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Spatial and temporal dynamics of ammonia oxidizers in the sediments of the Gulf of Finland, Baltic Sea

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Abstract

The diversity and dynamics of ammonia-oxidizing bacteria (AOB) and archaea (AOA) nitrifying communities in the sediments of the eutrophic Gulf of Finland (GoF) were investigated. Using clone libraries of ammonia monooxygenase (*amoA*) gene fragments and terminal restriction fragment length polymorphism (TRFLP), we found a low richness of both AOB and AOA. The AOB *amoA* phylogeny matched that of AOB 16S ribosomal genes from the same samples. AOA communities were characterized by strong spatial variation while AOB communities showed notable temporal patterns. At open sea sites, where transient anoxic conditions prevail, richness of both AOA and AOB was lowest and communities were dominated by organisms with gene signatures unique to the GoF. Given the importance of nitrification as a link between the fixation of nitrogen and its removal from aquatic environments, the low diversity of ammonia-oxidizing microbes across the GoF could be of relevance for ecosystem resilience in the face of rapid global environmental changes.

Authors' contribution

All authors contributed extensively to the work presented in this paper. SH and EL conceived the study. AV designed the sampling strategy, carried out the molecular work, analysed the data and prepared the manuscript. SH helped collecting the samples and collected the environmental data. EL and SH participated in the analysis and interpretation of the data as well as writing of the manuscript.

1 Introduction

Eutrophication is threatening coastal areas and estuaries globally and may lead to hypoxia and anoxia across vast areas (Diaz and Rosenberg, 2008). Concomitant with eutrophication, raising nutrient concentrations and changes in nitrogen cycling have been identified as a major concern for global ecosystems (Rockstrom et al., 2009). In the Baltic Sea, one of the largest brackish water bodies in the world, annual inputs of inorganic nitrogen from rivers have been averaging 500 kt over the last decade (Helsinki Commission, 2010). Eutrophication is strongest in the easternmost sub-basin, the Gulf of Finland (GoF), which is under heavy pressure from the Neva river runoffs (Kuuppo et al., 2006). The deepest areas of the GoF lie under a semi-permanent halocline extending from the Baltic Proper and the bottom waters are often anoxic (Conley et al., 2009). Coastal areas in the GoF do not suffer from such extreme oxygen conditions, but high primary productivity in summer can lead to hypoxia near the sediment and interfere with nitrification, which controls the extent of nitrogen losses through denitrification (Jäntti et al., 2011), accounting for the removal of a third of the nitrogen input to the GoF (Hietanen and Kuparinen, 2008).

Nitrification is mediated by several groups of microbes comprising both bacterial (Purkhold et al., 2000) and archaeal taxa (Brochier-Armanet et al., 2008). These microbes gain energy for their metabolism by oxidizing ammonia to nitrite in a two-step reaction that requires oxygen to proceed. Nitrite oxidizing bacteria constitute the second portion of the nitrification process, where the nitrite produced by ammonia oxidizers is converted to nitrate. Nitrate and nitrite can subsequently be reduced by nitrate-respiring microbes as well as other microorganism and removed from aquatic ecosystems as nitrogenous gases in the anammox and denitrification pathways (Knowles, 1982, Schmid et al., 2007).

Ammonia-oxidizing organisms are restricted in their metabolic capabilities, and they are controlled by a relatively limited set of variables with major effects from temperature,

salinity and substrate availability (Bouskill et al., 2012). Therefore the variation in community structure is rather predictable in estuaries, where steep environmental gradients prevail (Bernhard and Bollmann, 2010). Similar to estuarine environments, the GoF is brackish. However, the conditions in GoF do not display oscillations in physico-chemical variables typical to estuaries with direct contact to oceans. Instead, there are substantial seasonal changes in temperature, light, and nutrient concentrations. Furthermore, the presence of a semi-permanent halocline in the depth of 60 – 80 m prevents complete mixing of the water column, causing long-term hypoxia and even anoxia in the open sea sediments (Wulff et al., 1990). In the southern basins of the Baltic Sea, ammonia-oxidizing assemblages are phylogenetically related to those found in estuaries (Nicolaisen and Ramsing, 2002, Kim et al., 2008, Junier et al., 2009), but they remain uncharacterized in the sediments of northern basins such as the GoF.

Our aims were to study the diversity and factors controlling the community composition of betaproteobacterial ammonia-oxidizing bacteria (referred thereafter as AOB) and ammonia-oxidizing archaea (AOA) using the *amoA* gene, which encodes the α subunit of ammonia monooxygenase, the enzyme that catalyzes the first step in the oxidation of ammonia in both groups, as a phylogenetic marker. We used previously published pyrosequencing data to compare the *amoA* and the 16S ribosomal gene phylogeny for the same samples (Vetterli et al., 2015). We also investigated the factors affecting the community composition of AOB and AOA in time and space by using terminal restriction fragment length polymorphism (TRFLP) of *amoA* gene fragments.

2 Materials and methods

2.1 Sampling and study area

The sampling area covered the western and central part of the GoF, with two coastal sites on the south-western coast of Finland, 5 sites at mid-range between the coastal zones and the open sea area and 5 sites in the deeper, open sea area (Fig. 1). Of the coastal sites, Muncken (M) is a shallow (11 m) transportation area where sediments are composed of soft mud and fine-grained sand. The second site, Storfjärden (S), is a deeper accumulation basin with soft mud sediment. The open sea samples were collected from five stations along the deep east–west transect in the middle of the GoF (Fig. 1, LL9, LL1, GF1, GF2, XV-1). The rest of the samples were taken from four stations southeast of Helsinki at mid-range between the coastal and open sea, with varying bottom oxygen conditions (Fig. 1, ST1-5, Table 1).

For molecular analyses, intact sediment samples were taken using a Gemax twin corer (Ø 9 cm). Three cores were taken at each sampling site and time. The mid-range and open sea sites were sampled in May and June 2008 while the two coastal sites were sampled in April, August, and November 2008 and April, August and December 2009 (Table 1). The samples from the coastal sites collected in April are referred to as 'Spring samples', the samples collected in August as 'Summer samples' and the ones collected in late November and early December are referred to as 'Autumn samples'. Five subsamples (1 ml) from the sediment surface (0.5 cm) were transferred into micro centrifuge tubes using cut-off pipet tips. The rest of the surface layer was sliced to a polypropylene bag. Samples were placed on dry ice until they could be stored in a deep freezer (within two hours of sampling).

2.2 Physical and chemical analyses

Temperature and salinity were recorded using a conductivity, temperature and depth device (SiS CTD plus 100, SiS Sensoren Instrumente Systeme, Schwentinental, Germany and SeaBird 911 Plus, Sea-Bird Electronics Inc, Bellevue, WA, USA). Oxygen, NO_x (combined nitrite and nitrate), and NH_4^+ (ammonium) concentrations in the bottom water were measured

from water samples collected 5 cm above the sediment surface. Oxygen concentration was determined by titration. NH_4^+ and NOx were measured by ion chromatography (Lachat QuickChem, Hach Company, Loveland, Colorado, United States of America (USA)). The amount of carbon and nitrogen in the samples were determined from 4 mg of homogenized and dried sediment using an element analyzer (TruSpec Micro, LECO Corporation, St. Joseph, Michigan, USA), the data is presented as the ratio (C:N) of their masses. Sediment organic content was determined as loss on ignition (LOI) by combusting desiccated subsamples (105°C, 12 h) of the top 1 cm slice at 550°C for 3 hours.

2.3 DNA extraction

Each 1 ml subsample was briefly centrifuged to remove water before processing. Deoxyribonucleic Acid (DNA) was extracted using the Hexadecyltrimethylammonium bromide (CTAB) method as modified by Griffiths (Griffiths et al., 2000). Concentration and purity of the DNA were determined by spectrometry (Nanodrop, Thermo Scientific, Wilmington, Delaware, USA). Triplicate DNA samples from all open sea sites and both coastal sites from spring 2008 were normalized and combined into DNA pools to build clone libraries.

2.4 PCR amplification of *amoA* and clone libraries

AOB *amoA* gene fragments of 491 bp were amplified by polymerase chain reaction (PCR) optimizing the cycling conditions for the DyNAzyme II polymerase (Thermo Scientific) using the amoA-1F (5'-GGG GTT TCT ACT GGT GGT-3') and amoA-2R-TC (5'-CCC CTC TGCAAA GCC TTC TTC-3') primers (Rotthauwe et al., 1997, Nicolaisen and Ramsing, 2002). Briefly, reactions were done with 1.5 mM $MgCl_2$, 0.3 µM of each primer, 400 µM of nucleotides, 30-50 ng of template and 0.5 units of polymerase in a volume of 25 µl. The

cycling conditions for the amoA-1F and amoA-2R-TC primer pair were as following: an initial denaturation step at 94ºC for 2 min, followed by 25 cycles with denaturation at 94 ºC for 30 s, annealing at 57 ºC for 30 s, extension at 72 ºC for 45 s and a final extension step at 72 ºC for 5 min. AOA *amoA* fragments (635 bp) were amplified with the primers Arch-amoAF (5'-STA ATG GTC TGG CTT AGA CG-3') and Arch-amoAR (5'-GCG GCC ATC CAT CTG TAT GT-3') (Francis et al., 2005). AOA PCR reactions contained 1.5 mM $MgCl_2$, 0.3 µM of each primer, 400 µM of nucleotides and 0.5 units of polymerase in a volume of 25 µl. The cycling conditions were as following: an initial denaturation step at 94ºC for 2 min, followed by 25 cycles with denaturation at 94 ºC for 30 s, annealing at 53 ºC for 30 s, extension at 72 ºC for 50 s and a final extension step at 72 ºC for 5 min.

Single clone libraries were built for AOB and AOA *amoA* by using combined DNA extractions from all 2008 samples originating from both coastal and open sea sediments. Replicate PCR products were combined into a main PCR pool and cloned into the vector pDRIVE (Qiagen, Hilden, Germany). In total, 215 AOB and 106 AOA clones were sequenced using the vector primers SP6 and T7 (Qiagen) in a capillary sequencer (Applied Biosystems, Life Technologies, Carlsbad, California, USA).

2.5 TRFLP analysis

The software Cleaver (Jarman, 2006) was used to screen the clone sequences and identify the restriction enzymes that would allow the best resolution of the libraries. The *amoA* fragments for the analysis were amplified using 6-carboxyfluorescein (FAM) labeled primers at the 5′ terminus (FAM-amoA-1F and FAM-arch-amoAF) in the same PCR as described above except that 28 cycles were used. Triplicate DNA extractions were combined and used as template and only the labeled primers were present and 1 unit of polymerase was used in each reaction. Purified labeled PCR products from four independent PCR were combined in pairs and

digested with the restriction enzyme BfaI for AOB *amoA* fragments and BseDI for AOA *amoA* fragments in two independent reactions according to instructions given by the manufacturer (Thermo Scientific) for 4 hours at 37°C. Digested samples and undigested controls were mixed with size standards (GeneScan-500 for AOB *amoA* and MapMarker1000 for AOA *amoA*) and run on an ABI Genetic Analyzer 3130XL according to instructions given by the manufacturer (Applied Biosystems). The results were visualized using the Peak Scanner software (Applied Biosystems) and peak heights of terminal restriction fragments (TRFs) with expected sizes were recorded manually. The clone libraries were subsequently used to identify the fragments found in the samples.

2.6 16S rDNA sequences of *Nitrosospira* and *Nitrosomonas*

In essence, the same DNA material that was used in the TRFLP analysis was used for next-generation sequencing but only coastal sites samples were used. A total of 12 samples were sequenced, for both Storfjärden and Muncken for two consecutive years and three different time point during each year. Fragments spanning the V1, V2 and V3 regions of bacterial 16S rRNA genes were amplified and sequenced using a 454 GS FLX titanium pyrosequencing platform (454 Life Sciences, Branford, CT, USA), and the pyrosequencing data were processed and quality filtered as described in Vetterli et al. (2015). The data included altogether 177 sequences belonging to *Nitrosospira* and *Nitrosomonas*. This 16S rDNA data was used for comparison with the results obtained using the functional gene *amoA*.

2.7 Phylogenetic analyses

Sequences were manually aligned using Jalview (Waterhouse et al., 2009). The Bellerophon software (Huber et al., 2004) identified 16 chimeric AOB sequences. No chimeric AOA sequences were found in the data and the final datasets contained 199 AOB and 106 AOA

clones. The program MOTHUR (Schloss et al., 2009) was used to select representative sequences for each *amoA* operational taxonomic unit (OTU) and to calculate the rarefaction curves. Phylogenetic trees were constructed from Olsen distance matrices using the ARB Neighbor-Joining method combined with bootstrapping (1000 replicates for *amoA* and 10000 replicates for 16S rDNA sequences) in ARB (Ludwig et al., 2004). OTUs were based on a 95% sequence identity for *amoA* genes and 97% for 16S rDNA genes. AOB ribosomal gene sequence fragments were obtained from 16S rDNA genes pyrosequencing libraries as described in (Vetterli et al., 2015).

2.8 Data ordination and statistical analyses

All analyses were conducted in R statistical software package. Non-metric multidimensional scaling (NMDS) was performed with the function metaMDS (with k=2) in the *vegan* package (Oksanen et al., 2010) on Bray-Curtis dissimilarity matrices calculated from averaged relative abundances of TRFs. Correlation of environmental variables, rates and ordination data was achieved with the ENVFIT function, which produces a goodness-of-fit correlation coefficient (r^2) and a p-value based on 999 permutations. The ENVFIT data are shown on each ordination plot by the means of arrows that indicate the direction and strength of the gradient for the environmental variables. For arrows with a significant correlation, the gradient runs perpendicularly to the arrow in a linear or near-linear fashion. The effects of location and sampling time on community composition were calculated from dissimilarity matrices of the raw data by ANOSIM with the function *anosim*. Significance with *anosim* was estimated from 199 permutations. Spearman correlation coefficients were calculated with the *rcorr* function.

2.9 Nucleotide sequences

The AOA and AOB *amoA* sequences reported here are available in GenBank under POPSET identification numbers 270272467 and 291360448, respectively. The *Nitrosomonadaceae* 16S ribosomal DNA sequences of are available under the GenBank identification numbers KT873457 to KT873461. The raw pyrosequencing dataset is available online at www.ebi.ac.uk under study accession number PRJEB7690.

3 Results

3.1 Physico-chemical characteristics

At both coastal stations (Fig. 1), salinity at the sediment-water interface was stable throughout the sampling period (5.75 ± 0.65 sd). Seasonal variation was pronounced for temperature and LOI values, both of which peaked during the summer sampling points at both sites. The sediment at the deeper site (Storfjärden) contained twice as much organic matter compared to the shallow site (Muncken) throughout the sampling period with 17.1±8.4 and 7.6±1.3% LOI, respectively. Inversely, O_2 and NO_x concentrations were lowest during summer. NH_4^+ showed more interannual variability with generally higher concentrations in 2008 than in 2009 and on average concentrations were highest at the deep site but never exceeded 6.2 µM (Table 1).

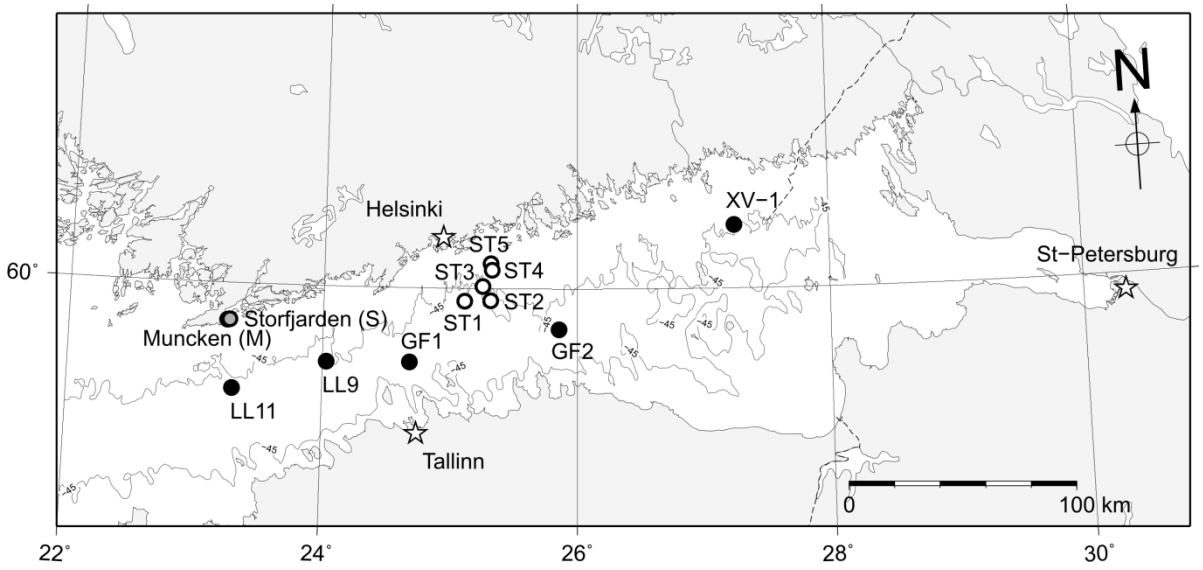


Figure 1. Sampling sites in the Gulf of Finland, Baltic Sea. The two coastal sites are show in grey. The mid-range sites , which span a bottom oxygen gradient, are in white and the open sea sites which stretch from the east of the GoF to the entrance of the Baltic proper are in black.

Site	Lat	Lon	Zone	Season	Date	Depth	T	S	O_2	NH_4^+	NO_x	LOI	C:N
S	59°51.31N	23°15.81E	coast	Spring	Apr-08	33	2.4	5.4	410	2.5	2.6	17.7	8.2
				Spring	Apr-09		0.8	5.3	393	0.1	6.9	13.2	8.7
				Summer	Aug-08		16.2	5.9	205	6.2	1	21	8.5
				Summer	Aug-09		5.8	6.6	178	1.8	3.5	31.6	10.5
				Autumn	Nov-08		6.4	6.5	424	0.3	5.3	10.8	8.4
				Autumn	Dec-09		7	6.3	290	1.4	4	8.6	9.1
M	59°51.14N	23°14.70E	coast	Spring	Apr-08	11	2.7	5.3	410	0	1.1	7.5	8.1
				Spring	Apr-09		1.7	5.1	424	0.2	4	5.2	8.8
				Summer	Aug-08		16.6	5.9	223	4.9	0.7	9.3	8.6
				Summer	Sep-09		14.3	5.8	267	1.4	2.2	7.8	6.9
				Autumn	Nov-08		6.3	6.5	360	0.2	5.4	7.6	8.4
				Autumn	Dec-09		5.5	4.5	360	0.1	4.3	8.6	9
ST1	59°56.96N	25°06.58E	mid		May-08	69	4.9	9.3	45	6.1	8.2	12.6	9.7
ST2	59°57.10N	25°19.00E	mid		May-08	63	4.4	8.7	112	6.5	2	10.9	8.9
ST3	59°58.93N	25°10.16E	mid		May-08	62	4.3	8.2	162	1.2	7.1	17.9	8.7
ST4	60°05.96N	25°18.97E	mid		May-08	46	3.5	7.2	210	2	2.8	12.3	8.9
ST5	60°04.43N	25°19.41E	mid		May-08	59	3.7	7.4	183	4.2	1.2	9.9	9.3
LL11	59°42.01N	24°01.81E	open		Jun-08	66	5.3	8.8	53	0.2	6	1.52	9.8
LL9	59°35.01N	23°17.81E	open		Jun-08	66	4.9	9.3	98	0	10.7	4.9	8.6
GF1	59°42.30N	24°40.93E	open		Jun-08	83	5.4	10	40	1.3	1.5	14.8	10.5
GF2	59°50.31N	25°51.41E	open		Jun-08	83	5.2	9.8	31	1.5	5.1	18.2	8.7
XV-1	60°15.00N	27°14.82E	open		Jun-08	64	3.5	6.9	241	6.7	8.7	23.8	8.4

Table 1. Physico-chemical variables for each sampling time. Depth in meters, temperature (T) in °C, Salinity (S), oxygen (O_2) in µM, NH_4^+ in µM, NO_x in µM and loss on ignition (LOI) in % and C:N stands for the carbon to nitrogen ratio.

	Depth	T	S	O_2	NH_4^+	NO_x	LOI	C:N
Depth		0.41	0	0	0.3	0.14	0.12	0.34
T	-0.19		0.78	0.43	0.47	0.3	0.89	0.59
S	**0.89**	0.06		0	0.33	0.31	0.4	0.07
O_2	**-0.8**	-0.18	**-0.9**		0.21	0.84	0.28	0.05
NH_4^+	0.24	0.17	0.22	-0.28		0.11	0	0.98
NO_3^-	0.33	-0.24	0.23	-0.05	-0.36		0.68	0.48
LOI	0.35	-0.03	0.2	-0.25	**0.64**	-0.1		0.23
C:N	0.22	0.13	0.4	**-0.4**	-0.01	-0.16	-0.27	

Table 2. Spearman correlation coefficients for environmental variables. The coefficients and their p-values are in the lower and upper portions of the matrix, respectively. Significant values are highlighted. Abbreviations for the variables are the same as those in Table 1.

At the mid-range and open sea sites (Fig. 1), many variables were depth-dependent (Table 2). The depth of the open sea sites correlated positively with salinity and negatively with oxygen concentration across all sites. This is in line the extent of the anoxic zone reported by HELCOM for the sampling years (Axe, 2010). Half of these sites had oxygen concentration below 100 µM. The easternmost site XV-1 had more oxygen (241 µM) than any other open sea site, yet it contained more NH_4^+ and organic matter. Overall, organic matter and NH_4^+ were well correlated (Table 2) because the former is the primary source of NH_4^+. At the deepest sites LL9, LL11, GF-1 and GF-2, the trend was not as sharp, probably because the rate of organic matter mineralization was limited by oxygen availability.

The extent of the halocline is a moving target in the GoF because it depends on inflows from the North Sea feeding the deep waters of the Baltic Proper and where it usually sits around 50-80 m of depth. At our sites, salinity was higher towards the mouth of the GoF in the west, up to 10 units, where bottom waters are in direct contact with the halocline of the Baltic Proper. According to water column salinity profiles (data not shown), sites GF1 and GF2, which are deep local basins, where below the halocline. Sites LL9, LL11 and ST1 were

either inside or just below the halocline. The lowest salinity was found at the easternmost site XV-1, closest to the Neva estuary. The mid-range sites ST1 to ST5 were characterized by overall higher oxygen concentration 5 cm above sediment (100-200 µM) but with salinity nearing the conditions found at the deep open sea sites.

3.2 Phylogenetic analysis of AOB *amoA* and 16S rDNA gene fragments

AOB clones isolated from sediments in the GoF were related to *Nitrosomonas* and *Nitrosospira* genera, with similarities to AOB commonly found in estuaries (Fig. 2). At a 95% sequence similarity threshold, 7 OTUs were identified in the AOB *amoA* clone library (Fig. 2). Four of these OTUs belonged to the *Nitrosomonas* genera and were all related to AOB found in other estuarine environments. *Nitrosomonas* sequences from the GoF shared up to 99% identity at the amino acid level with phylotypes isolated from the southern Baltic Sea (Nicolaisen and Ramsing, 2002, Risgaard-Petersen et al., 2004, Kim et al., 2008). Three OTUs belonged to the major 'Estuarine/marine' *Nitrosomonas*-like cluster first described by Francis and colleagues (2003), while the fourth OTU was associated with a cluster of estuarine sequences affiliated with *Nitrosomonas marina* and *Nitrosomonas aestuarii*. The latter OTU, which contained 5% of the AOB clones, formed the monophyletic group '*GoF Nitrosomonas-like*' among sequences from other estuaries (Fig. 2) and seemed to represent a novel *amoA* sequence type with a closest nucleotide identity of 90% with AOB sequences from estuaries.

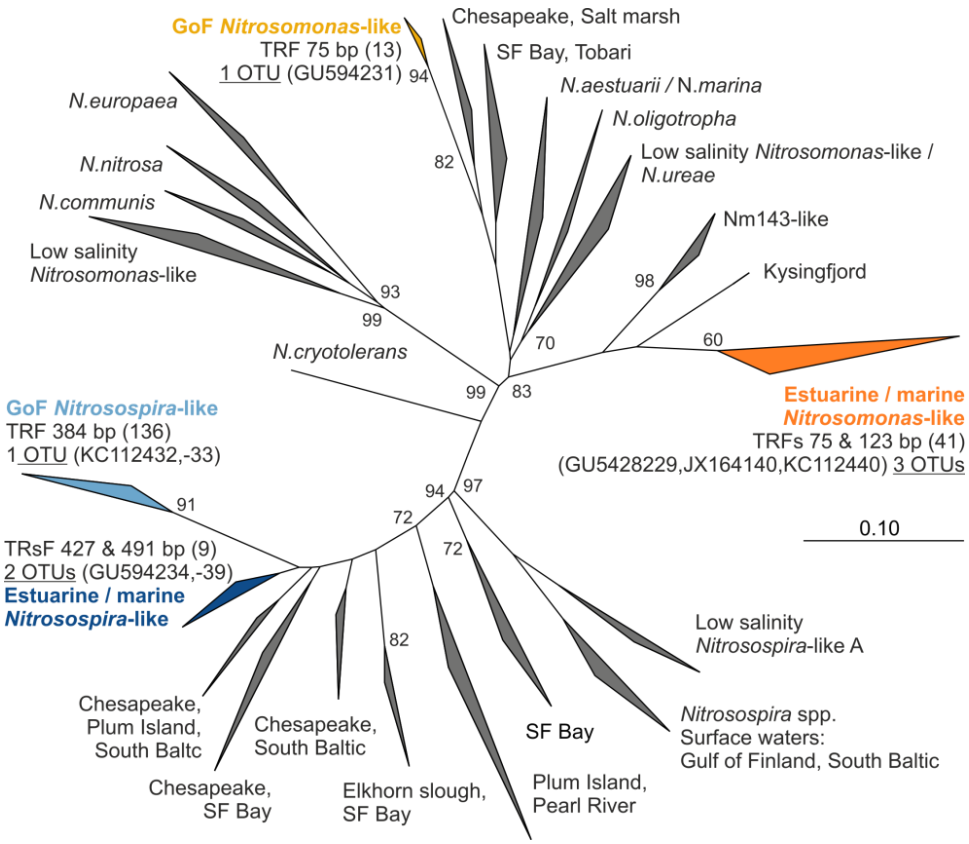
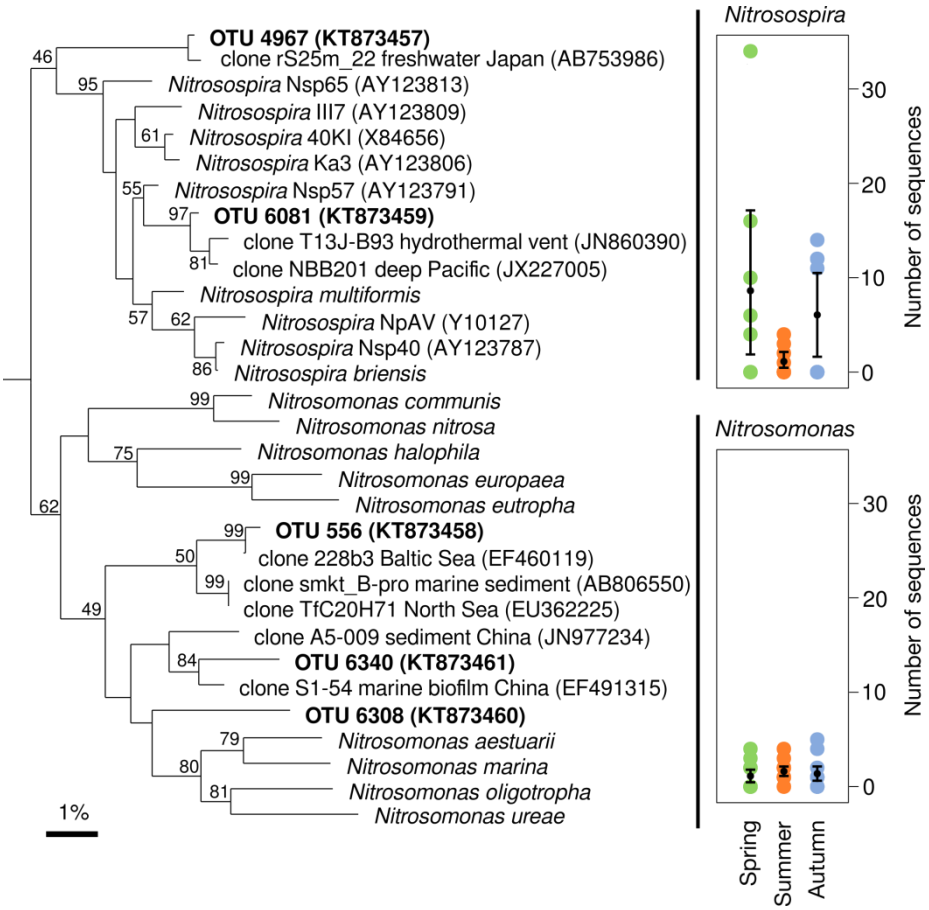


Figure 2. Unrooted neighbor-joining phylogenetic of AOB *amoA* sequences from the GoF and other environments (fragment length for the clones: 436 bp). Bootstrap support values (1000 replicates) lower than 60 are not shown. For clones from the GoF, the sizes of BfaI TRFs, followed by the number of clones in parenthesis, the accession number of reference sequences and the number of OTUs are shown. Colors highlighting the *Nitrosospira*/ *Nitrosomonas* found in the GoF correspond to the colors used for TRFs' identification in figure 5A. Subclusters unique to the GoF are labeled "GoF".

The *Nitrosospira*-like sequences from the GoF belonged to three subclusters associated with a cluster previously denoted as '*Nitrosospira*-like B' (Fig. 2), which encompasses sequences from estuaries and brackish environments (Kim et al., 2008, Bernhard and Bollmann, 2010, Lage et al., 2010). The majority of the *Nitrosospira*-like sequences (136 clones) grouped into a clade with a single OTU (denoted 'GoF *Nitrosospira*-like' in the tree,

Fig. 2). This subcluster formed a monophyletic group with a maximum sequence identity of 91% to the closest related sequences in databanks.

The pyrosequencing data revealed a total of five 16S rDNA OTUs for the coastal samples, two *Nitrosospira*-like OTUs (129 sequence reads) and three OTUs (48 sequence reads) belonging to the *Nitrosomonas* genus. The 16S rDNA sequences from the pyrosequencing data and the *amoA* sequences identified approximately the same number of OTUs and they grouped in the same *Nitrosospira* and *Nitrosomonas* clusters (Fig. 3). This suggests that both *amoA* and 16S rDNA approaches identified similar OTU richness and diversity in the samples. The most abundant AOB sequence in the pyrosequencing dataset (122 out of 177 AOB reads) belonged to an OTU in the *Nitrosospira*-like genus, as did the most abundant *amoA* clones. These two dominant OTUs might represent the same organisms.



313 **Figure 3.** Neighbor-joining phylogenetic tree of 16S rDNA fragments obtained by
314 pyrosequencing. The sequences were 400 bp long on average and covered the hypervariable
315 regions V1 to V3. Bootstrap support values (10000 replicates) lower than 50 are not shown.
316 The bar represents 1% sequence divergence. The tree is rooted to the betaproteobacterium
317 *Thiobacillus* spp and the OTU retrieved for this study are in bold. The number of
318 *Nitrosomonas* and *Nitrosospira* 16S rDNA sequence fragments in the pyrosequencing dataset
319 are shown to the right of the tree. Data from the two coastal sites Storfjärden and Muncken
320 are merged for this analysis. At each season, the data points represent the number of 16S
321 rDNA sequences for two sites, two years and for three (*Nitrosomonas*, n=12) or two
322 (*Nitrosopira*, n=8) OTUs. The bars shows the 95% confidence intervals inferred from 1000
323 bootstrap.
324
325 **3.3 Phylogenetic analysis of AOA *amoA***
326 Phylogenetic analysis indicated that the sequences retrieved in our study belonged to 5 OTUs
327 at a 95% sequence similarity cut-off (Fig. 4) falling into 2 of the 5 recently recognized major
328 AOA clusters (Pester et al., 2012). Sequences assigned to the major *Nitrosopumilus* cluster
329 dominated the clone library. The sequences in this cluster segregated into the *Nitrosoarcheum*
330 subcluster, which contains low-salinity AOA (Mosier and Francis, 2008) and into the 'GoF
331 sediments' subcluster, more closely related to *N.maritimus* and to sequences from sites with
332 variable salinity in other estuaries such as the Elkhorn slough (Francis et al., 2005, Wankel et
333 al., 2011), Plum Island (Bernhard et al., 2010) and the San Francisco Bay (Francis et al., 2005,
334 Mosier and Francis, 2008). The diversity of our AOA sequences was greater in the
335 *Nitrosoarcheum* subcluster than in the other AOA clusters, yet it only contained 11% of our
336 AOA clones. At a 95% sequence similarity cut-off, these sequences grouped into 3 OTUs, two
337 of which grouped with either of the two *Nitrosoarcheum* candidate species *N.limnia* (Mosier

et al., 2012a) and *N.koreensis* (Kim et al., 2011). Our clones were totally absent from many of the subclusters where other estuarine AOA sequences are commonly found with the same primer set (Francis et al., 2005); they were also missing from the *Nitrosotalea* cluster, which regroups sequences from soil environments but also contains many estuarine AOA sequences. A minority of 4 GoF clones belonged to the *Nitrososphaera* 'sister' cluster, which is related to the cultured soil *Nitrososphaera* AOA (Tourna et al., 2011).

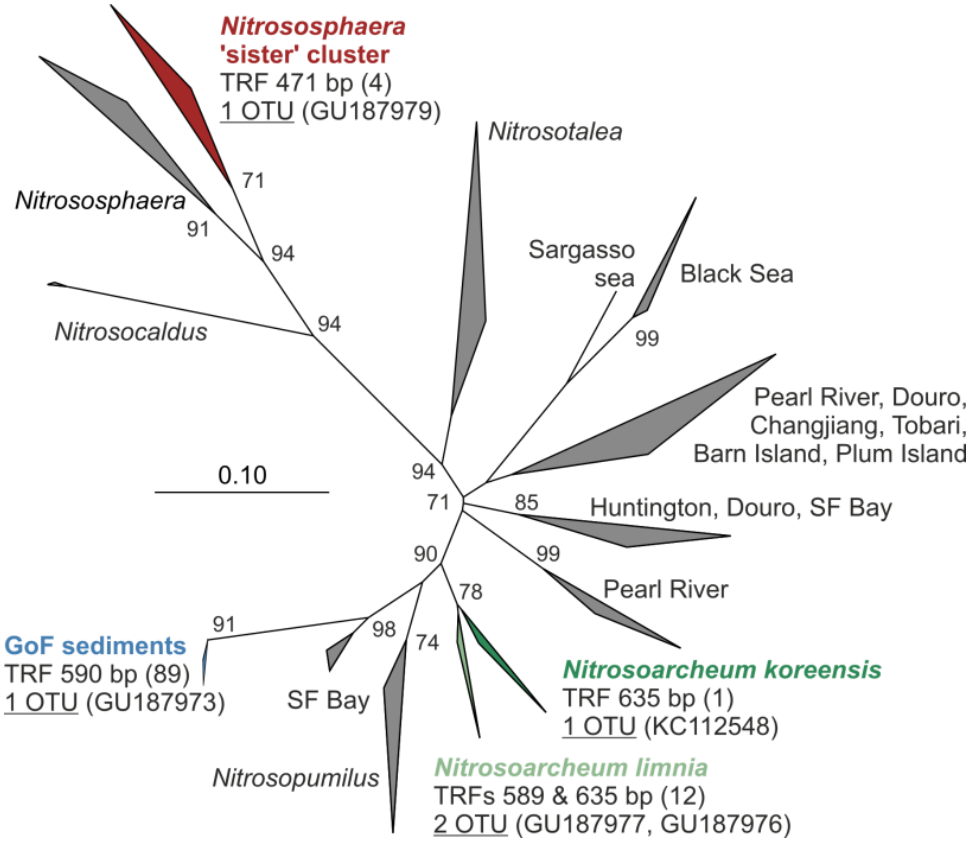


Figure 4. Unrooted neighbor-joining phylogenetic tree of AOA *amoA* sequences from the GoF and other environments (fragment length for the clones: 560 bp). Bootstrap support values (1000 replicates) lower than 60 are not shown. For clones from the GoF, the sizes of BseDI TRFs, followed by the number of clones in parenthesis, the accession number of reference sequences and the number of OTUs are shown. Colors of the groups correspond to the colors used for TRFs identification in figure 6A. Subclusters unique to the GoF are labeled "GoF".

353

354 **3.4 TRFLP of AOB *amoA***

355 A total of 5 BfaI TRFs were found (Fig. 5A). The predominant *Nitrosospira*-like sequences in
356 the library were represented by a TRF of 384 bp, which was also the major TRF in most
357 samples. In the estuarine and marine *Nitrosospira*-like group, two TRFs (427 and 491 bp)
358 accounted for up to two thirds of all *Nitrosospira*-related TRFs at our westernmost sampling
359 sites. The *Nitrosospira*-like TRFs were always dominant except for the summer samplings at
360 the deeper coastal site Storfjärden, where the 75 bp *Nitrosomonas*-like TRF was found in
361 approximately equal proportions. This 75 bp TRF corresponded to two OTUs in the
362 'Estuarine/marine' *Nitrosomonas*-like cluster and to a third OTU in the 'GoF *Nitrosomonas*-
363 like' cluster encompassing estuarine sequences and related to *Nitrosomonas aestuarii* and
364 *N.marina* (Fig. 2). The 'Estuarine/marine' cluster also contained a 123 bp which was scarce in
365 all samples (Fig. 5A).

366

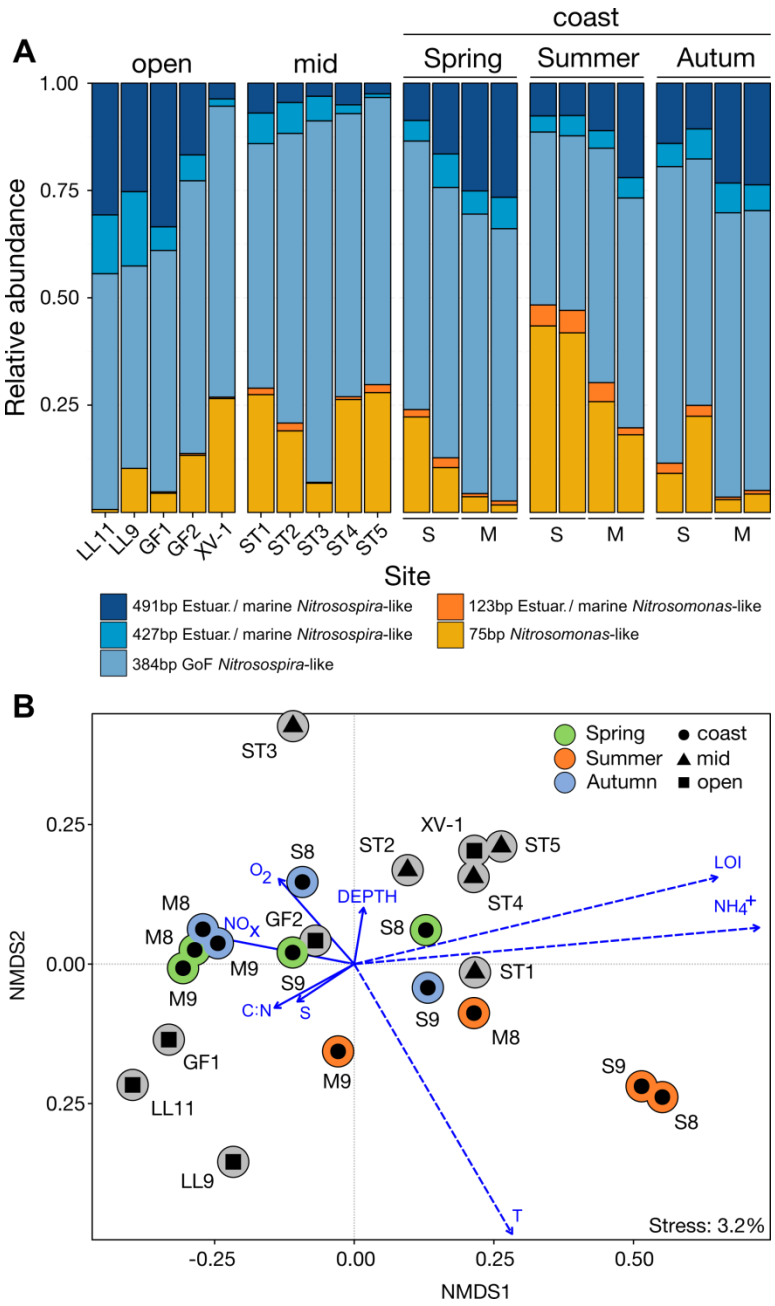


Figure 5. Panel A. AOB Community profiles as represented by the relative abundance of TRFs, the mean values for duplicate profiles are shown. The colors of the bars in panel A match the colors of their repsective clusters in the AOB tree (Fig. 2). Profiles for consecutive sampling years at the coastal sites (S and M) are juxtaposed and profiles for both sites are grouped by season. Panel B. NMDS ordination plot of AOB community TRFLP profiles and environmental variables. The samples are arranged according to the region from where they

were sampled: ‘open’, ‘mid’ and ‘coast’ for samples, taken from the deep open sea sediments, for samples taken halfway between the open sea and the coastal range and for samples taken near the coast, respectively. For coastal samples, S and M represent the deeper and shallower site, respectively. Their data points are labeled with a letter followed by a digit that refers to the sampling year (2008 and 2009).Variables with statistically significant correlation to the community ordination are shown as dashed arrows. Abbreviations for the variables are the same as those in Table 1.

Interannual variation of AOB TRFLP profiles at coastal sites was moderate, while the seasonal variation in community composition was notable as revealed by the ordination analysis (Fig. 5B). ANOSIM indicated that the variation in community structure was significant between seasons ($R=0.31$, $p=0.02$, $n=24$). The community structure differed at the two coastal sites Muncken and Storfjärden, but their variation followed a similar seasonal trend ($R=0.26$, $p=0.01$, $n=24$). The seasonal effect was strongest in summer with an increase in the relative abundance of *Nitrosomonas* TRFs (75 and 123 bp TRFs) during both sampling years and at both sites (Fig. 5A). Pairwise ANOSIM confirmed that most of the differences were found between the summer samples and the two other seasons (summer vs. spring, $R=0.49$, $p<0.01$; summer vs. Autumn $R= 0.52$, $p<0.01$, $n=16$ for each combination) and that there were no differences between the spring and autumn communities ($R=-0.08$, $p=0.89$, $n=16$). The shift to higher proportions of *Nitrosomonas* fragments correlated with higher sediment organic content and higher ammonium concentration in the water above the sediment (Fig. 5B) as well as bottom water temperature. The 123 bp TRF was not detected at all at the two westernmost open sea sites LL9 and LL11 and increased in abundance during summer, similarly to the 75 bp TRF (Figs 5A). In the open sea, the sites ST1 to ST5, which are closer to the coastal range, displayed higher abundances of the 75 bp *Nitrosomonas* TRF

400 (Fig. 5A). Overall, the factors that contributed the most in explaining the AOB community
401 composition were the organic content of the sediment, the temperature and the ammonium
402 concentration in bottom water (Table 3).
403

	r^2	
	AOB	AOA
Depth	0	0.84**
T	0.33*	0.04
S	0.03	0.8
O_2	0.09	0.75**
NH_4^+	0.53*	0.13
NO_3^-	0.06	0.09
LOI	0.46*	0.16
C:N	0.02	0.14

404

405 **Table 3.** Goodness of fit between environmental variables and the community datasets
406 computed with the function *envfit*. Factors explaining the community composition in a
407 significant manner are shown with *($p<0.05$) and **($p<0.001$). Abbreviations for the
408 variables are the same as in Table 1.
409

410 **3.5 TRFLP of AOA *amoA***

411 A striking pattern was observed in the distribution of the 4 AOA TRFs in our data (Fig.4, Fig.
412 6A). There was a clear distinction between the coastal and open sea sites as illustrated by both
413 the ordination analysis and the relative abundances of TRFs in the communities (Fig. 6A and
414 B). The AOA of the *Nitrosoarcheum* cluster (589 and 635 bp) were highly dominant at the
415 coastal sites, while the AOA of the cluster unique to the GoF (590 bp) dominated the open
416 sites (Fig. 6A) as well as the clone library (Fig. 4). The sequences associated with the
417 *Nitrososphaera* 'sister' cluster had a TRF of 471 bp, this fragment was found in low
418 abundance in many samples and totally absent at many of the open sea sites. Interestingly,

proximate sites such as GF1, GF2 and ST1, ST2 showed highly similar community patterns despite being almost 50 km apart.

Pairwise ANOSIM confirmed that the metacommunity at the mid-range and open sea sites differed significantly from the coastal communities. (R=1, p=0.01, n=42), but no significant differences were found between the seasons or sites at the coastal locations. The ordination analysis indicated that the AOA community composition correlated significantly with oxygen concentration and salinity (Fig. 6B, Table 3), both of which are dependent on depth (Table 2).

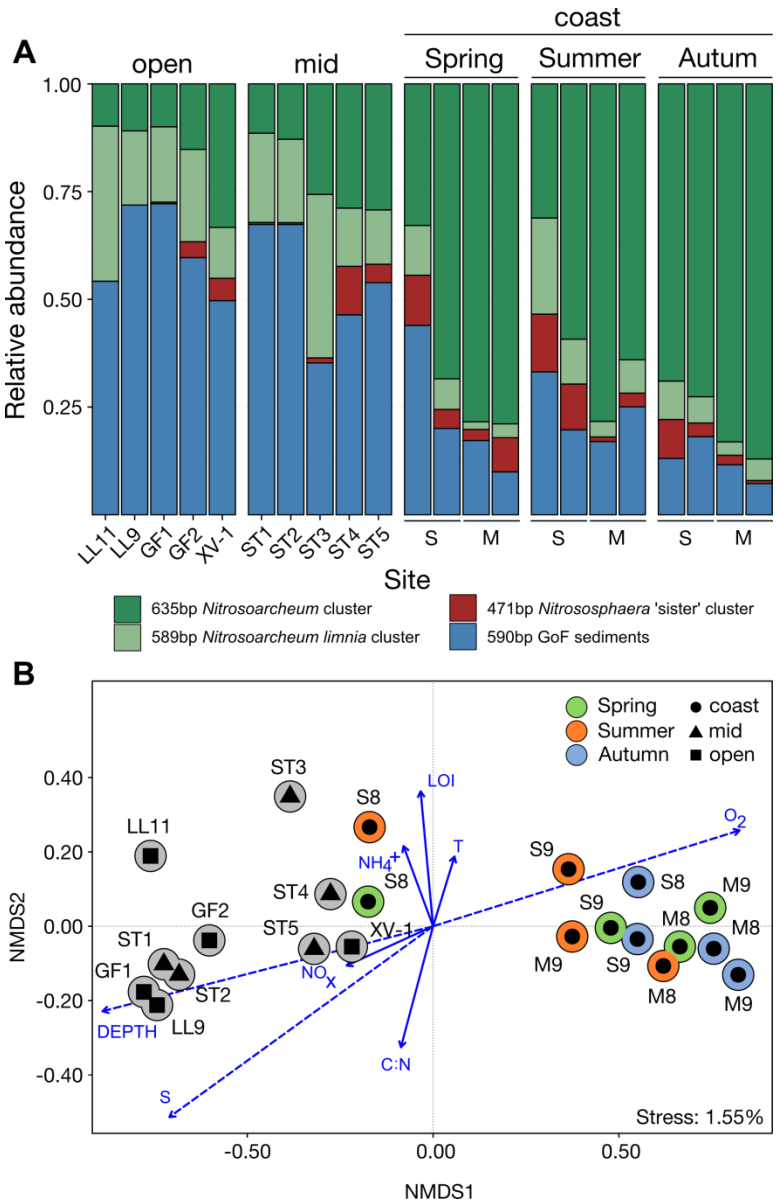
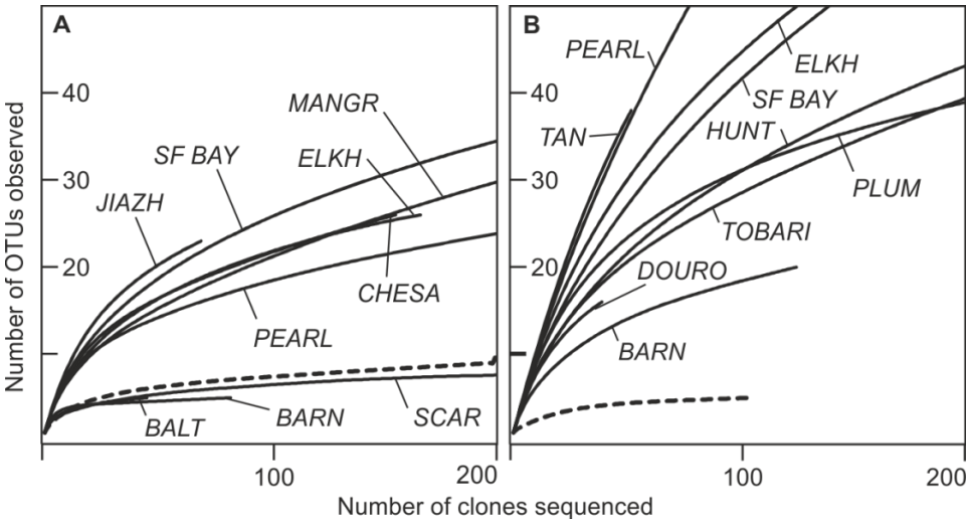


Figure 6. Panel A. AOA Community profiles as represented by the relative abundance of TRFs. The colors of the bars in panel A match the colors of their respective clusters in the AOA tree (Fig. 4). Panel B. NMDS ordination plot of AOA community TRFLP profiles and environmental variables. See the legend of figure 5 for details.

4 Discussion

4.1 Diversity of ammonia-oxidizing microbes in the GoF

According to the *amoA* marker, the diversity of both AOA and AOB communities in the GoF was lower than in most other sediments studied (Fig. 7), despite the large geographical area covered in this work, but in agreement with the notion that more diverse habitats harbor higher biodiversity. Indeed, our sampling sites included mostly muddy sediment and the geochemical conditions spanned short gradients (e.g. salinity 4.5 – 10). Comparable AOB richness has been observed at estuarine sites with similar geochemical characteristics, like those in the southern Baltic Sea (Fig. 7) (Kim et al., 2008), Chesapeake Bay (Francis et al., 2003) and northern San Francisco Bay (Mosier and Francis, 2008). There was, however, no notable geochemical similarities between our sites and the Barn Island and Scarborough tidal marshes sampling sites (Lage et al., 2010, Moin et al., 2008) although the AOB diversity was closest to that of the GoF (Fig. 7). We also observed a lower richness of AOA than AOB *amoA* fragments, although this is generally the opposite in natural sediments (Beman and Francis, 2006, Santoro et al., 2008, Moin et al., 2009). The nitrogen-rich nature of the GoF could favor AOB over AOA, which are thought to thrive in nitrogen-poor environments (Erguder et al., 2009).



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452

453 **Figure 7.** Rarefaction analysis of the clones from the Gulf of Finland (dashed curves) for the
454 AOB (panel A) and AOA (panel B) libraries as well as *amoA* libraries representing various
455 estuarine sediments. Rarefaction was calculated using a 95% sequence similarity threshold.
456 The abbreviations refer to BALT - Southern Baltic Sea, SCAR - Scarborough and BARN -
457 Barn Island salt marshes, SF BAY - San Francisco Bay, CHESA - Chesapeake Bay, ELKH -
458 Elkhorn Slough, JIAZH - Jiaozhou Bay, PEARL - Pearl River estuary, MANGR - a polluted
459 mangrove forest, TAN - Tanoura Bay, DOURO - Douro River estuary, TOBARI - Bahia del
460 Tobari, PLUM - Plum Island Sound estuary and HUNT - Huntington beach subterranean
461 estuary. Analyses were donw in MOTHUR.

462

463 The sediment communities of both AOB and AOA differed greatly from the water
464 column communities in the Baltic Sea. For instance, we did not find the same freshwater-
465 related *Nitrosospira* sequences that were amplified from surface waters of the GoF
466 (Tuomainen et al., 2003). We did not find any AOA sequences related to *Nitrosopumilus*
467 either, while they are known to dominate the water column of the central Baltic Sea (Labrenz
468 et al., 2010). In the *Nitrosoarcheum* cluster (Fig. 4), our sequences showed closest affinity to
469 clones isolated from the sediment of the North San Francisco Bay (Mosier and Francis, 2008),

in an area with the same level of salinity as the sampling sites in the GoF. Earlier studies have suggested that the predominant AOA in the North San Francisco Bay may represent a low-salinity AOA ecotype (Francis et al., 2005, 2008). This was confirmed by genetic and physiological characterization of Candidatus *Nitrosoarcheum limnia*, a low-salinity AOA (Mosier et al., 2012a, Mosier et al., 2012c) and thus it is not surprising that TRFs related to *Nitrosoarcheum* are widespread in the GoF (Fig. 6A).

We also found apparent northern Baltic Sea ecotypes of both AOB and AOA *amoA* gene (AOB sequences KC112432 and GU594231 in Fig. 2 and AOA sequence GU187973 in Fig. 4). These sequences formed monophyletic clusters with closest sequence similarity of 85 to 91% to other *amoA* sequences in public databases and had low diversity within their clade. They were among the most abundant clones and TRFs suggesting that they may represent organisms adapted to the specific environmental conditions in the open sea sediments of the GoF. The phylogenetic analyses of the 16S rDNA AOB sequences confirmed these observations for the *Nitrosomonas*-like clones to a large extent (Fig. 3). However, the 16S rDNA OTU that matched the non-dominant *amoA Nitrosospira*-like OTU was nearly identical (>99.7% identity, 1 base change in a 483 bp fragment) to freshwater sequences from Japan (Fig.3), suggesting that these sequence types are not unique to the Gulf of Finland.

4.2 Temporal and spatial variation

4.2.1 Dynamics of AOB communities

At the coastal sites, the AOB metacommunity in the GoF was dominated by *Nitrosospira*-like over *Nitrosomonas* during most of the sampling period (Fig. 5A). This is also supported by the pyrosequencing data (Vetterli et al., 2015) where the absolute amount of *Nitrosospira* reads was higher than that of *Nitrosomonas* (Fig. 3).

494 At the open sea sites, it could not clearly be established whether the low abundance of
495 *Nitrosomonas* OTUs (Fig. 5A) was caused by lack of oxygen, by the higher salinity or by both
496 due to the intercorrelation of many environmental variables with depth (Table 2).
497 Furthermore, these two variables explained only a smaller portion of the variation in the
498 community dataset (Table 3). Salinity is an important regulator of AOB community
499 composition in estuaries (de Bie et al., 2001, Freitag et al., 2006) and *Nitrosospira*-like
500 sequences are dominating the water column and sediment communities at sites with higher
501 salinity in many estuaries (Francis et al., 2003, Bernhard et al., 2005, Wankel et al., 2011).
502 The salinity between coastal and open sea sites varied from 4.5 to 10 (Table 1), while studies
503 elsewhere have looked at variation of 10 salinity units or more between sites. Thus it seems
504 likely that other factors in combination with salinity are responsible for shaping AOB
505 communities at the deepest sites of the open sea.
506 In addition to salinity, the AOB community composition was explained significantly
507 by the amount of organic matter (LOI), NH_4^+ concentration and the bottom water temperature
508 interface across all our sites (Fig. 5B, Table 3). The increase in the relative abundance of
509 *Nitrosomonas* AOB TRFs at coastal sites in summer specifically matched the increase in these
510 three variables. Similar observations were made in the Chesapeake Bay, where a positive
511 correlation was found between NH_4^+ and the abundance of sequences related to *Nitrosomonas*
512 *aestuarii* in surface waters (Bouskill et al., 2011); in New England salt marshes, where a
513 positive trend was shown between long-term fertilization and *Nitrosomonas* OTUs (Peng et
514 al., 2012); and in the United Kingdom where an increase in NH_4^+ concentration in summer
515 corresponded to an increase in *Nitrosomonas* 16S rDNA PCR fragments in an oligotrophic
516 lake (Whitby et al., 1999). The *N. aestuarii* clade was also shown to be favored by NH_4^+
517 amendments and more oxic conditions in the sediment communities of a mangrove forest
518 (Laanbroek et al., 2013) and indeed, part of our sequences represented by the 75 bp TRF,

519 which was most abundant in our summer samples (Fig. 5A), are related to the
520 *N.aestuarii*/*N.marina* clade (Fig. 2). This is further supported by the higher proportion of
521 *Nitrosomonas* TRFs at sites in the mid-range (Zone 'mid', Table 1) where temperature is
522 lower and NH_4^+ concentrations higher (Fig. 5A). Stations XV-1 and ST-1 to ST-5 had on
523 average 5.1µM NH_4^+ whereas in the open sea, the deepest stations LL11, LL9 GF1 and GF2
524 had on average 0.3 µM NH_4^+ at the sediment-water interface (Table 1) and the latter were
525 almost devoid of *Nitrosomonas* TRFs. These results are consistent with the trait-based model
526 presented by Bouskill and colleagues with regards to temperature and substrate availability
527 (Bouskill et al., 2012).
528 The TRFLP data presented here cannot, however, determine whether the absolute
529 abundance of *Nitrosomonas* TRFs increased in summer samples or if the *Nitrosospira* TRFs
530 became less abundant. According to pyrosequencing data obtained in our earlier study
531 (Vetterli et al., 2015), the latter is more likely (Fig.3) although the low amount of reads
532 retrieved for *Nitrosomonas*-like 16S rDNA fragments prevents us from drawing any strict
533 conclusion. What can be derived from the pyrosequencing data is that AOB, specifically
534 *Nitrosospira*, are most likely outcompeted by the rest of the microbial community at the
535 surface of coastal sediment during summer. The nitrification potential in these sediments is
536 highest during cold periods when oxygen availability is high, but the lack of ammonium
537 keeps the realized rates low. In warmer, ammonium–rich periods, limited oxygen availability
538 lowers the potential while the realized rates are higher than during cold periods (Jäntti et al.,
539 2011). Taken together with the AOB community composition data, this suggest that
540 *Nitrosospira*-like AOB contribute to an important fraction of the bacterial nitrification in the
541 coastal GoF as their patterns follows the potential for nitrification. Both global warming and
542 continuing eutrophication negatively affect oxygen conditions, which may lead to inhibition
543 of nitrification in the GoF in summer. In addition, organic matter is produced in higher

quantities in warmer periods and most known denitrifying microbes require organic matter to reduce nitrite and nitrate to gaseous N_2. Combined, these effects would lead to the temporal uncoupling of nitrification and denitrification and thus further enhance the accumulation of nutrients in the ecosystem.

4.2.2 Dynamics of AOA communities

The AOA assemblages in the sediment of GoF did not display any clear temporal variation in contrast to what has been observed in marine waters (Pitcher et al., 2011, Hollibaugh et al., 2014). This might be due to environmental AOA generally thriving in more oligotrophic conditions than AOB (Erguder et al., 2009) and having ammonia oxidation capacity per cell an order of magnitude below that of many AOB, as well as lower substrate requirements (Pester et al., 2011). Under these assumptions, the energy requirements of AOA would be fulfilled in the sediment environment of the coastal GoF and the coastal AOA population structure would be stable throughout the seasons, regardless of nutrients availability.

On the other hand, there was a strong spatial pattern between coastal and open sea AOA communities, with depth, salinity and oxygen being important community composition predictors in the ordination analysis (Table 3, Fig. 6B). Clear global distribution patterns of AOA phylotypes have been found between water column and the sediment (Francis et al., 2005, Prosser and Nicol, 2008) as well as between different water depths (Beman et al., 2008, Tolar et al., 2013). However, no obvious segregation between AOA phylotypes have been found in sediments at different water depths (Biller et al., 2012). In the GoF we observed that AOA sequences dominating coastal sites had similarity with sequences belonging to the 'Lower Salinity Environment' cluster identified by Cao et al. (2013), which also includes the halotolerant archaeon Candidatus *Nitrosoarchaeum limnia* (Mosier et al., 2012a). In culture experiments it was shown that *N.limnia* is able to subsist at higher salinities, but with a slower

569 growth rate (Mosier et al., 2012c). The sequences in the 'GoF sediments' cluster that
570 dominated the deeper saline sites in the GoF had more resemblance to the marine archaeon
571 Candidatus *Nitrosopumilus salaria* (Mosier et al., 2012b), which is found in abundance at a
572 site with mixed salinity in the San Francisco Bay (Mosier and Francis, 2008). Physiological
573 differences between the AOA dominating the sediments in shallow and deep water habitats
574 may be of relevance for future nitrification activity of AOA in the sediments of the Baltic Sea
575 where there is a long-term trend of decreasing salinity.

576 In addition to eutrophication-induced oxygen fluctuations, global climate changes
577 have been predicted to change precipitation patterns, causing changes in salinity and nutrient
578 loading. Taken together these changes are likely to affect nitrogen processing, an important
579 ecosystem service, especially in the coastal and estuarine areas of the GoF that have so far
580 been in less critical state than the open sea anoxic sediments. To protect the nitrification it is
581 important to be aware of the factors that control the process. According to our results, changes
582 in salinity are less critical than changes in nutrient loading, highlighting the importance of
583 eutrophication control.

584

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592

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