



## **Metagenomic evidence of a novel family of anammox bacteria in a subsea environment**

Downloaded from: <https://research.chalmers.se>, 2025-12-04 22:42 UTC

Citation for the original published paper (version of record):

Suarez, C., Martins, P., Jetten, M. et al (2022). Metagenomic evidence of a novel family of anammox bacteria in a subsea environment. *Environmental Microbiology*, 24(5): 2348-2360.  
<http://dx.doi.org/10.1111/1462-2920.16006>

N.B. When citing this work, cite the original published paper.

# Metagenomic evidence of a novel family of anammox bacteria in a subsea environment

Carolina Suarez,<sup>1,2</sup> Paula Dalcin Martins,<sup>3</sup> Mike S.M. Jetten,<sup>3</sup> Sabina Karačić,<sup>4</sup> Britt Marie Wilén,<sup>4</sup> Oskar Modin,<sup>4</sup> Per Hagelia,<sup>5</sup> Malte Hermansson<sup>2</sup> and Frank Persson<sup>1b,4\*</sup>

<sup>1</sup>Division of Water Resources Engineering, Faculty of Engineering LTH, Lund University, Lund, Sweden.

<sup>2</sup>Department of Chemistry and Molecular Biology, University of Gothenburg, Gothenburg, Sweden.

<sup>3</sup>Department of Microbiology, RIBES, Radboud Institute for Biological and Environmental Sciences, Radboud University, Nijmegen, The Netherlands.

<sup>4</sup>Division of Water Environment Technology, Department of Architecture and Civil Engineering, Chalmers University of Technology, Gothenburg, Sweden.

<sup>5</sup>Construction Division, The Norwegian Public Roads Administration, Oslo, Norway.

## Summary

Bacteria in the order ‘*Candidatus Brocadiales*’ within the phylum *Planctomycetes* (*Planctomycetota*) have the remarkable ability to perform anaerobic ammonium oxidation (anammox). Two families of anammox bacteria with different biogeographical distributions have been reported, marine *Ca. Scalinduaceae* and freshwater *Ca. Brocadiaceae*. Here we report evidence of three new species within a novel genus and family of anammox bacteria, which were discovered in biofilms of a subsea road tunnel under a fjord in Norway. In this particular ecosystem, the nitrogen cycle is likely fuelled by ammonia from organic matter degradation in the fjord sediments and the rock mass above the tunnel, resulting in the growth of biofilms where anammox bacteria can thrive under oxygen limitation. We resolved several metagenome-assembled genomes (MAGs) of anammox bacteria, including three *Ca. Brocadiales* MAGs that could not be classified at the family level. MAGs of this novel family had all the diagnostic genes for a full anaerobic ammonium oxidation pathway in which nitrite

was probably reduced by a NirK-like reductase. A survey of published molecular data indicated that this new family of anammox bacteria occurs in many marine sediments, where its members presumably would contribute to nitrogen loss.

## Introduction

In 1995, our understanding of the nitrogen cycle changed, when the anaerobic ammonium oxidation (anammox) process was uncovered in an anaerobic pilot plant (Mulder *et al.*, 1995). Anammox bacteria were found to couple the oxidation of ammonium to nitrite reduction, resulting in the production of nitrogen gas (van de Graaf *et al.*, 1995). Since then, a comprehensive picture of these bacteria and their relevance to the global nitrogen cycle has emerged (Kuypers *et al.*, 2018). Microorganisms capable of performing the anammox process were identified as novel members of the phylum *Planctomycetes* (Strous *et al.*, 1999), with all known anammox bacteria so far being part of the order ‘*Candidatus Brocadiales*’ (Jetten *et al.*, 2010). Anammox bacteria have been shown to contribute to nitrogen turnover in many oxygen-limited natural environments (Kuypers *et al.*, 2003), and their unique metabolism has resulted in new biotechnologies for more sustainable biological nitrogen removal in wastewater treatment plants (Jetten *et al.*, 1997; van Dongen *et al.*, 2001; Lackner *et al.*, 2014). Anammox bacteria have quite a versatile metabolism, for example being able to use several organic acids (Kartal *et al.*, 2013) and various electron acceptors such as nitrate, nitrite, metal oxides, nitric oxide (NO) and electrodes (Hu *et al.*, 2019; Shaw *et al.*, 2020).

To this date, anammox bacteria are yet to be isolated. Nonetheless, enrichments and molecular approaches have revealed the existence of two anammox families, *Ca. Scalinduaceae* and *Ca. Brocadiaceae*. These families seem to occupy different ecological niches, with salinity largely influencing their biogeography (Sonthiphand *et al.*, 2014). *Ca. Scalinduaceae* is commonly associated with marine environments, with *Ca. Scalindua* as the only described genus. *Ca. Brocadiaceae* are frequently observed in freshwater

Received 7 December, 2021; accepted 7 April, 2022. \*For correspondence. E-mail frank.persson@chalmers.se; Tel. +46-31-7722160.

communities and wastewater bioreactors. Four genera of *Ca. Brocadia* are known: *Ca. Jettenia* (Quan *et al.*, 2008), *Ca. Brocadia* (Strous *et al.*, 1999), *Ca. Anammoxoglobus* (Kartal *et al.*, 2007) and the model anammox bacterium *Ca. Kuenenia* (Strous *et al.*, 2006).

New, undescribed groups of anammox bacteria might exist, and there are hints of their existence from environmental surveys. For example, in the SILVA 138 database (Quast *et al.*, 2013) there is a group of 16S rRNA gene sequences named as GWA2-50-13, and in the GTDB 06-RS202 taxonomy (Parks *et al.*, 2018, 2020) there is a family with placeholder name f\_\_2-02-FULL-50-16-A. In phylogenetic trees, these groups cluster within the anammox bacteria. Most anammox bacteria have been described from enrichments, but culture-independent methods such as single-amplified genomes and metagenome-assembled genomes (MAGs) are complementary approaches that can be used to unravel novel microorganisms (Rinke *et al.*, 2013; Hug *et al.*, 2016).

One of the first MAGs ever resolved was the genome of *Ca. Kuenenia stuttgartiensis* (Strous *et al.*, 2006). MAGs of anammox bacteria had been recovered and described from enrichments (Strous *et al.*, 2006; Oshiki *et al.*, 2015), wastewater treatment plants (Speth *et al.*, 2016; Park *et al.*, 2017), but also from environmental surveys (Speth *et al.*, 2017; Zhao *et al.*, 2020). MAGs can be used to predict anammox metabolism based on phylogenetic information and the presence of genes linked to the anammox process. Although, to this date (2022-02-22), there are 122 *Ca. Brocadiales* genomes at the NCBI assembly database, many of them were sampled in engineered environments like wastewater bioreactors. Thus, databases are likely biased towards relatively fast-growing anammox bacteria which are adapted to nitrogen-rich and low-salinity conditions.

Anammox bacteria are obligate anaerobes, and thus they can occur in habitats with limited oxygen supply such as thick biofilms (Suarez *et al.*, 2019) and marine oxygen minimum zones (Woebken *et al.*, 2008). Other largely unexplored anoxic environments could harbour novel anammox bacteria. For example, in marine sediments, oxygen is rapidly used as terminal electron acceptor, with anoxic conditions just a few millimetres below the sediment surface (Glud, 2008). Deep seafloor sediments are poorly surveyed environments and despite their nutrient limitation, rich and distinct microbial communities, including anammox bacteria, can thrive in these ecosystems (Parkes *et al.*, 1994; Kallmeyer *et al.*, 2012; Inagaki *et al.*, 2015; Hoshino and Inagaki, 2019; Zhao *et al.*, 2020). Sampling of seafloor communities can be challenging as underwater drilling is required. In sub-sea road tunnels, however, microbial seafloor communities are accessible as water seepage from surrounding bedrock occurs (Grønhaug, 1978;

Hagelia, 2011). One such example is the Oslofjord tunnel in Norway, a subsea road tunnel with a maximum depth of 134 m below sea level, where saline water seepage from cracks in the bedrocks results in growth of microbial biofilms on the rough sprayed concrete surfaces used for rock support of the tunnel. The biofilms are rich in manganese oxide and iron hydroxide biominerals. Redox reactions within the biofilms involve the sulfur, carbon, nitrogen, iron and manganese biogeochemical cycles, sometimes leading to acidification (Hagelia, 2007, 2011; Karačić *et al.*, 2018).

Surveys of the Oslofjord site using 16S rRNA gene sequencing showed a microbial community where nitrogen-transforming microorganisms are abundant (Karačić *et al.*, 2018). Both ammonium-oxidizing bacteria and ammonium-oxidizing archaea are present and presumably oxidize ammonium to nitrite. Amplicon sequence variants classified as the marine anammox *Ca. Scalindua* were present in these datasets. In addition, a group of unclassified *Ca. Brocadiales* were the second most abundant taxa in some samples (Karačić *et al.*, 2018). It is unclear if these unclassified sequences belonged to anammox bacteria, as their potential function was only inferred from 16S rRNA gene taxonomy. To explore the *Ca. Brocadiales* community in the Oslofjord site, and describe their taxonomy and potential functions, we used shotgun metagenomic sequencing with the aim of resolving MAGs of these unclassified potentially new anammox bacteria.

## Results and discussion

### *Three MAGs represent a novel family of anammox*

Analysis of MAGs from two sites in the tunnel yielded seven Oslofjord Tunnel MAGs (OFTMs) that, with GTDB-Tk, were classified within the order *Ca. Brocadiales*: OFTM134, OFTM214, OFTM301, OFTM1, OFTM180, OFTM351 and OFTM256. They were detected in five out of eight samples, with the sum of their relative abundance ranging between 0.05% and 5.37%. OFTM134, OFTM214 and OFTM301 could not be classified at family level and were 80%–95% complete (Table 1), while the other four MAGs were affiliated to the genus *Ca. Scalindua*. These seven MAGs were placed in a phylogenetic tree of 92 concatenated core bacterial genes (Fig. 1), which revealed that the three unclassified MAGs (OFTM134, OFTM214 and OFTM301) may represent novel anammox taxa.

In order to investigate the level of taxonomic novelty, average amino acid identity (AAI) was computed for a set of genomes including the three novel MAGs (OFTM134, OFTM214 and OFTM301), representatives of the four known anammox genera, and two non-anammox

**Table 1.** Summary of genomic information of three anammox MAGs (completeness and contamination was determined by checkM; rRNA molecules and number of scaffolds by DRAM).

MAG	Scaffolds	Completeness (%)	Contamination (%)	5S rRNA	16S rRNA scaffold (begin-end bp)	23S rRNA scaffold (begin-end bp)	tRNA count
OFTM134	114	95.1	3.4	None	OFTM134_10 (2548–3239)	OFTM134_10, (2–2148)	48
OFTM214	142	93.9	5.7	None	OFTM214_0 (6–843)	None	45
OFTM301	769	82.8	9.7	None	none	None	32

*Planctomycetes* genomes (Fig. 2). AAI values among the three OFTMs ranged from 60% to 63%. Between the three OFTMs and genomes representative of the four known anammox genera, AAI ranged from 48% to 50%. Interestingly, AAI between non-anammox and anammox genomes was 38%–39%. Taking into consideration previously reported AAI thresholds of 60%–80% AAI for genus level and >85% AAI at the species level (Luo *et al.*, 2014), these results indicate that OFTM134, OFTM214 and OFTM301 may represent a novel family of anammox bacteria, and that each genome may represent a distinct species within this new genus.

#### *The novel MAGs have genomic potential for full anammox metabolism*

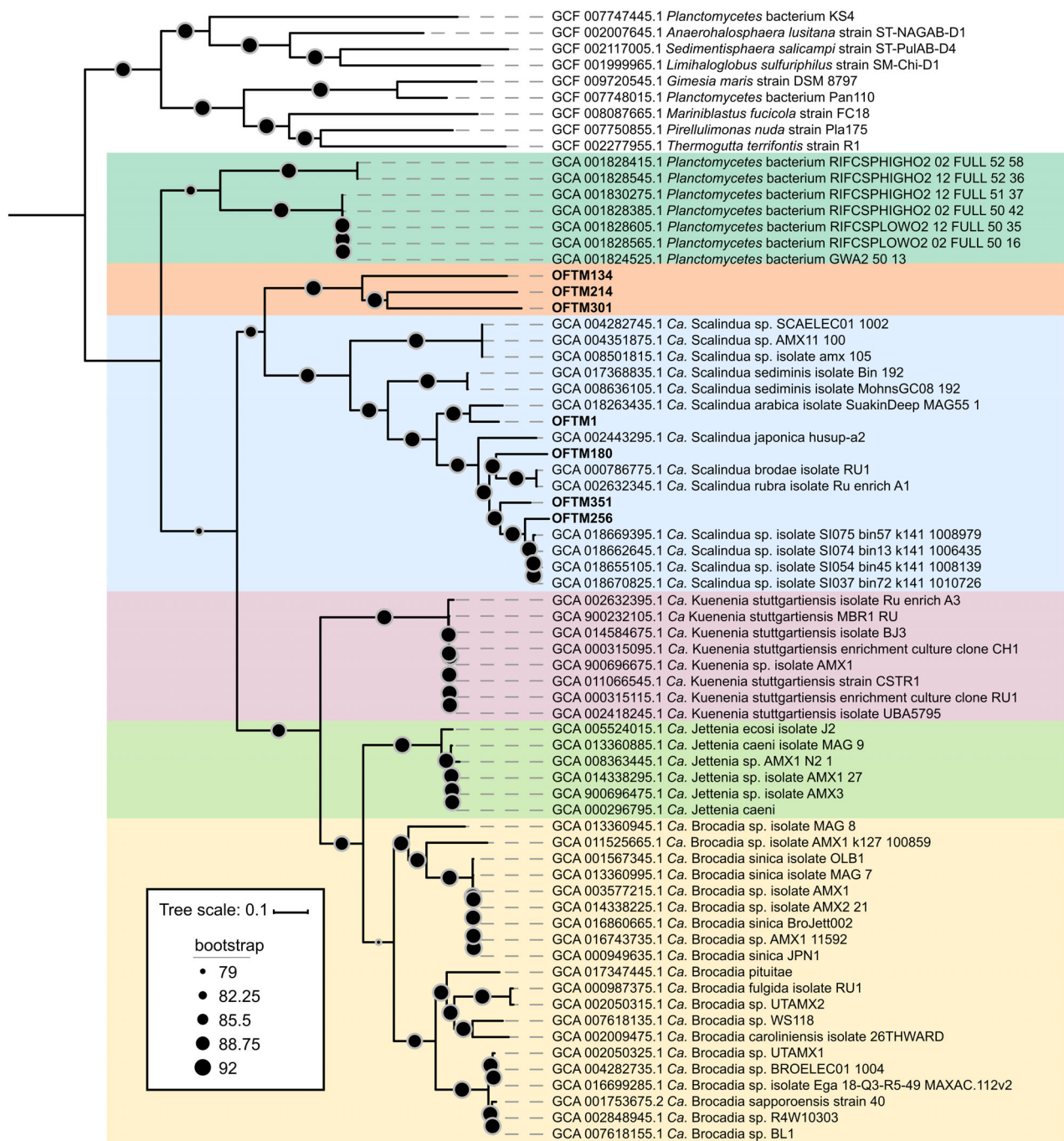
Genomic potential for the full anaerobic ammonium oxidation pathway as well as components of electron transport chain were identified in OFTM134, OFTM214 and OFTM301 (Fig. 3). Genes encoding nitrate reductase/nitrite oxidoreductase (*nrxABC*) were identified in the three MAGs. This protein complex may catalyse nitrite oxidation to nitrate (van de Graaf *et al.*, 1996; Chicano *et al.*, 2021). Nitrite could be reduced to NO via a NirK-type nitrite reductase identified in OFTM134 and OFTM301. Copper-containing NirK proteins have been reported in *Ca. Jettenia* (Hira *et al.*, 2012; Mardanov *et al.*, 2019) and *Ca. Brocadia caroliniensis* (Park *et al.*, 2017), while *Ca. Kuenenia stuttgartiensis*, and *Ca. Scalindua* harbour a heme-iron NirS-type nitrite reductase (Strous *et al.*, 2006; van de Vossenberg *et al.*, 2013; Oshiki *et al.*, 2017) (Fig. S3). Both NirK and NirS were absent in OFTM214, as also reported for several *Ca. Brocadia* (Oshiki *et al.*, 2015; Narita *et al.*, 2017; Okubo *et al.*, 2021) (Fig. S3). A multiheme hydroxylamine oxidoreductase (HAO)-encoding gene, present in this MAG, might catalyse this reaction (Ferousi *et al.*, 2021). Alternatively, the MAG, which is ~94% complete, might be missing a nitrite reductase-encoding gene.

In the next step, NO and ammonium can then serve as substrates for a hydrazine synthase (*hzsABC*). Genes encoding HzsABC were identified in OFTM134, OFTM214 and OFTM301. Hydrazine can finally be

converted to dinitrogen gas by a hydrazine dehydrogenase (HDH). Interestingly, each MAG had several genes encoding proteins with multiheme motifs annotated as HDH or HAO (DOI: 10.5281/zenodo.5524859). Of six genes annotated as HAO/HDH in OFTM134, one had a best BLASTP hit to kustc0694 HDH, four to kustc1061 hydroxylamine oxidase (HOX), and one to HAO kustc4574, part of the Rieske complex R/b-3. In OFTM214, one gene had a best BLASTP hit to HDH and one to HOX, while in OFTM301, one gene had a best BLASTP hit to HDH and two to HOX. Moreover, the three MAGs had a hydroxylamine reductase (EC: 1.7.99.1, K05601) gene (*hcp*), encoding a protein hypothesized to be used for ammonification – the disproportionation of hydroxylamine in the absence of nitrite into dinitrogen gas and ammonium (van der Star *et al.*, 2008).

Almost complete electron transport chains were identified in OFTM134, OFTM214 and OFTM301 (DOI: 10.5281/zenodo.5524859). All genes encoding subunits of the complex I, NADH dehydrogenase (*nuoA-N*), were present, except *nuoG*. Two genes were annotated as *nuoF* in OFTM134 and OFTM301, and *nuoE* was absent in OFTM301. A series of genes were concomitantly annotated as subunits of both the sodium-pumping NADH:quinone oxidoreductase (NQR) and the RNF complex, and scattered genes in the genomes had significant BLASTP hits to NQR subunits, but no MAGs had all genes. Given the high homology between the two complexes and the previous miss-annotation of *RnfEA* as *NqrDE* (de Almeida *et al.*, 2016), and that *nqrA* was missing in the three MAGs, but all subunits of the RNF complex were present in all MAGs, we assume that these six genes are *rnfABCDEG* in each of the three MAGs. That would mean that NQR is absent in these organisms. However, as genomic evidence is inconclusive, physiological investigation would be required to elucidate the potential presence of NQR. Succinate dehydrogenase genes *sdhABC* (complex II) were present in OFTM134 and OFTM214, while OFTM301 only harboured *sdhC*.

There are three Rieske/cyt<sub>b</sub> complexes (complex III) in *K. stuttgartiensis*: R/b-1, R/b-2 and R/b-3 (Kartal *et al.*, 2011, 2013; de Almeida *et al.*, 2016). The Rieske complex R/b-1 (identified via BLASTP analyses using



**Fig. 1.** Phylogenetic tree built with the UBCG pipeline using 92 concatenated core bacterial genes retrieved from 70 genomes. Non-anammox *Planctomycetes* genomes form a clade at the top of the tree. The GTDB “f\_2-02-FULL-50-16-A” clade is highlighted in darker green. Novel anammox (this study) are highlighted in orange, while known genera *Ca. Scalindua*, *Ca. Kuenenia*, *Ca. Jettienia* and *Ca. Brocadia* are highlighted in blue, pink, light green and yellow respectively. Reference genomes start with GCA or GCF reference numbers, and MAGs from this study start with OFTM.

kuste3096-7) was identified in OFTM134, OFTM214 and OFTM301: three copies in OFTM134, three copies in OFTM214 and five copies in OFTM301. A *c*-type cytochrome-encoding gene was frequently present upstream or downstream of the two R/b-1 subunits. In

several instances, this gene was a homologue of cytochrome *c*<sub>554</sub>, which accepts electrons from HAO in the ammonium oxidizer *Nitrosomonas europaea* (Yamanaka and Shinra, 1974). OFTM134 and OFTM214 had five out of six genes encoding subunits of R/b-2 (kustd1480-85);

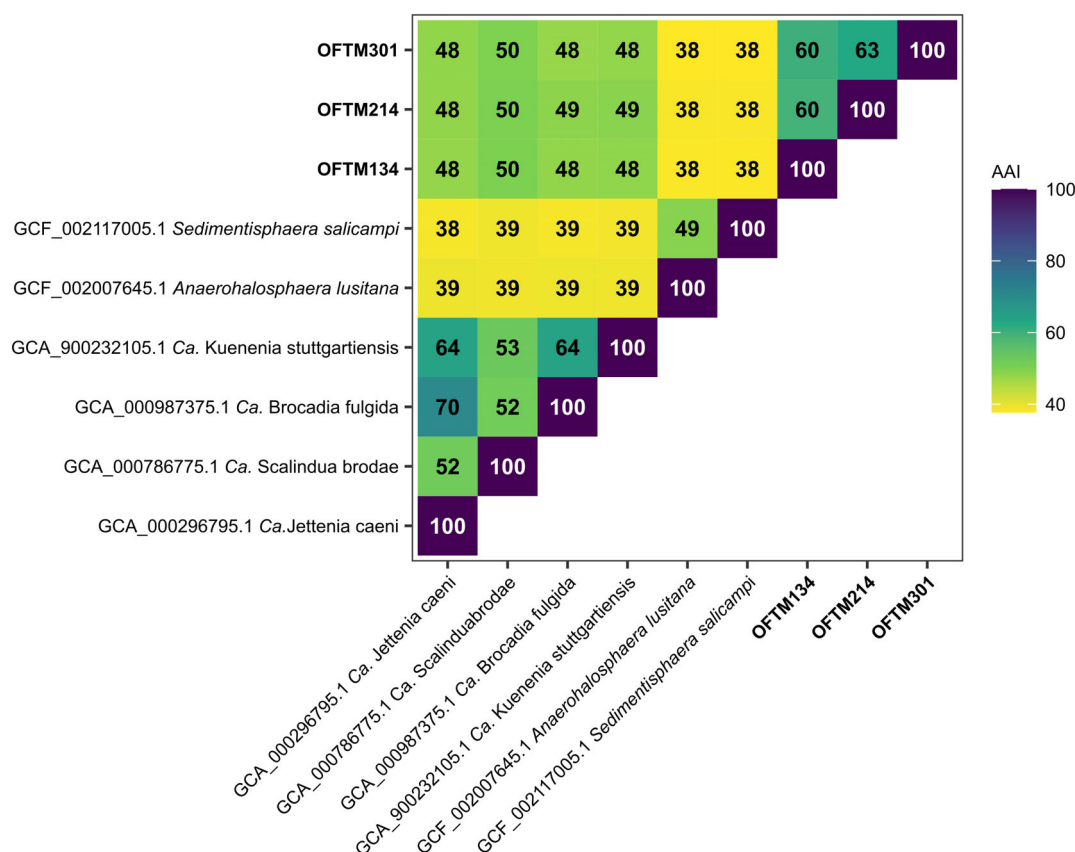


Fig. 2. Average AAI matrix. Numbers express percent of AAI similarity between genome pairs.

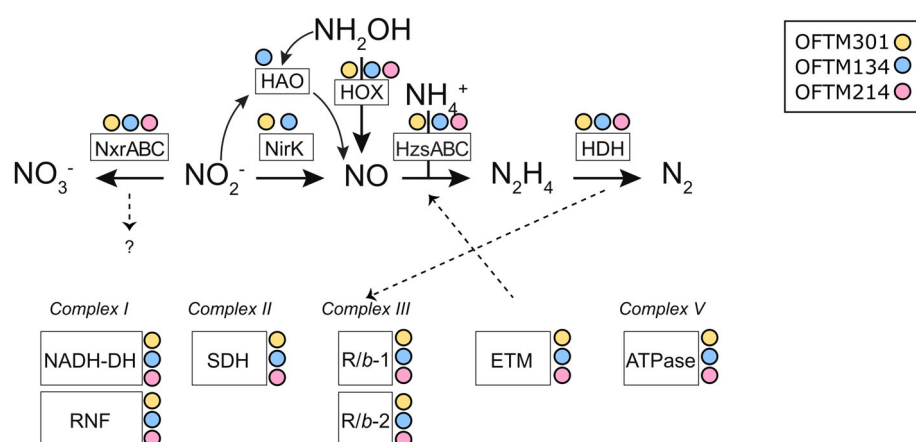


Fig. 3. Summary of metabolic potential identified in the three MAGs. The upper section highlights proteins identified in the novel MAGs involved in the pathway for anaerobic ammonium oxidation, and the bottom section displays proteins involved in the electron transport chain. Solid arrows indicate the flux of substrates, and dashed arrows indicate the flow of electrons. NxrABC, nitrate reductase/nitrite oxidoreductase (EC:1.7.5.1 1.7.99.-); NirK, NO-forming nitrite reductase (EC:1.7.2.1); HzsABC, hydrazine synthase (EC:1.7.2.7); HDH, hydrazine dehydrogenase (EC:1.7.2.8); HAO, hydroxylamine oxidoreductase (EC:1.7.2.6); HOX, hydroxylamine oxidase (homologue to kustc1061); NADH-DH, NADH dehydrogenase *nuoA-N* (EC:7.1.1.2); RNF, Rnf complex *mfaBCDEG* (EC:7.2.1.2); SDH, succinate dehydrogenase (EC:1.3.5.1), R/b-1-2: Rieske-heme b complexes 1 and 2; ETM, electron transfer module.

the only gene missing was a homologue of kustd1482, a gene of unknown function. OFTM301 had four out of six genes encoding R/b-2; kustd1480 and kustd1482 were

missing. Finally, genes encoding subunits of R/b-3 (homologues to kustc4569-74) were not detected. Out of the six R/b-3 genes, significant BLASTP hits to gene

kuste4570 were identified in the three MAGs, and OFTM134 also had a hit to kuste4574, the subunit with high similarity to HAO.

Both subunits of the electron transfer module (ETM; kuste2855-6) were identified in OFTM134, OFTM214 and OFTM301 (two copies in OFTM301). In each MAG, a gene encoding a cytochrome *c*<sub>554</sub> was present upstream or downstream of the two ETM subunits. Interestingly, genes encoding the cytochrome *bd* complex subunits I and II (*cydAB*) were present: *cydA* was present in two to three copies in each MAG, and *cydB* was present in one copy in OFTM134 and OFTM301 (in OFTM214 it was missing). *CydAB* might be used to counteract oxidative and nitrosative stress (Giuffrè *et al.*, 2014). The gene *cydA* has been previously identified in anammox bacteria (de Almeida *et al.*, 2016); however, given that *cydX* was missing in all three MAGs, it is unlikely that these organisms would have cytochrome *bd* oxidase activity (VanOrsdel *et al.*, 2013). Finally, a complete F-type ATPase was present in OFTM134 and OFTM214, while in OFTM301, six of the eight subunits were present. MAG OFTM134 also had a complete V-/A-type ATPase. Complete MAG annotation, BLASTP results, and gene nucleotide and amino sequences are available at DOI: 10.5281/zenodo.5524859.

The presence of the most important diagnostic genes associated to the anammox process and the phylogenetic affiliation of OFTM134, OFTM301 and OFTM214 within *Ca. Brocadiales* strongly suggest that these are new bacteria, able to perform the anammox process.

#### *Potential metabolic versatility was identified in the novel MAGs*

Besides genes encoding proteins involved in anammox metabolism, the MAGs had potential for sulfur, hydrogen and an alternative nitrogen cycle pathway. OFTM134, OFTM214 and OFTM301 had a nickel-dependent hydrogenase and corresponding maturation protein-encoding genes, as well as a sulfhydrogenase-encoding gene (*hydB*) involved in polysulfide reduction to sulfide (Ma *et al.*, 1993), which seems to be absent in some *Ca. Brocadia* (Fig. S3). OFTM134 also had a sulfide:quinone oxidoreductase-encoding gene (*sqr*) for sulfide oxidation to elemental sulfur (Cherney *et al.*, 2010) and a thiosulfate reductase/polysulfide reductase chain A-encoding gene (*phsA*) for thiosulfate reduction to sulfide (Hinsley and Berks, 2002). The *sqr* gene is lacking in most anammox bacteria, with the exception of *Ca. Brocadia sinica*, while *phsA* is only observed in *Ca. Scalindua sediminis* and *Ca. Kuenenia* (Fig. S3). OFTM134 and OFTM214 had a *sorA* gene encoding a cytochrome-dependent sulfite dehydrogenase for sulfite oxidation to sulfate (Kappler *et al.*, 2000).

OFTM134, OFTM214 and OFTM301 had one or two copies of a histidine ammonia-lyase-encoding gene (*hutH*) for amino acid utilization and ammonia production (Schwede *et al.*, 1999), which could potentially enter the ammonium oxidation pathway. This gene is also present in *Ca. Scalinduaceae* and in some *Ca. Brocadia* (Fig. S3). OFTM214 had a D-lactate dehydrogenase-encoding gene (*dld*), potentially involved in D-lactate utilization (Pinchuk *et al.*, 2009). Finally, OFTM214 and OFTM301 had periplasmic nitrate reductase-encoding genes (*napAB*), for potential nitrate reduction to nitrite (Jepson *et al.*, 2007). Reflecting the potential metabolic versatility of these organisms, heterodisulfide reductase-encoding genes (*hdr*) and Methylviologen-reducing hydrogenase-encoding genes (*mvh*) were identified: *hdrABC* in OFTM134, *hdrD* and *mvhD* in OFTM214, and *hdrABCD* and *mvhD* in OFTM301. Both *hdrABC* and *mvhD* are also observed among *Ca. Scalinduaceae*, but not among *Ca. Brocadiales* (Fig. S3).

For some anammox bacteria, the use of organic acids such as acetate has been reported (Kartal *et al.*, 2008; van de Vossenberg *et al.*, 2013). Bacteria incorporate acetate into acetyl-CoA via two main pathways: one uses an AMP-forming acetyl-CoA synthetase (ACS), and another requires both acetate kinase (AckA) and phosphate acetyltransferase (Pta), although this pathway can also be used for acetate formation (Wolfe, 2005). Genes with homology to kustc1128 ACS were found in OFTM134, OFTM214 and OFTM301. The AckA + Pta pathway was present in both OFTM301 and OFTM134. OFTM301 also had a putative acetate permease (ActP). However, at least for *K. stuttgartiensis*, the ability to incorporate acetate is still under discussion (Russ *et al.*, 2012; Lawson *et al.*, 2021). Additionally, OFTM214 had an ADP-forming acetyl-CoA synthetase, which has been linked to acetate production from acetyl-CoA in archaea and some bacteria (Schäfer *et al.*, 1993; Schmidt and Schönheit, 2013). Both *AckA* and *Pta* seem to be absent in *Ca. Kuenenia* and *Ca. Jettenia*, but they are observed in *Ca. Scalindua* and *Ca. Brocadia*; *ActP* is also observed in some *Ca. Scalindua* (Fig. S3).

#### *The novel MAGs have a limited geographical distribution*

We did a BLASTP search with ribosomal protein S3 sequences to resolve if similar novel anammox were present in published metagenomes. The highest hits had between 66% and 73% identity to the novel anammox protein sequences. When comparing 16S rRNA gene sequences, one hit with 95% identity to OFTM134 and 93% to OFTM214 was retrieved. This corresponds to the NCBI accession number KM019039.1, which was sampled at brine-seawater interface of the Kebrit Deep brine pool at the Red Sea (Guan *et al.*, 2015). This is an

environment characterized by its anoxic and hypersaline conditions. Additional *Ca. Brocadiales* 16S rRNA genes were recovered with a targeted assembly of the 16S rRNA gene from the metagenome (Gruber-Vodicka *et al.*, 2020); although some of them grouped with *Ca. Scalinduaceae*, others were part of the OFTM134, OFTM214 and KM019039.1 groups (Fig. S4). The latter were part of a new clade, which was different to all *Ca. Brocadiales* families in the SILVA 138 database (Fig. S4). Thus, based on 16S rRNA gene phylogeny, there is evidence of four major clades among *Ca. Brocadiales*: *Ca. Scalinduaceae*, *Ca. Brocadiaceae*, GWA2-50-13 and the novel family from this study.

The near absence of this family in databases suggests that the novel MAGs could be rare, have a limited geographical distribution or thrive in poorly sampled ecosystems. Standard 16S rRNA V3–V4 and V4 primers can detect these anammox bacteria (see Supporting Information). However, a search against the 16S rRNA amplicon datasets at the NCBI resulted in similar sequences only being observed in 49 SRA samples. Of them, 25 corresponded to ‘marine sediment metagenome’ samples, 11 to ‘sediment metagenome’ and eight to ‘marine metagenome’ samples.

Other phylogenetic markers for anammox bacteria exist. One of them is HDH, a gene where PCR primers had been used for environmental surveys (Yang *et al.*, 2020). For OFTM134, two sequences with around 99% similarity were found. These and two other related sequences are from surveys in Jiaozhou Bay sediments, suggesting that these might be similar species (Fig. 4). These HDH sequences seem to correspond to the anammox novel clade II reported in Dang *et al.* (2010). A lower similarity was observed for OFTM301 and OFTM214, but they seem to be part of the same clade (Fig. 4). The poor representation of these sequences in HDH clone libraries could be in part due to multiple mismatches in commonly used HDH primers (See Supporting Information).

In the absence of adequate primers, metagenomics improves the identification of HDH in environmental samples. One approach that could be used is protein-level assembly (Steinegger *et al.*, 2019). We used this to successfully recover several HDHs that clustered either with *Ca. Scalinduaceae* or with the novel anammox clade (Fig. 4). HDH recovered from MAGs in other surveys suggests that other undescribed anammox clades may exist (Fig. 4), as also suggested by 16S rRNA and concatenated protein trees (Fig. S4, Fig. 1). Primer mismatches in HDH amplicon studies and misclassification in 16S rRNA amplicon studies could lead to an underestimation of anammox bacteria in marine sediments, which could affect estimations of total nitrogen turnover.

Since the novel anammox family appears to be present in marine sediments, this would indicate adaptations to salinity. Indeed, OpuC, encoding a transporter for glycine betaine and proline, was present in all three MAGs. OMTF214 harboured a gene encoding a BCCT transporter, which is involved in the uptake of several compatible solutes (Ziegler *et al.*, 2010). OpuC appears to be absent in *Ca. Brocadia* and BCCT transporters are only observed in *Ca. Kuenenia* and *Ca. Scalindua arabica* (Fig. S3). Instead of using compatible solutes, another adaptation to salinity could be to maintain high intracellular salt concentrations, as it has been suggested for *Ca. Scalindua rubra* (Speth *et al.*, 2017).

#### *Distribution of anammox MAGs in the Oslofjord biofilms*

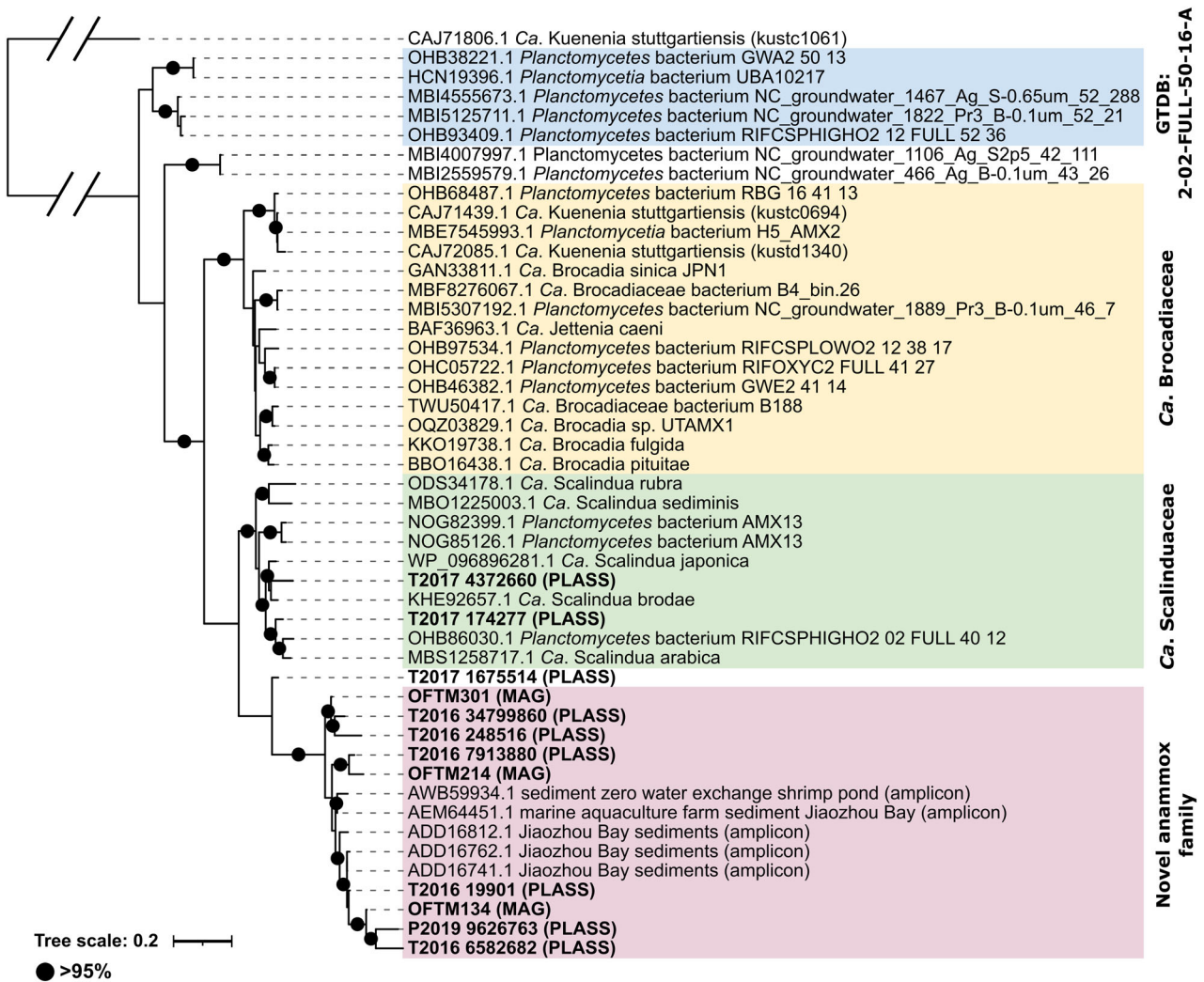
Anammox bacteria often coexist with nitrifiers, and such was also the case for the microbial community in the Oslofjord biofilms. MAGs classified as the nitrifiers *Nitrosomonas*, *Nitrosopumilus*, *Nitrospirales* and *Nitrospirota* were recovered (data not shown). This confirms the results of Karačić *et al.* (2018), where nitrifiers were also observed in the amplicon dataset.

Both *Ca. Scalindua* and the novel anammox MAGs coexisted in the Oslofjord biofilm (Fig. 5). In this study, two separate locations were sampled in four different years, but it seems as if OFTM134, OFTM214 and OFTM301 were not represented in all samples (Fig. 5). It is unclear what factors affected the abundance and distribution of anammox bacteria in the Oslofjord tunnel. Local variations in the microbial communities between biofilms and within the biofilms could lead to distinct nitrogen-cycling processes, and thus nitrogen loss by the anammox process could be localized to certain spots.

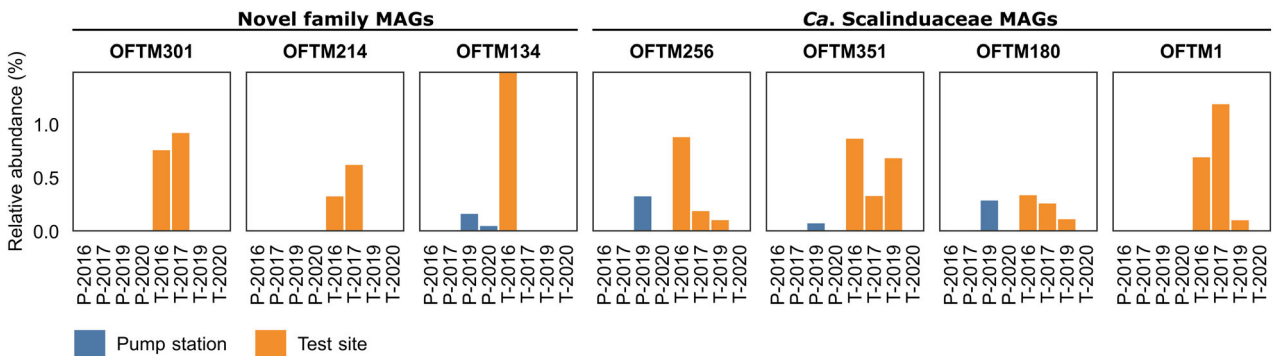
#### *Oslofjord tunnel biofilms*

Biofilm growth has been observed in the Oslofjord sub-sea tunnel and is clearly linked to concrete biodeterioration (Hagelia, 2011; Karačić *et al.*, 2018). The communities sampled represent biofilms associated with water seeps on the tunnel walls, the mode of occurrence being similar to observations of biofilm growth on exposed rock walls in underground mines (Bond *et al.*, 2000; Sjöberg *et al.*, 2020). Given that anammox bacteria have been identified in sediments (Dang *et al.*, 2010; Prokopenko *et al.*, 2013; Devol, 2015; Zhao *et al.*, 2020), we hypothesize that the origin of anammox bacteria in the tunnel is seawater, seeping through cracks in the bedrock, from overlying marine sediments into the tunnel.

Results from a previous study at the Oslofjord tunnel suggest that nitrogen transformation processes occur in



**Fig. 4.** Phylogenetic tree of hydrazine dehydrogenase (HDH) amino acid sequences. Circles shown Ultrafast bootstrap support higher than 95%. The HOX kustc1061 of *Ca. Kuenenia stuttgartiensis* was used as outgroup.



**Fig. 5.** Relative abundances of *Ca. Brocadiaceae* MAGs at the two sites in the Oslofjord tunnel.

the tunnel biofilms (Hagelia, 2011). Ammonium loss from water coming from bedrock, passing through biofilms, and falling into ditches below was inferred for some

locations (Fig. S1) (Hagelia, 2011). For example, in water collected from rock joints at location V5, the ammonium concentration was 1.7 mg N/L, but in the ditch below it

was 0.003 mg N/L (Hagelia, 2011), potentially due to nitrification and/or anammox activity.

The main source of ammonium in the tunnel could have been organic matter degradation in the marine sediments above the tunnel. In sediments, oxygen is rapidly used as electron acceptor, while ammonium accumulates with increasing depth as organic matter is degraded (Devol, 2015). Data from Hagelia (2011) show that in the water column above the tunnel, at 60 m sea depth, the ammonium concentration was below detection limit at <0.005 mg N/L. However, in the deeper zone of the tunnel, water collected from rock joints typically had about 1–1.5 mg N/L ammonium (Fig. S1). Ammonium concentrations in the tunnel were positively correlated with local sediment thickness (Fig. S2). This supports that the chemical composition of the water changed when passing through sediments, before arriving to the tunnel.

In the absence of oxygen, nitrate and nitrite in the sediments above the tunnel would have been used by denitrifiers and anammox bacteria. Hagelia (2011) observed that for rock joints in the tunnel, nitrate + nitrite concentrations were around 0.006 mg N/L, while the concentration in the Oslofjord water column was 0.15 mg N/L (Fig. S1). Despite the incoming water to the tunnel being poor in nitrate + nitrite, in the tunnel, the oxic environment would favour aerobic ammonia oxidation and further nitrite oxidation. The newly produced nitrite could sustain other nitrogen-converting processes such as anammox.

## Conclusions

Here we described three novel species within a novel genus and family of anammox bacteria, which were found in metagenomes from a subseafloor biofilm. We suggest the name *Ca. Anammoxibacter* for this new genus and *Ca. Anammoxibacteraceae* for the family, referring to their predicted potential for the anammox process. This study adds to our understanding of the largely unexplored biodiversity of subseafloor environments. Future exploration of other anaerobic communities where ammonium is present could lead to discovery of more anammox bacteria, with implications to the global nitrogen cycle and potential novel biotechnological applications.

## Experimental procedures

The Oslofjord subsea road tunnel is located near Drøbak, Norway (59.66472 N, 10.61306 E). In locations where water seepage occurred, biofilms with orange or black colour were observed. The locations with biofilm are

shielded from tunnel traffic exhaust fumes by a continuous inner lining of cast concrete elements. The sea depths above the investigated part of the tunnel vary from about 30 to 45 m, with sediment thicknesses of 10–55 m and rock covers ranging from 35 to 75 m. The sediments consist of mud and clays in upper parts and are frequently dominated by fluvio-glacial deposits (sand and gravel) in the deeper parts (Backer and Blindheim, 1999; Haverkamp *et al.*, 2014). The bedrock here consists mainly of granitic gneiss and granite pegmatite, being variably affected by deep weathering along rock joint systems with associated clay minerals (illite, kaolinite, montmorillonite), calcite, chlorite and subordinate sulfide (pyrite) (NPRA, unpublished data).

Two separate places in the tunnel, referred to as Pump-station and Test-site, were sampled on four different occasions over a period of 5 years. Sampling and DNA extraction were conducted as previously described (Karačić *et al.*, 2018). Shotgun metagenomic sequencing was done in an Illumina NovaSeq6000 platform, generating 150 bp paired-end reads. Reads were normalized to 100× coverage using BBNorm in the BBTools package 38.61b (<https://sourceforge.net/projects/bbmap>). Co-assembly of normalized reads was performed with Megahit 1.2.9 (Li *et al.*, 2015), followed by mapping the reads to the assembly with Bowtie v2.3.5.1 (Langmead and Salzberg, 2012). Metagenomic binning was conducted with both MetaBAT2 v2.15 (Kang *et al.*, 2019) and BinSanity v0.5.3 (Graham *et al.*, 2017). MAGs obtained with both methods were dereplicated with DASTool v1.1.2 (Sieber *et al.*, 2018), resulting in 401 MAGs with less than 10% contamination and completeness higher than 50% as determined with CheckM (Parks *et al.*, 2015). Using GTDB-Tk v1.5.0 (Chaumeil *et al.*, 2020) with the GTDB 06-RS202 taxonomy (Parks *et al.*, 2018, 2020), seven MAGs were classified as *Ca. Brocadiales*. The relative abundance of MAGs was estimated with coverM v0.6.1 (<https://github.com/wwood/CoverM>) with the *relative\_abundance* parameter in *genome* mode using BWA-MEM (Li, 2013).

MAGs were annotated with DRAM v1.0 (Shaffer *et al.*, 2020) with default options, except *-min\_contig\_size* 1000, and genes of interest were searched in annotation files. While the gene encoding hydrazine synthase subunit A (*hzsA*) was annotated via the PFAM hit to PF18582, subunits B and C, present downstream, were identified via BLASTP analyses and manual inspection of CxxCH motifs. Genes encoding proteins involved in the anammox electron transport chain were searched both via annotation files and via BLASTP using previously identified reference sequences from *Ca. Kuenenia stuttgartiensis* (de Almeida *et al.*, 2016; Kartal and Keltjens, 2016).

A UBCG v3.0 (Na *et al.*, 2018) phylogenetic tree with 92 genes was constructed to place the MAGs. For that, reference genomes were downloaded from NCBI, and after checkM v1.1.3 inspection, 56 high quality (>90% complete and <5% contaminated) reference genomes were selected. Average AAI between selected genomes was calculated using the Kostas Lab tool (<http://enve-omics.ce.gatech.edu/g-matrix/index>). For this, genomes were gene-called with Prodigal v2.6.3 (Hyatt *et al.*, 2010), and amino acid fasta files were used as input.

The identification of organisms related to the MAGs in this study in other databases deposited on NCBI was attempted via BLASTP using as query sequences the ribosomal protein S3 (RpsC) and hydroxylamine dehydrogenase (HDH) in this study's MAGs, and via BLASTN using as query sequences fragments of the 16S rRNA gene extracted with SSU-align v0.1.1 (Nawrocki, 2009). For HDH, identified and reference amino acid sequences were aligned with MAFFT v7.487 using the L-INS-i option (Katoh and Standley, 2013). This was used to construct a maximum likelihood tree with IQ-TREE v2.1.4 (Minh *et al.*, 2020), using 1000 replicates for bootstrap support with ultrafast bootstrap (Hoang *et al.*, 2018), and a WAG + R3 model chosen by ModelFinder (Kalyaanamoorthy *et al.*, 2017).

### Acknowledgements

The authors acknowledge support from the National Genomics Infrastructure in Stockholm funded by Science for Life Laboratory, the Knut and Alice Wallenberg Foundation and the Swedish Research Council, and SNIC/Uppsala Multi-disciplinary Center for Advanced Computational Science for assistance with massively parallel sequencing and access to the UPPMAX computational infrastructure. Assembly and binning were done with resources provided by SNIC through UPPMAX under the projects SNIC 2021-22-112 and 2021-23-111. M.S.M.J. and P.D.M. were supported by N.W.O./OCW SIAM 024002002 and ERC Synergy MARIX 854088; M.H. and C.S. were supported by FORMAS (2018-01423) and the University of Gothenburg; S.K., B.M.W., P.H. and F.P. were supported by Future Infrastructures, a research agreement between Chalmers and the Norwegian Public Road Administration.

### Data Availability

MAGs from this study have been deposited into NCBI (accession numbers: JAIQZO000000000, JAIQZQ000000000, JAIQZP000000000). Metagenome reads and other MAGs from the Oslofjord tunnel biofilms can be found in the BioProject PRJNA755678. Annotated gene and protein files, as well as the DRAM annotation spreadsheet and other DRAM output files, have been deposited in Zenodo (DOI:10.5281/zenodo.5524859), available at <https://zenodo.org/record/5524859#.Yg5RqJTMJaQ>.

### References

- Backer, L., and Blindheim, O.T. (1999) The Oslofjord subsea road tunnel. Crossing a weakness zone under high water pressure by freezing. In *Proceedings of the World Tunnel Congress: Challenges of the 21st Century*, Alten, T., Backer, L., Bollingmo, P., Broch, E., Holmøy, K., Holter, K. G., and Nielsen, K. (eds). Oslo, Norway: Balkema, pp. 309–316.
- Bond, p.L., Smriga, S.P., and Banfield, J.F. (2000) Phylogeny of microorganisms populating a thick, subaerial, predominantly lithotrophic biofilm at an extreme acid mine drainage site. *Appl Environ Microbiol* **66**: 3842–3849.
- Chaumeil, P.-A., Mussig, A.J., Hugenholtz, P., and Parks, D. H. (2020) GTDB-Tk: a toolkit to classify genomes with the Genome Taxonomy Database. *Bioinformatics* **36**: 1925–1927.
- Cherney, M.M., Zhang, Y., Solomonson, M., Weiner, J.H., and James, M.N.G. (2010) Crystal structure of sulfide:Quinone oxidoreductase from *Acidithiobacillus ferrooxidans*: insights into sulfidotrophic respiration and detoxification. *J Mol Biol* **398**: 292–305.
- Chicano, T.M., Dietrich, L., de Almeida, N.M., Akram, M., Hartmann, E., Leidreiter, F., *et al.* (2021) Structural and functional characterization of the intracellular filament-forming nitrite oxidoreductase multiprotein complex. *Nat Microbiol* **6**: 1129–1139.
- Dang, H., Chen, R., Wang, L., Lizhong, G., Chen, P., Tang, Z., *et al.* (2010) Environmental factors shape sediment anammox bacterial communities in hypernutrified Jiaozhou Bay, China. *Appl Environ Microbiol* **76**: 7036–7047.
- de Almeida, N.M., Wessels, H.J.C.T., de Graaf, R.M., Ferousi, C., Jetten, M.S.M., Keltjens, J.T., and Kartal, B. (2016) Membrane-bound electron transport systems of an anammox bacterium: a complexome analysis. *Biochim Biophys Acta Bioenerg* **1857**: 1694–1704.
- Devol, A.H. (2015) Denitrification, anammox, and N<sub>2</sub> production in marine sediments. *Ann Rev Mar Sci* **7**: 403–423.
- Ferousi, C., Schmitz, R.A., Maalcke, W.J., Lindhoud, S., Versantvoort, W., Jetten, M.S.M., *et al.* (2021) Characterization of a nitrite-reducing octaheme hydroxylamine oxidoreductase that lacks the tyrosine cross-link. *J Biol Chem* **296**: 100476.
- Giuffrè, A., Borisov, V.B., Arese, M., Sarti, P., and Forte, E. (2014) Cytochrome bd oxidase and bacterial tolerance to oxidative and nitrosative stress. *Biochim Biophys Acta Bioenerg* **1837**: 1178–1187.
- Glud, R.N. (2008) Oxygen dynamics of marine sediments. *Mar Biol Res* **4**: 243–289.
- Graham, E.D., Heidelberg, J.F., and Tully, B.J. (2017) BinSanity: unsupervised clustering of environmental microbial assemblies using coverage and affinity propagation. *PeerJ* **5**: e3035.
- Gronhaug, A. (1978) Requirements of geological studies for undersea tunnels. In *Rock Mechanics*, Müller, L. (ed). Vienna: Springer, pp. 27–40.
- Gruber-Vodicka, H.R., Seah, B.K.B., and Pruesse, E. (2020) phyloFlash: rapid small-subunit rRNA profiling and targeted assembly from metagenomes. *mSystems* **5**: e00920-20.

- Guan, Y., Hikmawan, T., Antunes, A., Ngugi, D., and Stingl, U. (2015) Diversity of methanogens and sulfate-reducing bacteria in the interfaces of five deep-sea anoxic brines of the Red Sea. *Res Microbiol* **166**: 688–699.
- Hagelia, P. (2007) Sprayed concrete deterioration influenced by saline ground water and Mn- and Fe-biomineralisation in subsea road tunnel. In *Mechanical Effects on Reactive Systems. The 20th Kongsberg Seminar*, Jamtveit, B. (ed). Norway: Kongsberg, p. 26.
- Hagelia, P. (2011) Deterioration mechanisms and durability of sprayed concrete for rock support in tunnels. PhD Thesis. Delft, the Netherlands: TU Delft.
- Haverkamp, T.H.A., Hammer, Ø., and Jakobsen, K.S. (2014) Linking geology and microbiology: inactive pockmarks affect sediment microbial community structure. *PLoS One* **9**: e85990.
- Hinsley, A.P., and Berks, B.C. (2002) Specificity of respiratory pathways involved in the reduction of sulfur compounds by *Salmonella enterica*. *Microbiology* **148**: 3631–3638.
- Hira, D., Toh, H., Migita, C.T., Okubo, H., Nishiyama, T., Hattori, M., et al. (2012) Anammox organism KSU-1 expresses a NirK-type copper-containing nitrite reductase instead of a NirS-type with cytochrome cd1. *FEBS Lett* **586**: 1658–1663.
- Hoang, D.T., Chernomor, O., von Haeseler, A., Minh, B.Q., and Vinh, L.S. (2018) UFBoot2: improving the ultrafast bootstrap approximation. *Mol Biol Evol* **35**: 518–522.
- Hoshino, T., and Inagaki, F. (2019) Abundance and distribution of archaea in the seafloor sedimentary biosphere. *ISME J* **13**: 227–231.
- Hu, Z., Wessels, H.J.C.T., van Alen, T., Jetten, M.S.M., and Kartal, B. (2019) Nitric oxide-dependent anaerobic ammonium oxidation. *Nat Commun* **10**: 1244.
- Hug, L.A., Baker, B.J., Anantharaman, K., Brown, C.T., Probst, A.J., Castelle, C.J., et al. (2016) A new view of the tree of life. *Nat Microbiol* **1**: 16048.
- Hyatt, D., Chen, G.-L., LoCascio, p.F., Land, M.L., Larimer, F.W., and Hauser, L.J. (2010) Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics* **11**: 119.
- Inagaki, F., Hinrichs, K.-U., Kubo, Y., Bowles, M.W., Heuer, V.B., Hong, W.-L., et al. (2015) Exploring deep microbial life in coal-bearing sediment down to ~2.5 km below the ocean floor. *Science* **349**: 420–424.
- Jepson, B.J.N., Mohan, S., Clarke, T.A., Gates, A.J., Cole, J. A., Butler, C.S., et al. (2007) Spectropotentiometric and structural analysis of the periplasmic nitrate reductase from *Escherichia coli*. *J Biol Chem* **282**: 6425–6437.
- Jetten, M.S.M., den Camp, H.J.M.O., Kuenen, J.G., and Strous, M. (2010) “Candidatus Brocadiaaceae” fam. nov. In *Bergey’s Manual of Systematics of Archaea and Bacteria*, Krieg, N.R., Staley, J.T., Brown, D.R., Hedlund, B.P., and Paster, B.J. (eds). New York: Springer, pp. 918–925.
- Jetten, M.S.M., Horn, S.J., and van Loosdrecht, M.C.M. (1997) Towards a more sustainable municipal wastewater treatment system. *Water Sci Technol* **35**: 171–180.
- Kallmeyer, J., Pockalny, R., Adhikari, R.R., Smith, D.C., and D’Hondt, S. (2012) Global distribution of microbial abundance and biomass in seafloor sediment. *Proc Natl Acad Sci U S A* **109**: 16213–16216.
- Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., von Haeseler, A., and Jermini, L.S. (2017) ModelFinder: fast model selection for accurate phylogenetic estimates. *Nat Methods* **14**: 587–589.
- Kang, D.D., Li, F., Kirton, E., Thomas, A., Egan, R., An, H., and Wang, Z. (2019) MetaBAT 2: an adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. *PeerJ* **7**: e7359.
- Kappler, U., Bennett, B., Rethmeier, J., Schwarz, G., Deutzmann, R., McEwan, A.G., and Dahl, C. (2000) Sulfite: cytochrome c oxidoreductase from *Thiobacillus novellus*: purification, characterization, and molecular biology of a heterodimeric member of the sulfite oxidase family. *J Biol Chem* **275**: 13202–13212.
- Karačić, S., Wilén, B.-M., Suarez, C., Hagelia, P., and Persson, F. (2018) Subsea tunnel reinforced sprayed concrete subjected to deterioration harbours distinct microbial communities. *Biofouling* **34**: 1161–1174.
- Kartal, B., de Almeida, N.M., Maalcke, W.J., Op den Camp, H.J.M., Jetten, M.S.M., and Keltjens, J.T. (2013) How to make a living from anaerobic ammonium oxidation. *FEMS Microbiol Rev* **37**: 428–461.
- Kartal, B., and Keltjens, J.T. (2016) Anammox biochemistry: a tale of heme c proteins. *Trends Biochem Sci* **41**: 998–1011.
- Kartal, B., Maalcke, W.J., de Almeida, N.M., Cirpus, I., Gloerich, J., Geerts, W., et al. (2011) Molecular mechanism of anaerobic ammonium oxidation. *Nature* **479**: 127–130.
- Kartal, B., Rattray, J., van Niftrik, L.A., van de Vossenberg, J., Schmid, M.C., Webb, R.I., et al. (2007) Candidatus “Anammoxoglobus propionicus” a new propionate oxidizing species of anaerobic ammonium oxidizing bacteria. *Syst Appl Microbiol* **30**: 39–49.
- Kartal, B., Van Niftrik, L., Rattray, J., Van De Vossenberg, J. L.C.M., Schmid, M.C., Sinnighe Damsté, J., et al. (2008) Candidatus ‘Brocadia fulgida’: an autofluorescent anaerobic ammonium oxidizing bacterium. *FEMS Microbiol Ecol* **63**: 46–55.
- Katoh, K., and Standley, D.M. (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* **30**: 772–780.
- Kuypers, M.M.M., Marchant, H.K., and Kartal, B. (2018) The microbial nitrogen-cycling network. *Nat Rev Microbiol* **16**: 263–276.
- Kuypers, M.M.M., Sliekers, A.O., Lavik, G., Schmid, M., Jørgensen, B.B., Kuenen, J.G., et al. (2003) Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. *Nature* **422**: 608–611.
- Lackner, S., Gilbert, E.M., Vlaeminck, S.E., Joss, A., Horn, H., and van Loosdrecht, M.C.M. (2014) Full-scale partial nitrification/anammox experiences – An application survey. *Water Res* **55**: 292–303.
- Langmead, B., and Salzberg, S.L. (2012) Fast gapped-read alignment with bowtie 2. *Nat Methods* **9**: 357–359.
- Lawson, C.E., Nuijten, G.H.L., de Graaf, R.M., Jacobson, T. B., Pabst, M., Stevenson, D.M., et al. (2021) Autotrophic and mixotrophic metabolism of an anammox bacterium revealed by in vivo <sup>13</sup>C and <sup>2</sup>H metabolic network mapping. *ISME J* **15**: 673–687.
- Li, D., Liu, C.-M., Luo, R., Sadakane, K., and Lam, T.-W. (2015) MEGAHIT: an ultra-fast single-node solution for

- large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics* **31**: 1674–1676.
- Li, H. (2013) Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM arXiv:13033997 [q-bio].
- Luo, C., Rodriguez-R, L.M., and Konstantinidis, K.T. (2014) MyTaxa: an advanced taxonomic classifier for genomic and metagenomic sequences. *Nucleic Acids Res* **42**: e73.
- Ma, K., Schicho, R.N., Kelly, R.M., and Adams, M.W. (1993) Hydrogenase of the hyperthermophile *Pyrococcus furiosus* is an elemental sulfur reductase or sulfhydrogenase: evidence for a sulfur-reducing hydrogenase ancestor. *Proc Natl Acad Sci U S A* **90**: 5341–5344.
- Mardanov, A.V., Beletsky, A.V., Ravin, N.V., Botchkova, E. A., Litt, Y.V., and Nozhevnikova, A.N. (2019) Genome of a novel bacterium “*Candidatus Jettenia ecosi*” reconstructed from the metagenome of an anammox bioreactor. *Front Microbiol* **10**: 2442.
- Minh, B.Q., Schmidt, H.A., Chernomor, O., Schrempf, D., Woodhams, M.D., von Haeseler, A., and Lanfear, R. (2020) IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. *Mol Biol Evol* **37**: 1530–1534.
- Mulder, A., van de Graaf, A.A., Robertson, L.A., and Kuenen, J.G. (1995) Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. *FEMS Microbiol Ecol* **16**: 177–183.
- Na, S.-I., Kim, Y.O., Yoon, S.-H., Ha, S., Baek, I., and Chun, J. (2018) UBCG: up-to-date bacterial core gene set and pipeline for phylogenomic tree reconstruction. *J Microbiol* **56**: 280–285.
- Narita, Y., Zhang, L., Kimura, Z., Ali, M., Fujii, T., and Okabe, S. (2017) Enrichment and physiological characterization of an anaerobic ammonium-oxidizing bacterium ‘*Candidatus Brocadia sapporoensis*’. *Syst Appl Microbiol* **40**: 448–457.
- Nawrocki, E. (2009) Structural RNA homology search and alignment using covariance models. PhD Dissertation.
- Okubo, T., Toyoda, A., Fukuhara, K., Uchiyama, I., Harigaya, Y., Kuroiwa, M., et al. (2021) The physiological potential of anammox bacteria as revealed by their core genome structure. *DNA Res* **28**: dsaa028.
- Oshiki, M., Mizuto, K., Kimura, Z., Kindaichi, T., Satoh, H., and Okabe, S. (2017) Genetic diversity of marine anaerobic ammonium-oxidizing bacteria as revealed by genomic and proteomic analyses of ‘*Candidatus Scalindua japonica*’. *Environ Microbiol Rep* **9**: 550–561.
- Oshiki, M., Shinyako-Hata, K., Satoh, H., and Okabe, S. (2015) Draft Genome Sequence of an Anaerobic Ammonium-Oxidizing Bacterium, “*Candidatus Brocadia sinica*”. *Genome Announc* **3**: e00267-15.
- Park, H., Brotto, A.C., van Loosdrecht, M.C.M., and Chandran, K. (2017) Discovery and metagenomic analysis of an anammox bacterial enrichment related to *Candidatus “Brocadia caroliniensis”* in a full-scale glycerol-fed nitrification-denitrification separate centrate treatment process. *Water Res* **111**: 265–273.
- Parkes, R.J., Cragg, B.A., Bale, S.J., Getliff, J.M., Goodman, K., Rochelle, P.A., et al. (1994) Deep bacterial biosphere in Pacific Ocean sediments. *Nature* **371**: 410–413.
- Parks, D.H., Chuvochina, M., Chaumeil, P.-A., Rinke, C., Mussig, A.J., and Hugenholtz, P. (2020) A complete domain-to-species taxonomy for bacteria and archaea. *Nat Biotechnol* **38**: 1079–1086.
- Parks, D.H., Chuvochina, M., Waite, D.W., Rinke, C., Skarshewski, A., Chaumeil, P.-A., and Hugenholtz, P. (2018) A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life. *Nat Biotechnol* **36**: 996–1004.
- Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., and Tyson, G.W. (2015) CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res* **25**: 1043–1055.
- Pinchuk, G.E., Rodionov, D.A., Yang, C., Li, X., Osterman, A.L., Dervyn, E., et al. (2009) Genomic reconstruction of *Shewanella oneidensis* MR-1 metabolism reveals a previously uncharacterized machinery for lactate utilization. *Proc Natl Acad Sci U S A* **106**: 2874–2879.
- Prokopenko, M.G., Hirst, M.B., De Brabandere, L., Lawrence, D.J.P., Berelson, W.M., Granger, J., et al. (2013) Nitrogen losses in anoxic marine sediments driven by *Thioploca*–anammox bacterial consortia. *Nature* **500**: 194–198.
- Quan, Z.-X., Rhee, S.-K., Zuo, J.-E., Yang, Y., Bae, J.-W., Park, J.R., et al. (2008) Diversity of ammonium-oxidizing bacteria in a granular sludge anaerobic ammonium-oxidizing (anammox) reactor. *Environ Microbiol* **10**: 3130–3139.
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* **41**: D590–D596.
- Rinke, C., Schwientek, P., Sczyrba, A., Ivanova, N.N., Anderson, I.J., Cheng, J.-F., et al. (2013) Insights into the phylogeny and coding potential of microbial dark matter. *Nature* **499**: 431–437.
- Russ, L., Harhangi, H.R., Schellekens, J., Verdellen, B., Kartal, B., den Camp, H.J.M.O., and Jetten, M.S.M. (2012) Genome analysis and heterologous expression of acetate-activating enzymes in the anammox bacterium *Kuenenia stuttgartiensis*. *Arch Microbiol* **194**: 943–948.
- Schäfer, T., Selig, M., and Schönheit, P. (1993) Acetyl-CoA synthetase (ADP forming) in archaea, a novel enzyme involved in acetate formation and ATP synthesis. *Arch Microbiol* **159**: 72–83.
- Schmidt, M., and Schönheit, P. (2013) Acetate formation in the photoheterotrophic bacterium *Chloroflexus aurantiacus* involves an archaeal type ADP-forming acetyl-CoA synthetase isoenzyme I. *FEMS Microbiol Lett* **349**: 171–179.
- Schwede, T.F., Rétey, J., and Schulz, G.E. (1999) Crystal structure of histidine ammonia-lyase revealing a novel polypeptide modification as the catalytic electrophile. *Biochemistry* **38**: 5355–5361.
- Shaffer, M., Borton, M.A., McGivern, B.B., Zayed, A.A., La Rosa, S.L., Solden, L.M., et al. (2020) DRAM for distilling microbial metabolism to automate the curation of microbiome function. *Nucleic Acids Res* **48**: 8883–8900.
- Shaw, D.R., Ali, M., Katuri, K.P., Gralnick, J.A., Reimann, J., Mesman, R., et al. (2020) Extracellular electron transfer-dependent anaerobic oxidation of ammonium by anammox bacteria. *Nat Commun* **11**: 2058.

- Sieber, C.M.K., Probst, A.J., Sharrar, A., Thomas, B.C., Hess, M., Tringe, S.G., and Banfield, J.F. (2018) Recovery of genomes from metagenomes via a dereplication, aggregation and scoring strategy. *Nat Microbiol* **3**: 836–843.
- Sjöberg, S., Stairs, C.W., Allard, B., Homa, F., Martin, T., Sjöberg, V., *et al.* (2020) Microbiomes in a manganese oxide producing ecosystem in the Ytterby mine, Sweden: impact on metal mobility. *FEMS Microbiol Ecol* **96**: fiae169.
- Sonthiphand, P., Hall, M.W., and Neufeld, J.D. (2014) Biogeography of anaerobic ammonia-oxidizing (anammox) bacteria. *Front Microbiol* **5**: 399.
- Speth, D.R., In 't Zandt, M.H., Guerrero-Cruz, S., Dutilh, B.E., and Jetten, M.S.M. (2016) Genome-based microbial ecology of anammox granules in a full-scale wastewater treatment system. *Nat Commun* **7**: 11172.
- Speth, D.R., Lagkourados, I., Wang, Y., Qian, P.-Y., Dutilh, B.E., and Jetten, M.S.M. (2017) Draft genome of *Scalindua rubra*, obtained from the interface above the discovery deep brine in the Red Sea, sheds light on potential salt adaptation strategies in anammox bacteria. *Microb Ecol* **74**: 1–5.
- Steinegger, M., Mirdita, M., and Söding, J. (2019) Protein-level assembly increases protein sequence recovery from metagenomic samples manifold. *Nat Methods* **16**: 603–606.
- Strous, M., Fuerst, J.A., Kramer, E.H.M., Logemann, S., Muyzer, G., van de Pas-Schoonen, K.T., *et al.* (1999) Missing lithotroph identified as new planctomycete. *Nature* **400**: 446–449.
- Strous, M., Pelletier, E., Mangenot, S., Rattei, T., Lehner, A., Taylor, M.W., *et al.* (2006) Deciphering the evolution and metabolism of an anammox bacterium from a community genome. *Nature* **440**: 790–794.
- Suarez, C., Piculell, M., Modin, O., Langenheder, S., Persson, F., and Hermansson, M. (2019) Thickness determines microbial community structure and function in nitrifying biofilms via deterministic assembly. *Sci Rep* **9**: 5110.
- van de Graaf, A.A., de Bruijn, P., Robertson, L.A., Jetten, M.S.M., and Kuenen, J.G. (1996) Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor. *Microbiology* **142**: 2187–2196.
- van de Graaf, A.A., Mulder, A., de Bruijn, P., Jetten, M., Robertson, L.A., and Kuenen, J.G. (1995) Anaerobic oxidation of ammonium is a biologically mediated process. *Appl Environ Microbiol* **61**: 1246–1251.
- van de Vossenberg, J., Woebken, D., Maalcke, W.J., Wessels, H.J.C.T., Dutilh, B.E., Kartal, B., *et al.* (2013) The metagenome of the marine anammox bacterium 'Candidatus *Scalindua profunda*' illustrates the versatility of this globally important nitrogen cycle bacterium. *Environ Microbiol* **15**: 1275–1289.
- van der Star, W.R.L., van de Graaf, M.J., Kartal, B., Picioreanu, C., Jetten, M.S.M., and van Loosdrecht, M.C.M. (2008) Response of anaerobic ammonium-oxidizing bacteria to hydroxylamine. *Appl Environ Microbiol* **74**: 4417–4426.
- van Dongen, U., Jetten, M.S.M., and van Loosdrecht, M.C.M. (2001) The SHARON®-Anammox® process for treatment of ammonium rich wastewater. *Water Sci Technol* **44**: 153–160.
- VanOrsdel, C.E., Bhatt, S., Allen, R.J., Brenner, E.P., Hobson, J.J., Jamil, A., *et al.* (2013) The *Escherichia coli* CydX protein is a member of the CydAB cytochrome bd oxidase complex and is required for cytochrome bd oxidase activity. *J Bacteriol* **195**: 3640–3650.
- Woebken, D., Lam, P., Kuypers, M.M.M., Naqvi, S.W.A., Kartal, B., Strous, M., *et al.* (2008) A microdiversity study of anammox bacteria reveals a novel *Candidatus Scalindua* phylotype in marine oxygen minimum zones. *Environ Microbiol* **10**: 3106–3119.
- Wolfe, A.J. (2005) The acetate switch. *Microbiol Mol Biol Rev* **69**: 12–50.
- Yamanaka, T., and Shinra, M. (1974) Cytochrome c-552 and cytochrome c-554 derived from *Nitrosomonas europaea*: purification, properties, and their function in hydroxylamine oxidation. *J Biochem* **75**: 1265–1273.
- Yang, Y., Li, M., Li, H., Li, X.-Y., Lin, J.-G., Denecke, M., and Gu, J.-D. (2020) Specific and effective detection of anammox bacteria using PCR primers targeting the 16S rRNA gene and functional genes. *Sci Total Environ* **734**: 139387.
- Zhao, R., Mogollón, J.M., Abby, S.S., Schleper, C., Biddle, J. F., Roerdink, D.L., *et al.* (2020) Geochemical transition zone powering microbial growth in subsurface sediments. *Proc Natl Acad Sci U S A* **117**: 32617–32626.
- Ziegler, C., Bremer, E., and Krämer, R. (2010) The BCCT family of carriers: from physiology to crystal structure. *Mol Microbiol* **78**: 13–34.

## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Appendix S1.** Supporting Information.