , functions as a NO dismutase (NOD) to convert two NO molecules to N_2 and O_2 (Ettwig *et al.*, 2012). Indeed, DAMO_2434 and 2437 have substitutions at key residues in both the quinol-binding and catalytic sites. The qNor sequences recovered here also lack conservation of the canonical quinol-binding motif and contain catalytic site substitutions identical to those of DAMO_2434 and 2437 (Figure 2). The identification of these candidate NODs raises the possibility of nitrite-dependent O_2 production in the OMZ.

Genes of CH_4 oxidation, including those encoding the pMMO enzyme, were not detected in the NC10 transcript pool identified by the lowest common ancestor. However, ETNP OMZ transcripts with top BLASTX matches to *Ca. M. oxyfera pmoB* were detected at low abundance in the ETNP, but not in

the GD (Supplementary Table S6). pMMO-encoding transcripts of aerobic methylotrophs were not detected in the ETNP but were recovered in the

upper GD OMZ (90, 100 m; data not shown), despite anoxic conditions in this zone. Low levels of NC10-assigned transcripts of CH₄ oxidation may

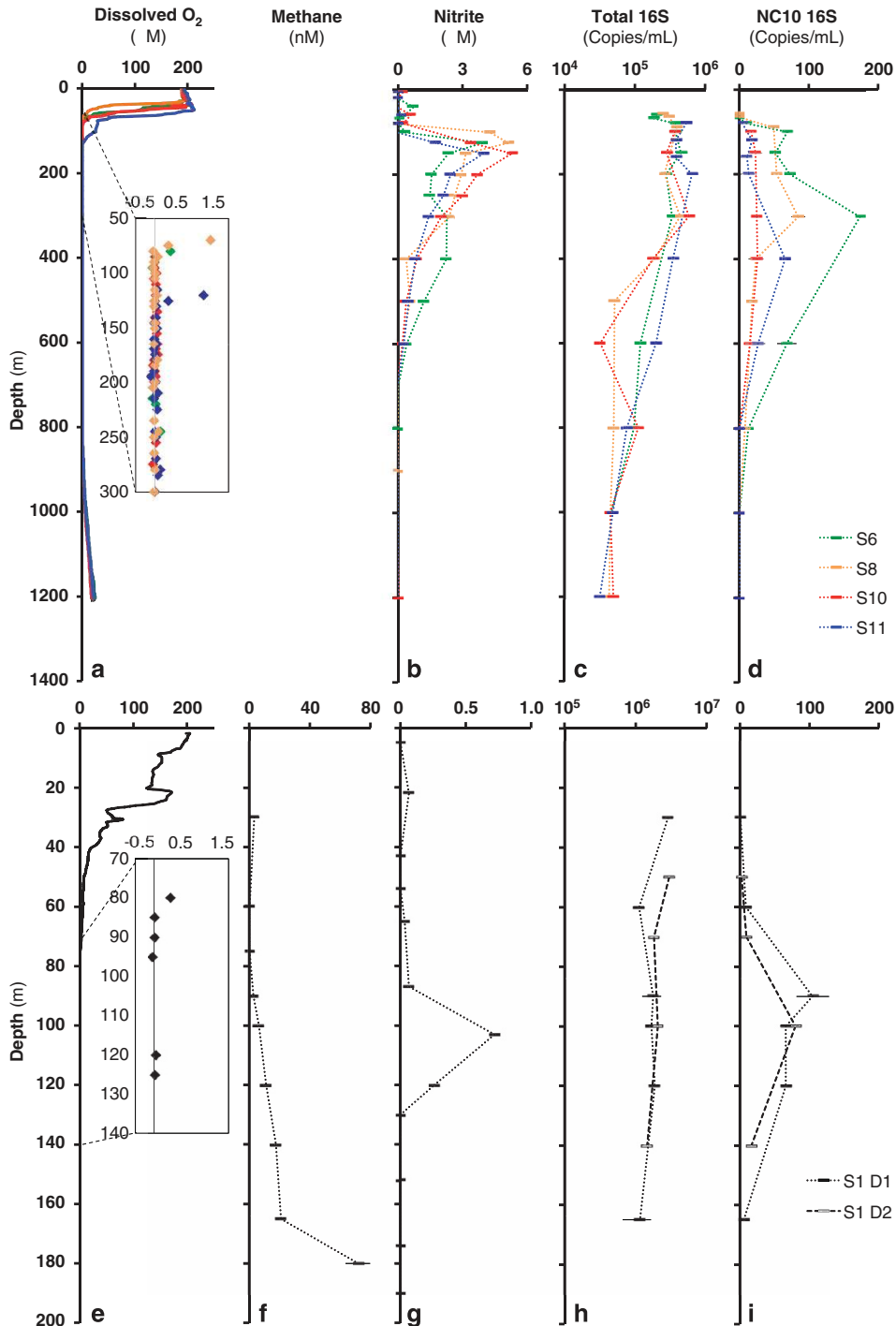


Figure 1 Water column chemistry and microbial biomass in the ETNP OMZ in May 2014 (a–d) and GD OMZ in January 2015 (e–i). ETNP stations (colors) span a nearshore (S6) to offshore (S11) gradient (Supplementary Figure S1). (a, e) Dissolved oxygen, based on standard oceanographic Clark type O₂ electrodes with an inset showing higher resolution STOX sensor measurements. (f) Methane. (b, g) Nitrite. (e–i) Total (c, h) and NC10-specific (d, i) 16S rRNA gene counts, based on quantitative PCR analyses of the 0.2–1.6 μm biomass fraction. Data from the ETNP reflect discrete collections (per depth) using a CTD/rosette or a pump-profiling system during mid May 2014. Data from GD reflect discrete collections (per depth) using a hand-deployed Niskin bottle on 21 January 2015 (D1), with additional samples for 16S counts obtained on 23 January (D2). Error bars are standard deviations among replicates (duplicate and triplicate water samples from the same cast for [CH₄] and [NO₂], respectively; triplicate qPCRs for 16S counts). [O₂] data are provided for representative casts at each station. Challenges in calibration prevented accurate [CH₄] measurements in the ETNP (see text).

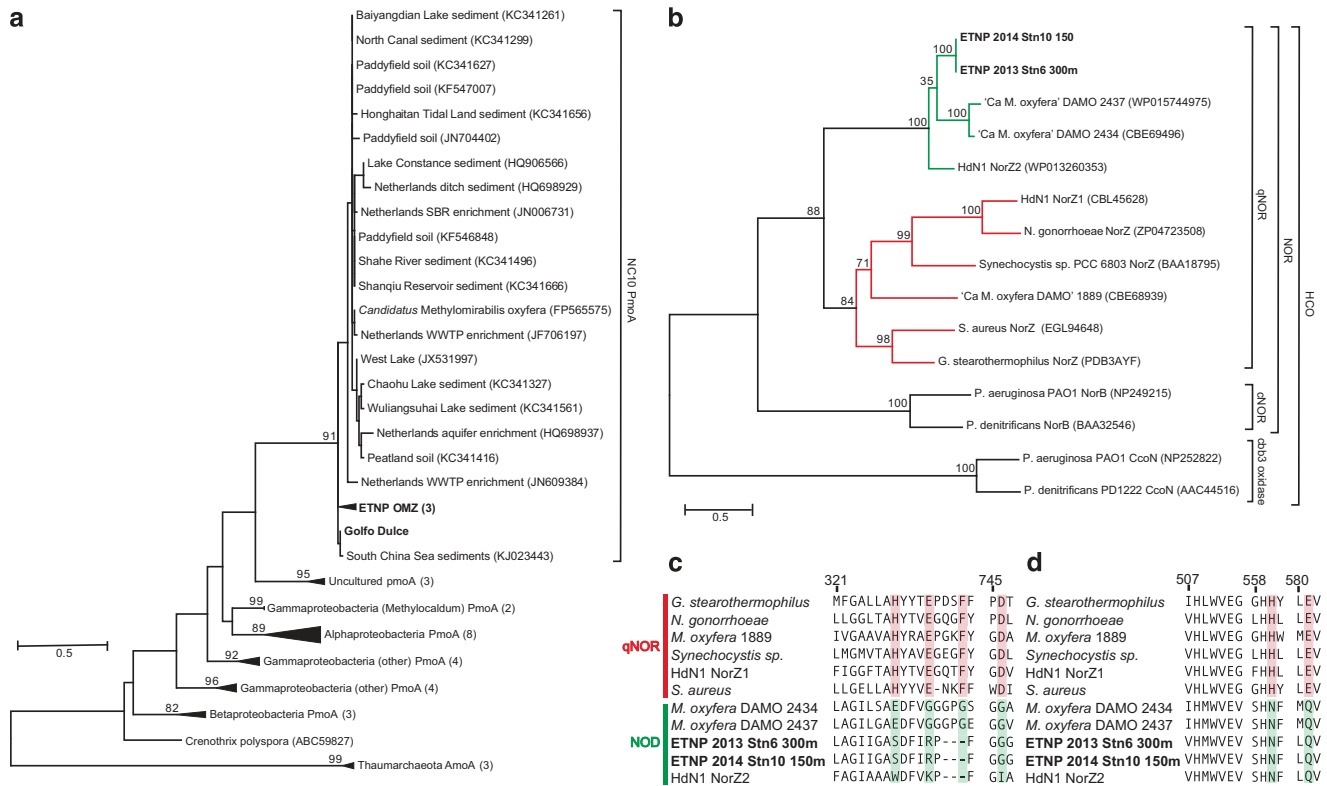


Figure 2 Phylogenetic evidence for the presence and transcriptional activity of NC10 in OMZs. (a) Particulate methane monooxygenase subunit A (PmoA) gene phylogeny. PmoA sequences ($n = 53$) recovered from the ETNP and GD are highlighted in bold within the larger clade of NC10-like sequences from other studies, separated from PmoA of aerobic methanotrophic clades and an ammonia monooxygenase (AmoA) outgroup. Phylogeny was inferred based on Maximum Likelihood (ML) analysis of 88 amino acids using the Dayhoff substitution model. Bootstrap values greater than 70 are shown, along with NCBI Accession numbers for database sequences. Numbers in parentheses represent the number of unique sequences included in each collapsed node—Taxon identifiers and Accession numbers for all sequences are in Supplementary Figure S2. The scale bar represents 50 amino-acid changes per 100 amino acids. (b) Heme-copper oxidases (HCO) phylogeny following that of Ettwig *et al.* (2012), with putative nitric oxide dismutases (NODs) shown in green, including two full-length sequences (labeled ETNP) assembled from OMZ NC10 transcript fragments. The sequences used for assembly were those with top matches (bit score > 50) to *Ca. M. oxyfera* via BLASTX against the NCBI-nr database. Phylogeny was inferred by ML analysis of 520 amino acids based on the Le and Gascuel_2008 model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. (c, d) Alignment of the qNor quinol-binding (c) and catalytic (d) sites showing putative NOD (green) compared with canonical qNor (red) sequences, following that of Ettwig *et al.* (2012). Numbering reflects residue numbers of *G. stearothermophilus*. Shading highlights conserved residues in canonical qNors where amino-acid replacement has occurred in putative NODs.

be due to low sequencing depth or sequence misclassification. Alternatively, OMZ NC10 bacteria may utilize energy substrates other than CH_4 .

To search for biogeochemical evidence of n-damo, we conducted anoxic 10-day incubations of OMZ water with $^{13}\text{CH}_4$ and $^{15}\text{NO}_2$ to measure rates of anaerobic CH_4 oxidation and N_2 production. CH_4 oxidation rates were below the detection limit (0.6 nmol d^{-1}) at all analyzed depths in the ETNP, and at two of the three analyzed depths in the GD (100, 120 m) (Supplementary Table S7), consistent with very low published rates from the ETNP (Pack *et al.*, 2015) and with low NC10 gene counts at both ETNP and GD sites. In contrast, CH_4 oxidation was measured at $2.6 \pm 0.7 \text{ nmol d}^{-1}$ at 90 m in the GD and was inhibited with the addition of acetylene, a pMMO inhibitor, suggesting OMZ CH_4 consumption by pMMO-catalyzed AOM. However, the recovery of MMO transcripts from putative aerobic methylotrophs at this depth

suggests that methanotrophic activity was likely only to a minor extent due to NC10. N_2 production rates greatly exceeded the CH_4 oxidation rates in the GD, further indicating a minor contribution of NC10 to fixed nitrogen loss (Supplementary Table S7).

Together, these results identify pelagic OMZs as a niche for NC10 bacteria. Water residence time in the ETNP OMZ core is estimated at 3.9 ± 0.8 years (DeVries *et al.*, 2012). Given this estimate, and the observed decline in NC10 16S gene abundance with proximity to the sediment–water interface in the GD, it is unlikely that the DNA and RNA sequences detected here represent allochthonous cells advected into the system from sediment. Rather, the transcription of key diagnostic enzymes of n-damo, notably the putative NOD encoded by qNor, suggests a role for NC10 bacteria in OMZ N and O_2 transformations. N_2 production by NC10 would represent a route of N loss in addition to that of classical denitrification and anammox, although the latter are undoubtedly the

dominant N sinks in OMZs. The recovery of NC10 *pmoA* genes and the vertical distribution of NC10 genes and transcripts relative to CH₄ and NO₂⁻ maxima raise the possibility for NC10 CH₄ consumption in OMZs, although this prediction remains to be validated. Given the observed enrichment of CH₄ in OMZs, originating either from advection from sediments (Pack *et al.*, 2015) or potentially from OMZ methanogens (Supplementary Information, Supplementary Table S8, Supplementary Figure S5), even low rates of CH₄ consumption by OMZ NC10 bacteria could have an important role in the open-ocean methane budget.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

We thank the crew of the *R/V New Horizon* for help in sample collection, Niels Peter Revsbech and Morten Larsen for oxygen concentration analysis in the GD, and Alvaro Morales for coordinating and supporting work at the GD field site. This work was supported by the National Science Foundation (1151698 to FJS), the Sloan Foundation (RC944 to FJS), the Danish National Research Foundation DNR53, the Danish Council of Independent Research, and the European Research Council 'Oxygen' grant (267233; supporting LAB and BT).

References

- Babbin AR, Keil RG, Devol AH, Ward BB. (2014). Organic matter stoichiometry, flux, and oxygen control nitrogen loss in the ocean. *Science* **344**: 406–408.
- Chen J, Jiang XW, Gu JD. (2015). Existence of novel phylotypes of nitrite-dependent anaerobic methane-oxidizing bacteria in surface and subsurface sediments of the South China Sea. *Geomicrobiol J* **32**: 1–9.
- Dalsgaard T, Stewart FJ, Thamdrup B, De Brabandere L, Revsbech NP, Ulloa O *et al.* (2014). Oxygen at nanomolar levels reversibly suppresses process rates and gene expression in anammox and denitrification in the oxygen minimum zone off Northern Chile. *Mbio* **5**: 10–1128.
- Deutzmann JS, Stief P, Brandes J, Schink B. (2014). Anaerobic methane oxidation coupled to denitrification is the dominant methane sink in a deep lake. *Proc Natl Acad Sci USA* **111**: 18273–18278.
- DeVries T, Deutsch C, Primeau F, Chang B, Devol A. (2012). Global rates of water-column denitrification derived from nitrogen gas measurements. *Nat Geosci* **5**: 547–550.
- Ettwig KF, Butler MK, Le Paslier D, Pelletier E, Manganot S, Kuypers MMM *et al.* (2010). Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* **464**: 543–548.
- Ettwig KF, Speth DR, Reimann J, Wu ML, Jetten MS, Keltjens JT. (2012). Bacterial oxygen production in the dark. *Front Microbiol* **3**: 273.
- Ettwig KF, van Alen T, van de Pas-Schoonen KT, Jetten MSM, Strous M. (2009). Enrichment and molecular detection of denitrifying methanotrophic bacteria of the NC10 Phylum. *Appl Environ Microbiol* **75**: 3656–3662.
- Ganesh S, Bristow LA, Larsen M, Sarode N, Thamdrup B, Stewart FJ. (2015). Size-fraction partitioning of community gene transcription and nitrogen metabolism in a marine oxygen minimum zone. *ISME J* **9**: 2682–2696.
- Haroon MF, Hu S, Shi Y, Imelfort M, Keller J, Hugenholtz P *et al.* (2013). Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. *Nature* **500**: 567–570.
- Hu BL, Shen LD, Lian X, Zhu Q, Liu S, Huang Q *et al.* (2014). Evidence for nitrite-dependent anaerobic methane oxidation as a previously overlooked microbial methane sink in wetlands. *Proc Natl Acad Sci USA* **111**: 4495–4500.
- Naqvi SWA, Bange HW, Farias L, Monteiro PMS, Scranton MI, Zhang J. (2010). Marine hypoxia/anoxia as a source of CH₄ and N₂O. *Biogeosciences* **7**: 2159–2190.
- Norđi KA, Thamdrup B. (2014). Nitrate-dependent anaerobic methane oxidation in a freshwater sediment. *Geochim Cosmochim Acta* **132**: 141–150.
- Pack MA, Heintz MB, Reeburgh WS, Trumbore SE, Valentine DL, Xu X *et al.* (2015). Methane oxidation in the eastern tropical North Pacific Ocean water column. *J Geophys Res Biogeosci* **120**: 1078–1092.
- Raghoebarsing AA, Pol A, van de Pas-Schoonen KT, Smolders AJP, Ettwig KF, Rijpstra WIC *et al.* (2006). A microbial consortium couples anaerobic methane oxidation to denitrification. *Nature* **440**: 918–921.
- Reeburgh WS. (2007). Oceanic methane biogeochemistry. *Chem Rev* **107**: 486–513.
- Sansone FJ, Popp BN, Gasc A, Graham AW, Rust TM. (2001). Highly elevated methane in the eastern tropical North Pacific and associated isotopically enriched fluxes to the atmosphere. *Geophys Res Lett* **28**: 4567–4570.
- Shen LD, Wu HS, Gao ZQ. (2015). Distribution and environmental significance of nitrite-dependent anaerobic methane-oxidising bacteria in natural ecosystems. *Appl Microbiol Biotechnol* **99**: 133–142.
- Thamdrup B, Dalsgaard T, Revsbech NP. (2012). Widespread functional anoxia in the oxygen minimum zone of the Eastern South Pacific. *Deep Sea Res Pt I* **65**: 36–45.
- Tiano L, Garcia-Robledo E, Dalsgaard T, Devol AH, Ward BB, Ulloa O *et al.* (2014). Oxygen distribution and aerobic respiration in the north and southeastern tropical Pacific oxygen minimum zones. *Deep Sea Res Pt I* **94**: 173–183.

Supplementary Information accompanies this paper on The ISME Journal website (<http://www.nature.com/ismej>)