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Links between seawater flooding, soil ammonia oxidiser communities and their response to changes in salinity

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Keywords:	ammonia-oxidising archaea, ammonia-oxidising bacteria, nitrification, flooding, salinity, <i>Nitrosococcus</i>

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1 **Links between seawater flooding, soil ammonia oxidiser communities and**
2 **their response to changes in salinity**

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15

16 **Keywords:** ammonia-oxidising archaea; ammonia-oxidising bacteria; nitrification; flooding;
17 salinity; *Nitrosococcus*

18

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24 **Abstract**

25 Coastal areas worldwide are challenged by climate change-associated increases in sea level
26 and storm surge quantities that potentially lead to more frequent flooding of soil ecosystems.
27 Currently, little is known of the effects of inundation events on microorganisms controlling
28 nitrification in these ecosystems. The goal of this study was to investigate the impact of
29 seawater flooding on the abundance, community composition and salinity tolerance of soil
30 ammonia oxidisers. Topsoil was sampled from three islands flooded at different frequencies
31 by the Wadden Sea. Archaeal ammonia oxidiser *amoA* genes were more abundant than
32 their betaproteobacterial counterparts and the distribution of archaeal and bacterial ammonia
33 oxidiser *amoA* and 16S rRNA gene sequences significantly differed between the islands.
34 The findings indicate selection of ammonia oxidiser phylotypes with greater tolerance to high
35 salinity and slightly alkaline pH (e.g., *Nitrosopumilus* representatives) in frequently flooded
36 soils. A cluster phylogenetically related to gammaproteobacterial ammonia oxidisers was
37 detected in all samples analysed in this survey. Nevertheless, no gammaprotebacterial
38 *amoA* genes could be amplified via PCR and only betaproteobacterial ammonia oxidisers
39 were detected in enrichment cultures. A slurry-based experiment demonstrated tolerance of
40 both bacterial and archaeal ammonia oxidisers to a wide range of salinities (e.g., Wadden
41 Sea water salinity) in soil naturally exposed to seawater at a high frequency.

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Introduction

Flooding of the Earth's low-lying coastal land is expected to increase as a result of recent and predicted future increases in sea levels (Nicholls and Cazenave 2010; Haigh *et al.* 2014; Wong *et al.*, 2014) and estimated higher number and intensification of storm surge events (Dasgupta *et al*. 2009; Mousavi *et al.* 2011; Grinsted *et al.* 2013). Soil ecosystems that are directly exposed to seawater during inundation are particularly susceptible to the effects of flooding, such as osmotic stress. It has been reported that tsunami-induced seawater inundation significantly alters soil bacterial community structure (Asano *et al.* 2013). In addition, soil salinity, which can be affected by flooding events, was found to be strongly related to bacterial community structure in tidal wetlands (Morrissey *et al.* 2014). Currently, the impacts of temporal seawater inundation on specific functional microbial groups, essential for soil fertility and globally important biogeochemical cycles are poorly understood.

The initial step of nitrification, a key process within the nitrogen cycle, is mediated by ammonia oxidisers (AO) (Prosser and Nicol 2008), which comprise ammonia oxidising archaea (AOA) and bacteria (AOB). AOA outnumber their bacterial counterparts in various marine environments such as North Sea or Atlantic Ocean water (Wuchter *et al.* 2006). Furthermore, AOA and AOB cohabit most terrestrial ecosystems (Prosser 2011), but their relative abundances in soil show significant variations (Prosser and Nicol 2012). Previous studies indicate that soil pH represents a major driver of these variations, with AOA typically dominating acidic niches (Nicol *et al.* 2008; Gubry-Rangin *et al.* 2010; Yao *et al.* 2013). There is also limited evidence for greater sensitivity of AOA toward high ammonia concentration and their preference of soils with low ammonia supply (Prosser and Nicol 2012). Oxygen availability represents another parameter affecting AO communities and few studies indicate that AOA have a higher affinity for oxygen than AOB (Chen *et al*. 2008; Jung *et al*. 2011; Pitcher *et al.* 2011; Kim *et al*. 2012). In wet tropical soil, which experiences fluctuating oxygen availability, AOA tolerant of extended periods of anoxia were detected (Pett-Ridge *et al*. 2013). Moreover, relationships between salinity and sediment AOA or AOB

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75 distribution were reported (Coci *et al.* 2005; Sahan and Muyzer 2008; Bernhard *et al.* 2010;
76 Zheng *et al.* 2014; Zhang et al. 2015). For instance, it has been shown that salinity is a
77 steering factor in selecting *Nitrosomonas* and *Nitrosopumilus* representatives (Coci *et al.*
78 2005; Zhang *et al.* 2015).

79 A study based on high-throughput sequencing of *amoA* genes (encoding subunit A of
80 ammonia monooxygenase) indicates that AOA are dominated by the *Nitrososphaera* cluster
81 in numerous soil ecosystems, with the exception of acidic niches (Pester *et al.* 2012). In
82 contrast, members of the *Nitrosopumilus* cluster (also called thaumarchaeal group I.1a or
83 marine group) represent the dominant AOA in oligotrophic seawater, which is probably due
84 to high affinity for ammonia (Martens-Habbena *et al.* 2009). AOB comprise the
85 betaproteobacterial genera *Nitrosomonas* and *Nitrosospira*, collectively forming the family
86 *Nitrosomonadaceae*, as well as the gammaproteobacterial genera *Nitrosococcus* and
87 *Nitrosoglobus*. Different *Nitrosomonas* lineages, such as the *Nitrosomonas* marina lineage
88 and the *Nitrosomonas europaea*/*Nitrosomonas mobilis* lineage, are predominantly detected
89 in marine or non-marine environments (Prosser 2014). *Nitrosococcus* has been poorly
90 studied with molecular techniques but cultured representatives were exclusively derived from
91 salty environments (Campbell *et al.* 2011; Wang *et al.* 2017). Furthermore, the
92 gammaproteobacterial AOB *Nitrosoglobus terrae* TAO100 was recently isolated from an
93 acidic agricultural soil (Hayatsu *et al.* 2017).

94 Both, AOA and AOB, have been detected in coastal soil and sediment (Moin *et al.* 2009;
95 Marton *et al.* 2015; Bernhard *et al.* 2016). In New England salt marsh sites, up to
96 approximately 10^9 AOA *amoA* genes g^{-1} dry sediment were reported (Moin *et al.* 2009) and
97 higher abundances of AOA than AOB *amoA* genes were found in soil and sediment samples
98 from salt marshes (Moin *et al.* 2009; Marton *et al.* 2015). Nevertheless, information about
99 effects of different seawater flooding frequencies on ammonia oxidisers inhabiting coastal
100 soil is lacking. As representatives of AOA and AOB show salinity preferences (Koops *et al.*
101 1991; Campbell *et al.* 2011; Qin *et al.* 2014), it can be assumed that both groups are

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affected by inundation events. The German Halligen can be considered a model system for extensive analysis of flooding effects. These islands are surrounded by the Wadden Sea and are inundated at different frequencies during the year. Approximately 100 years ago, dykes were established for the Halligen Hooge and Langeness (Schindler *et al.* 2014a). Due to the dykes and closing tidal gates with rising tides, Hooge and Langeness need much higher gauge levels to be flooded than Hallig Nordstrandischmoor. Therefore, Nordstrandischmoor shows higher seawater inundation frequencies than the Halligen Hooge and Langeness.

In this study, the impacts of seawater flooding on abundance, diversity and community composition of soil AO were investigated. Flooding significantly affects physicochemical parameters such as salinity or oxygen level in soil. Here, we focused on salinity, which can show strong fluctuations in temporarily flooded soil over time. In order to explore the response of ammonia oxidisers to changes in sea salt concentration, a soil slurry experiment was performed. All analyses were carried out using topsoil samples derived from Hooge, Langeness and Nordstrandischmoor. The study was based on the following hypotheses: (1) flooding of soil ecosystems evokes changes in AOA and AOB abundance due to changes in salinity and/or invasion of AO in surrounding seawater, (2) representatives of AO inhabiting soil frequently inundated with seawater exhibit halotolerance and tolerance toward changing salt concentrations, (3) the abundance of thaumarchaeal group I.1a (marine group) is positively correlated with seawater flooding frequency and (4) within AOB, phylogenetic lineages typically encountered in marine habitats show higher abundance in frequently flooded soil than in soil rarely inundated with seawater.

Materials and Methods

Study sites and soil sampling

The German Halligen, ten small islands with a total area of 23 km^2, are surrounded by the North Frisian Wadden Sea. They represent relics of former mainland or larger islands. At the beginning of the 20th century, dykes were established to strengthen the marshland of Hooge

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and Langeness against storm surges. The dykes show an average height of 1.5 m (Hooge) and 1.0 m (Langeness) above the present 10-year (2001-2010) mean high water (Schindler *et al.* 2014b). Inundation of Hooge and Langeness marshland only occurs during heavy, westerly storm conditions (Schindler *et al.* 2014b). In contrast, the marshland of Nordstrandischmoor is not enclosed by a dyke and therefore exhibits a high inundation frequency, with an average of 15 events year^{-1} between 2001 and 2010 (Schindler *et al.* 2014b), while the ten-year average (2001-2010) for Hooge is two events year^{-1} (Schindler *et al.* 2014b). The flooding frequency of Langeness (10 events year^{-1}) is intermediate.

Topsoil was sampled from Hooge (Ho), Langeness (La) and Nordstrandischmoor (No) (Figure 1), with coordinates of sampling sites (Ho1, Ho2, Ho3, La1, La2, La3, La4, No1, No2, and No3) provided in Table S1. Triplicate samples at each sampling site (designated, e.g., Ho1a, Ho1b, and Ho1c) were removed at 1-m distances from each other. The upper 10 cm of the mineral soil was separated from adjacent soil material, homogenised and sieved to <3.5 mm. Soil samples for slurry-based analyses were stored at 4°C and all other samples were kept frozen (-80°C).

Basic soil analyses and DNA extraction

Subsamples (2 g) of field-moist soils were used to determine gravimetric water content by drying to constant weight at 105°C. Another subsample (5 g) was extracted in 25 ml 1 M KCl for analysis of ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) using a flow injection analyser "Quikchem QC85S5" (Lachat Instruments, Loveland, CO, USA). Air-dried soil was used to determine soil pH, electrical conductivity and C and N concentrations. Soil pH was measured in a 1:2.5 suspension of soil in distilled water. Electrical conductivity was measured in the same extract using a "Tetra Con 325" electrode (WTW, Weilheim, Germany). Ground subsamples were analysed for total carbon (TC) and nitrogen (TN) concentrations by dry combustion with a CN analyser “Vario Max” (Elementar Analysensysteme GmbH, Hanau, Germany). Inorganic carbon (IC) was determined after

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combustion of organic carbon in a muffle furnace (450°C for 16 h) and soil organic carbon (SOC) was calculated as the difference between TC and IC.

Genomic DNA was extracted from 0.5 g soil using the PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instruction. The concentration of extracted DNA was estimated using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Loughborough, UK).

Soil slurry incubations

Slurries containing soil derived from Hooge, Langeness, or Nordstrandischmoor and artificial seawater were prepared in 500-ml Duran bottles and consisted of 20 g soil (moisture content adjusted to 45% with sterile distilled water) in 100 ml artificial seawater. A wide range of salinity conditions were covered using artificial seawater (Subow 1931) with different salt concentrations (5 ‰, 10 ‰, 20 ‰, or 30 ‰). Ammonia was not added to prevent inhibition of ammonia-sensitive ammonia oxidizers by unnaturally high concentrations of ammonia. Triplicate slurries were incubated in the dark for 30 days at 26°C. Every third day, slurries were shaken and seals were removed for 5 min. Samples (8 ml) were collected following shaking, using a pipette with a 3-mm diameter tip, directly after establishing slurries and after incubation for 15 and 30 days. Samples were centrifuged in Falcon tubes at 18,500 × g and 4°C for 5 min and pellets were stored frozen until DNA extraction and supernatants were used for analysis of NH_4^+, NO_2^- and NO_3^-. Duran bottles containing soil slurries were weighed several times during incubation to calculate evaporation, which was found to be negligible.

Quantitative real-time PCR analysis of *amoA* genes

Quantitative real-time PCR (qPCR) was carried out using a BioRad MyIQ Single-Color Real-Time PCR Detection System (BioRad, Hertfordshire, UK) and SybrGreen as fluorescent dye. Primer sets CrenamoA23f/CrenamoA616r (Tourna *et al.* 2008) and amoA-1F/amoA-2R (Rotthauwe *et al.* 1997) were used for AOA and betaproteobacterial AOB *amoA*

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3 180 quantification, respectively. The PCR mixture (final volume, 25 µL per reaction) contained
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5 181 25 µg bovine serum albumin (BSA), 400 nM of each AOA or AOB *amoA*-specific primer,
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7 182 12.5 µL of QuantiFastTM qPCR master mix (Qiagen, Crawley, UK), and 25 ng of DNA. The
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9 183 following cycling conditions were used: 15 min at 95°C, 40 cycles of 15 s at 94°C, 1 min 30 s
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11 184 for AOA *amoA* and 1 min for AOB *amoA* at 60°C and plate fluorescence measurement after
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13 185 8 s at 80°C. Melting curve analysis and agarose gel electrophoresis were performed to
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15 186 assess specificity of amplification during PCR. Amplification efficiency ranged from 87.6% to
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17 187 102.5% and from 80.3% to 89.7% for AOA and betaproteobacterial AOB *amoA*, respectively,
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19 188 with r^2 values >0.98.
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22 189 **Pyrosequencing of bar-coded *amoA* and 16S rRNA gene amplicons**
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25 190 AOA *amoA* genes and the V3-V5 region of bacterial 16S rRNA genes were amplified by
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27 191 PCR using DNA extracts from the collected soil samples as template (samples derived from
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29 192 soil slurry incubations were not considered). Primer CrenamoA23f (Tourna *et al.* 2008),
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31 193 containing a sample-specific MID (extended multiplex identifier) and Roche 454
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33 194 pyrosequencing adaptor A, as well as primer CrenamoA616r (Tourna *et al.* 2008), containing
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35 195 Roche 454 pyrosequencing adaptor B, were used for amplification of AOA *amoA*. The
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37 196 forward and reverse primers V3for 5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-MID-
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39 197 TACGGRAGGCAGCAG-3' and V5rev 5'-
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41 198 CCTATCCCCTGTGTGCCTTGGCAGTCTCAGCCGTCAATTCMTTTGAGT-3' (Roche 454
42
43 199 pyrosequencing adaptors are underlined) (Liu *et al.* 2007; Wang and Qian 2009; Nacke *et al.*
44
45 200 2016) were used to amplify the V3-V5 region of bacterial 16S rRNA genes. The PCR
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47 201 reaction mixture (50 µL) contained 10 µL 5-fold reaction buffer (Phusion GC buffer; Thermo
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49 202 Fisher Scientific), 200 µM of each of the four deoxynucleoside triphosphates, 5% dimethyl
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51 203 sulfoxide (DMSO), 0.2 µM of each of the primers, 1 U Phusion high-fidelity DNA polymerase
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53 204 (Thermo Fisher Scientific) and 50 ng of DNA. The following PCR cycling conditions were
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55 205 used for amplification of AOA *amoA*: initial denaturation at 98°C for 30 s, 30 cycles of
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57 206 denaturation at 98°C for 10 s, annealing at 55°C for 30 s and extension at 72°C for 30 s,
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followed by a final extension step at 72°C for 5 min. PCR reactions for amplification of bacterial 16S rRNA genes were initiated at 98°C (30 s), followed by 25 cycles of 98°C (10 s), 65°C (30 s) and 72°C (30 s), and ended with incubation at 72°C for 5 min. All samples were amplified in triplicate, purified using the peqGold gel extraction kit (Peqlab Biotechnologie GmbH, Erlangen, Germany) and pooled in equal amounts. Sequences of *amoA* and 16S rRNA gene amplicons were determined using a Roche GS-FLX 454 pyrosequencer and titanium chemistry as recommended by the manufacturer.

Pyrosequencing data processing and analysis

AOA *amoA* sequences exhibiting an average quality value of <30, primer mismatches or long homopolymers (>8 bp) were removed using QIIME (version 1.7) (Caporaso *et al.* 2010). In addition, reads were truncated at the first ambiguous base encountered. After truncation of remaining primer sequences, noise resulting from amplicon pyrosequencing was removed using the Acacia error-correction tool (version 1.5) (Bragg *et al.* 2012). Chimeras were removed using Uchime (version 7.0) (Edgar *et al.* 2011) and using 735 *amoA* sequences comprising the currently known five major clusters of archaeal *amoA* as a reference database, obtained from the ARB (Ludwig *et al.* 2004) database created by Pester *et al.* (2012). Putative chimeras identified by Uchime were compared with the NCBI non-redundant database using BLASTn. Clear breaks in the local alignments affiliating queried *amoA* amplicons to two very distinct sequence entries in the NCBI database confirmed Uchime-based chimera detection. Sequences classified as chimeras by Uchime and manual inspection of local alignments were discarded completely. Subsequently, all sequences shorter than 592 bp were removed. Determination of operational taxonomic units (OTUs) at 85% genetic similarity was performed using Uclust (Edgar 2010). Singleton OTUs containing only one *amoA* sequence were removed using QIIME. A representative sequence for each OTU was added to the consensus tree for archaeal *amoA* phylogeny constructed by Pester et al. (2012) using the parsonomy tool in ARB (Ludwig *et al*. 2004). Furthermore, *amoA* sequences of *Nitrosocosmicus franklandus* (Lehtovirta-Morley *et al*. 2016), *Nitrosoarchaeum*

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234 *koreensis* MY1 (Jung *et al*. 2011), and *Nitrosotalea devanaterra* Nd1 (Lehtovirta-Morley *et al*. 235 2011) were added to this tree. OTU-based analyses were performed at the same level of 236 surveying effort (1,200 randomly selected sequences per sample). Calculation of the Chao1 237 index (Chao and Bunge 2002) and the Shannon index (Shannon 1948) was conducted using 238 QIIME.

239 Bacterial 16S rRNA gene sequences were pre-processed as described by Broszat *et al.* 240 (2014), except that sequences shorter than 300 bp were removed using QIIME. OTUs were 241 determined at a genetic distance of 3% using Uclust (Edgar 2010), implemented in QIIME 242 (Caporaso *et al.* 2010), and were classified by comparison of partial 16S rRNA gene 243 sequences using the SILVA SSU database (Pruesse *et al.* 2007) release 119. OTUs 244 classified as chloroplast or mitochondrion as well as unclassified OTUs were removed from 245 16S rRNA gene sequence datasets and rarefaction curves were generated using QIIME. A 246 Neighbor Joining phylogenetic tree was constructed in MEGA (version 7.0) (Kumar *et al*. 247 2016) using the Maximum Composite Likelihood model based on a ClustalW alignment of 248 sequences (truncated to a length of 457 bp) affiliated to selected OTUs, uncultured 249 *Gammaproteobacteria* closely related to these OTUs, *Chromatiaceae*, and *Acidithiobacillus*. 250 A total number of 500 bootstrap resamplings were carried out to test the tree topology.

251 **Statistical analysis**

252 Data were tested for normal distribution before performance of ANOVA. One-way ANOVA 253 and Tukey pair-wise comparisons were conducted to determine differences in basic soil 254 properties, *amoA* abundance and Shannon diversity between Hooge, Langeness, and 255 Nordstrandischmoor. The effect of salinity on *amoA* abundance was determined by ANOVA 256 of data from triplicate slurries followed by Tukey *post hoc* tests. The PAST statistical 257 package (Hammer *et al.* 2001) was used to perform Mann-Whitney U tests to identify taxa 258 showing significant differences in relative abundance between Hallig soils. Non-metric 259 multidimensional scaling was performed based on weighted Unifrac (Lozupone *et al*. 2011)

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3 260 distance matrices (genetic distance: 3%) using the vegan package (Oksanen *et al*. 2016) in
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5 261 R (R Development Core Team, 2015).
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8 262 **Enrichment of AO and analysis of cultivates**
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10
11 263 In order to enrich gammaproteobacterial AOB, potentially inhabiting temporarily flooded soil,
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13 264 sea salts-containing medium has been inoculated with sample material derived from the
14
15 265 Halligen. Incubation was carried out in 100-mL Duran bottles containing 50 mL enrichment
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17 266 medium without shaking at 28°C in the dark. Per bottle, 1 g of topsoil was used as inoculum.
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19 267 After autoclaving of 30% or 50% artificial seawater described by Stephen *et al*. (1996), the
20
21 268 following components were added aseptically (per liter) to obtain cultivation medium: 2 ml
22
23 269 1 M $NaHCO_3$, 1 ml 7.5 mM EDTA-Na_2, 1 ml non-chelated trace element mixture (Widdel and
24
25 270 Bak, 1992), 1 ml selenite-tungstate solution (Widdel and Bak, 1992), 1 ml vitamin solution
26
27 271 (Balch *et al*. 1979), and 0.2 g KH_2PO_4. Furthermore, 500 µM or 12.5 mM NH_4Cl was added.
28
29 272 The pH of the medium was adjusted to 7.8 by the addition of 5% (wt/vol) Na_2CO_3 before
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31 273 filter-sterilization through a 0.22 µM pore size bottle-top filter. Subculturing was carried out
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33 274 by transferring 5% culture into fresh enrichment medium. The pH was monitored and if
34
35 275 necessary adjusted. Growth of ammonia oxidizers was assessed by analysis of inorganic
36
37 276 nitrogen. Colorimetric ammonia and nitrite measurement was performed as described by
38
39 277 Lehtovirta-Morley *et al*. (2014). Enrichment cultures were continuously subcultured in the
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41 278 laboratory for approximately two years.
42
43
44 279 To analyse enriched bacteria, DNA was extracted and the V3-V4 region of 16S rRNA genes
45
46 280 amplified by PCR. Primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 (Klindworth *et*
47
48 281 *al*. 2013) containing modifications for Illumina MiSeq sequencing were employed. The PCR
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50 282 reaction mixture was prepared as described above for amplification of the V3-V5 region of
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52 283 bacterial 16S rRNA genes but it contained 3% instead of 5% DMSO. PCR reactions were
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54 284 initiated at 98°C (1 min), followed by 29 cycles of 98°C (45 s), 60°C (45 s) and 72°C (30 s),
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56 285 and ended with incubation at 72°C for 5 min. PCR products were purified using the
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286 NucleoMag 96 PCR kit (Macherey and Nagel, Düren, Germany) and sequences determined
287 using an Illumina MiSeq instrument. Demultiplexing and clipping of sequence adapters from
288 raw sequences was performed by employing CASAVA data analysis software (Illumina).
289 Paired-end sequences were merged using PEAR v0.9.10 (Zhang *et al*. 2014) with default
290 parameters. Subsequently, sequences with an average quality score lower than 20 and
291 containing unresolved bases were removed with the split_libraries_fastq.py script from
292 QIIME (Caporaso *et al*. 2010). Remaining primer sequences were truncated using the
293 cutadapt program (Martin, 2011) and we employed Usearch (Edgar, 2010) to sort out
294 sequences shorter than 400 bp. Chimeric sequences were removed using Uchime (Edgar *et*
295 *al*. 2011) in reference mode against the Greengenes Gold data set
296 gold_strains_gg16S_aligned.fasta (DeSantis *et al*. 2006). OTUs were determined at a
297 genetic distance of 3% using Uclust (Edgar 2010) and classified by comparison of partial
298 16S rRNA gene sequences with the SILVA SSU database (Pruesse *et al.* 2007).

299 **Sequence data deposition**

300 Sequence data were deposited in the Sequence Read Archive (SRA) of the National Center
301 for Biotechnology Information (NCBI) under the accession numbers SRP083741 and
302 SRP114677.

303 Results

304 Soil characteristics and ammonia oxidiser abundance

305 Salinity, measured as electrical conductivity, and pH were significantly higher in soils from
306 Nordstrandischmoor than in soils from Langeness or Hooge (Table 1). This is likely due to
307 the high frequency of flooding of Nordstrandischmoor soil with seawater with a pH of
308 approximately 8.1, increasing the soil pH to 7.1 - 7.9. Total N, organic C and total C did not
309 differ significantly between the three soils, but ammonium and nitrate concentrations were
310 significantly different (Table 1). Furthermore, ammonium concentrations were higher than
311 nitrate concentrations at each site (Table 1).

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312 AOA and betaproteobacterial AOB were detected in all Hallig soil samples by qPCR
313 amplification of *amoA* genes (Figure 2), with AOA and AOB *amoA* abundances, respectively,
314 ranging from 2.85×10^6 to 1.37×10^8 genes g^{-1} dry soil and 1.86×10^5 to 6.11×10^7 genes
315 g^{-1} dry soil. AOA *amoA* genes were significantly more abundant than betaproteobacterial
316 AOB *amoA* genes (P<0.05) but there was no obvious difference between the three Halligen
317 (Figure 2), although abundances of both genes were higher in the Nordstrandischmoor soil
318 than in soil from Hooge (P<0.001). No significant relationships between nitrate data and
319 AOA or AOB *amoA* gene abundance were detected.

320 **Response of ammonia oxidisers to salt concentration**

321 A soil slurry experiment was performed to assess whether soil AO inhabiting Hooge,
322 Langeness and Nordstrandischmoor, flooded by seawater at different frequencies, respond
323 differentially to salinity. AOA *amoA* abundance in Nordstrandischmoor soil slurries
324 significantly increased during incubation at 5‰ and 10‰ salinity (P<0.05, Figure 3a). In
325 addition, average AOA *amoA* abundance increased at the two highest salinities tested
326 (Figure 3a). In contrast, AOA *amoA* abundance in slurries containing Hooge or Langeness
327 soil significantly decreased after incubation with 30‰ salinity for 30 days (P<0.05, Figure
328 3a). There were no significant effects of time or salinity on betaproteobacterial AOB *amoA*
329 abundance in Nordstrandischmoor soil slurries, but there was evidence of decreases in
330 abundance in Langeness soil slurries, which were significant at 5‰ and 30‰ salinity (Figure
331 3b). In Hooge soil slurries, the only significant change was an increase in abundance at 20‰
332 after incubation for 30 days.

333 Changes in inorganic nitrogen in soil slurries are presented in Figure 4. Ammonium
334 concentration in Hooge and Langeness soil slurries was significantly greater after incubation
335 for 15 days than at 0 days. This increase is potentially due to mineralisation of existing
336 organic nitrogen. The smallest increase was detected in Hooge and Langeness soil slurries
337 containing artificial seawater with a salt concentration of 20 ‰, indicating again a preference

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for this salinity. Furthermore, combined nitrite plus nitrate concentrations significantly increased between day 15 and day 30 of incubation in Hooge slurries with an artificial seawater salinity of 20 ‰ (Figure 4). In contrast, ammonium concentration in Nordstrandischmoor slurries was significantly lower after incubation for 15 days than at 0 days at artificial seawater salinities of 10 ‰, 20 ‰ and 30 ‰.

Flooding effects on ammonia oxidiser diversity and community composition

Pyrosequencing of AOA *amoA* gene amplicons yielded 76,427 raw sequences, of which 54,122 sequences were used following quality control. These sequences contained eight OTUs, defined at 85% AOA *amoA* gene sequence identity, which has been suggested as a 'species' threshold for AOA based on phylogenetic analysis of a comprehensive set of publicly available *amoA* sequences (Pester *et al.* 2012). Comparison of rarefaction analyses with the number of OTUs calculated by the Chao1 richness estimator indicate that estimated richness was covered by the sequencing effort in 27 of the 30 analysed samples, with approximately 80% coverage in the remaining three samples, and individual samples contained 3 - 5 OTUs (Table S2). Shannon diversities for OTUs in the soils from the different Halligen were not significantly different (P=0.93, Table S2).

The dominant AOA OTUs were affiliated to *Nitrosopumilus* subcluster 1, *Nitrososphaera* subcluster 4 and *Nitrososphaera* subcluster 8. These subclusters were described previously by Pester *et al.* (2012). Strong variations in *Nitrosopumilus* subcluster 1 and *Nitrososphaera* subcluster 8 relative abundances were found between soils from the three Halligen (Figure 5). Relative abundances of *Nitrosopumilus amoA* genes were higher in Nordstrandischmoor than in Hooge soil samples (P<0.05). *Nitrososphaera* subcluster 8 relative abundances were lower in Nordstrandischmoor than in Hooge or Langeness samples (P<0.05). *Nitrososphaera* subcluster 4 relative abundance did not vary significantly between soils (Figure 5). Besides the dominant subclusters, the *Nitrosopumilus* subcluster 2, the Nitrososphaera subclusters 5 and 6 as well as the *Nitrososphaera* sister cluster were

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detected in low relative abundance (Figure S1 and S2). The overall AOA community composition differed most strongly between Hooge and Nordstrandischmoor soil samples (Figure 6).

AOB were characterised by pyrosequencing of 16S rRNA gene amplicons using primers allowing amplification of both betaproteobacterial and gammaproteobacterial AOB genes. A total of 302,395 raw sequences was obtained, 260,868 sequences of which were of suitable quality, providing 6,400 high-quality sequences per sample after normalisation (Table S3). Rarefaction curves were not saturated (Figure S3), but taxonomic analysis of OTUs revealed that the dominant bacterial phyla typically occurring in soil (*Acidobacteria*, *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia*, *Bacteroidetes*, *Firmicutes*, *Chloroflexi*, *Planctomycetes*, and *Gemmatimonadetes*) (Janssen, 2006) were detected. The 16S rRNA gene sequences comprised representatives of the betaproteobacterial genera *Nitrosospira* and *Nitrosomonas* (Figure 5). At some Hooge and Langeness sites no sequences belonging to these genera were found as *Nitrosospira* and *Nitrosomonas* were presumably below the detection limit. OTUs belonging to a cluster, which only comprises sequences of so far uncultured *Gammaproteobacteria* were detected (Figure 7). This cluster is phylogenetically closely related to known gammaproteobacterial AOB (Figure 7). For instance, "OTU_BacUnc_7", which was detected in soil derived from all Halligen, showed the highest similarity to *Nitrosococcus halophilus* strain Nc 4 (93% identity) (Koops et al. 1990) with respect to cultured bacteria. It is possible that some representatives of the mentioned cluster represent gammaproteobacterial AOB. Relative abundances of OTUs affiliated to this cluster were significantly greater in Nordstrandischmoor soil than Hooge or Langeness soils ($P<0.05$). We performed PCR using primers *amoA*-3F and *amoB*-4R as described by Purkhold *et al*. (2000) to detect gammaproteobacterial AOB but no products were obtained. Attempts were made to culture gammaproteobacterial AOB but after several subculturing steps only *Nitrosomonadales* 16S rRNA gene sequences were detected in enrichment cultures (Figure S4). The relative abundances of *Nitrosospira* and *Nitrosomonas* were significantly greater in

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391 Nordstrandischmoor soil (*P*<0.05). *Nitrosomonas* sequences derived from Hallig soils were 392 closely related to representatives of the *Nitrosomonadaceae* cluster 6b, cluster 9, and the 393 *Nitrosomonas cryotolerans* lineage. In contrast, *Nitrosospira* clusters were not consistently 394 recovered with treeing methods using 16S rRNA gene sequences to infer relationships 395 (Prosser *et al.* 2014), but *Nitrosospira* sequences detected were closely related to those 396 found in salt marsh sediment. Similar to AOA, the composition of bacterial communities 397 differed most strongly between Hooge and Nordstrandischmoor soil samples (Figure 6).

398 Discussion

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399 AOA outnumber betaproteobacterial AOB in temporarily flooded soil

400 Estimated *amoA* gene abundances are in the range of those determined for soil or sediment 401 derived from other coastal environments (Moin *et al.* 2009; Marton *et al.* 2015), except that 402 maximum AOA *amoA* gene abundances were approximately one order of magnitude higher 403 in some New England salt marsh sites (Moin *et al.* 2009). Similar to our study, Moin *et al.* 404 (2009) and Marton *et al.* (2015) also reported greater abundance of AOA than 405 betaproteobacterial AOB *amoA* genes in New England and Louisiana salt marsh samples. 406 High abundances of AOA in coastal soil and sediment suggest that these habitats represent 407 an important niche for archaeal nitrifiers. Nevertheless, AOA do not always outnumber AOB 408 in marine sediment, salt marshes or river mouths (Bernhard and Bollmann 2010; Li *et al.* 409 2015; Dini-Andreote *et al.* 2016). Besides AOA and betaproteobacterial AOB, a cluster 410 phylogenetically closely related to gammaproteobacterial AOB was detected in temporarily 411 flooded soil based on high-throughput amplicon sequencing of bacterial 16S rRNA genes. 412 The number of known complete *amoA* sequences from gammaproteobacterial AOB is very 413 limited (Junier *et al.* 2008), restricting the design of primers targeting total communities of 414 these microbes in environmental samples. Therefore, we did not perform *amoA*-specific 415 quantitative PCR to analyse the abundance of gammaproteobacterial AOB, potentially 416 existing in Hallig soil.

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417 **AO inhabiting frequently flooded soil tolerate a wide salinity range**

418 The salinity of the Wadden Sea, surrounding the German Halligen, is similar to that of
419 artificial seawater with a salt concentration of 30‰. Due to frequent flooding,
420 microorganisms inhabiting Nordstrandischmoor topsoil are often exposed to high salinities.
421 Accordingly, in contrast to Hooge and Langeness soil slurries, no decrease in AOA and AOB
422 *amoA* abundance was determined for Nordstrandischmoor soil slurries at the highest salinity
423 tested (30‰). Salt concentration in Nordstrandischmoor soil changes over time and can be
424 affected by environmental factors other than seawater inundation (e.g., rain events). Thus,
425 microorganisms colonising this dynamic habitat must tolerate a wide range of salinities. This
426 might explain why AO *amoA* abundances in Nordstrandischmoor soil slurries increased or
427 did not decrease at any of the tested salinities. Due to differences in Hallig-surrounding
428 protection constructions, Hooge and Langeness are less frequently flooded than
429 Nordstrandischmoor (Schindler *et al.* 2014a; Schindler *et al.* 2014b). This could be a major
430 reason why Nordstrandischmoor soil AO show better adaptation toward Wadden Sea water
431 salinity than those inhabiting Hooge or Langeness soil.

432 Similar to *amoA* abundances, measured ammonia and nitrite/nitrate concentrations indicated
433 a preference by Hooge and Langeness soil AO for an artificial seawater salinity of 20‰.
434 Ammonium concentration did not increase in Nordstrandischmoor soil slurries at any of the
435 salinities investigated. Considering previous studies focusing on coastal soil or sediment, an
436 inconsistency in relationships between AO abundance and ammonium or nitrite/nitrate
437 concentration and nitrification potential rate is evident (Dollhopf et al 2005, Marton et al
438 2015, Moin et al 2009). For instance, no significant correlations were identified at different
439 New England salt marsh sites between ammonium or nitrate concentrations and potential
440 nitrification rates, AOA *amoA* abundance, or betaproteobacterial AOB *amoA* abundance
441 (Moin *et al.* 2009). In contrast, Marton *et al.* (2015) found a significant correlation between
442 nitrification and betaproteobacterial AOB and AOA abundances when analysing Louisiana
443 salt marsh samples. Most previous studies of coastal soil or sediment do not assess or

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444 report the presence of gammaproteobacterial AOB. Here, we detected OTUs belonging to a
445 cluster, comprising so far only uncultured bacteria, which is phylogenetically closely related
446 to known gammaproteobacterial AOB. We could identify this cluster also in the phylogenetic
447 tree included in the SILVA (Quast et al. 2013) dataset
448 "SSURef_NR99_128_SILVA_07_09_16_opt.arb". It is possible that representatives of this
449 cluster contribute to nitrification in Hallig soil and other coastal soils or sediments, potentially
450 partly explaining the absence of consistent correlations between activity measurements and
451 AO abundance. Nevertheless, as we could not identify gammaproteobacterial AOB in
452 enrichment cultures and detected no gammaproteobacterial *amoA* genes, it is also possible
453 that the cluster comprises no AO.

454 **Selective association of study site and AO phylotype relative abundance**

455 Previous studies reported the absence or low abundance of *Nitrosopumilus amoA* genes at
456 a number of sites, including woodland, arable, spruce forest, and tundra soil (Nicol *et al.*
457 2008; Wessén *et al.* 2011; Pester *et al.* 2012), whereas all samples derived from the
458 German Halligen harboured *Nitrosopumilus amoA*. Different cultured *Nitrosopumilus*
459 representatives tolerate seawater salinities and preferably grow at near neutral or slightly
460 alkaline pH (Qin *et al.* 2014). In addition, genes encoding a mechanosensitive channel
461 protein and ectoine biosynthesis clusters were identified in microbes affiliated to
462 *Nitrosopumilus* (Walker *et al.* 2010; Blainey *et al.* 2011; Mosier *et al.* 2012).
463 Mechanosensitive channels protect cells from hypo-osmotic shock when conditions change
464 from e.g., high osmolarity to low osmolarity, and ectoine, a compatible solute, can be
465 produced when salinity is high to prevent influx of sea salt into the cell. Frequent flooding of
466 Nordstrandischmoor soil leads to temporarily high salinity and strong temporal fluctuations,
467 which potentially explain increased relative abundance of *Nitrosopumilus* in
468 Nordstrandischmoor samples compared to soil derived from Hooge and Langeness.
469 *Nitrososphaera* subcluster 8 has been detected in terrestrial environments such as upland
470 agricultural, pristine forest or meadow soil, and also in estuarine sediment (see consensus

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tree provided by Pester *et al.* (2012)), but information about ecophysiology of this subcluster is very limited and no cultured representatives are currently available. High relative abundances of *Nitrososphaera* subcluster 8 in Hooge and Langeness samples might be due to soil salinity and pH preferences. Sequences affiliated to *Nitrososphaera* subcluster 4 were found in various terrestrial and marine habitats such as sandy ecosystem soil, hot springs, and lake, deep sea or marsh sediment (see consensus tree provided by Pester *et al.* (2012)). Thus, *Nitrososphaera* subcluster 4 thrives in ecosystems covering a wide range of environmental conditions, which might explain similar relative abundances of this subcluster in Hooge, Langeness and Nordstrandischmoor soil.

Nitrosomonadaceae cluster 6b, cluster 9, and the *Nitrosomonas cryotolerans* lineage comprise microbes derived from marine habitats and cultured representatives with obligate salt requirements (optimal NaCl concentration: 300-400 mM) such as *Nitrosomonas marina* (Koops *et al.* 1991; Prosser *et al.* 2014). It can be assumed that increased abundances of the identified *Nitrosomonas* and *Nitrosospira* representatives in Nordstrandischmoor samples are associated with high soil salinities. Increased relative abundances of bacteria belonging to a cluster, which is phylogenetically closely related to known gammaproteobacterial AOB were detected in Nordstrandischmoor soil. This might be due to a preference for high salt concentration and pH values. Nevertheless, currently information on metabolic or physiological features of representatives affiliated to the mentioned cluster is lacking and no cultures are available. It is possible that isolation of the so far uncultured *Gammaproteobacteria* might require very specific culturing conditions.

Conclusions

AOA outnumbered their betaproteobacterial counterparts in rarely and rather frequently flooded soil but in contrast to our first hypothesis obvious differences in *amoA* gene abundance between the three islands were not detected. Nevertheless, apart from these microbial groups gammaproteobacterial AOB might contribute to nitrification in soil

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3 497 temporarily inundated with seawater. AO indigenous to soil naturally exposed to seawater at
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5 498 a high frequency tolerated a wide range of salinities (e.g., Wadden Sea water salinity),
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7 499 supporting our second hypothesis. Furthermore, the community composition of both AOA
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9 500 and AOB differed significantly between the islands. In addition to flooding, other factors such
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11 501 as specific site differences might have evoked the changes in community composition. In
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13 502 line with our third and fourth hypotheses, increased abundances of archaeal and bacterial
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15 503 ammonia oxidisers typically occurring in marine environments were detected in soil derived
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17 504 from the most frequently flooded island. Seawater flooding significantly affects soil salinity
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19 505 and pH which probably strongly contributed to selection of detected AO in the analysed
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21 506 samples. Besides salinity and pH other parameters such as oxygen availability, which were
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23 507 not determined in this study, are influenced by seawater flooding. Based on our data, we
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25 508 cannot analyse the proportional importance of these parameters with respect to the
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27 509 distribution of AO in temporarily flooded soil. Nevertheless, our results indicate that potential
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29 510 future sea level rise and an associated increase in flooding of coastal soils will enrich
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31 511 nitrifiers affiliated to e.g., *Nitrosopumilus*, comprising many representatives tolerating
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33 512 seawater salinity and slightly alkaline pH. To test if the changes in AO communities reported
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35 513 here also occur in other soils temporarily inundated with seawater, further sites representing
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37 514 gradients of flooding frequency in different geographic regions should be analysed in future
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39 515 surveys. A cluster comprising uncultured bacteria closely related to gammaproteobacterial
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41 516 AOB detected in this study has to be studied in more detail. For instance, single cell
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43 517 sequencing could be used to gain information on the metabolic potential of this cluster.
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45 518 Currently, no cultured representative from different microbial groups such as *Nitrososphaera*
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47 519 subcluster 4 inhabiting temporarily flooded soil are available. AO cultures from soil
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49 520 temporarily flooded by seawater allow investigation of mechanisms enabling tolerance to
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51 521 e.g., changes in salinity. Genomic analyses of such cultures are required for identification of
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53 522 genes potentially involved in responses to osmotic stress. In addition, physiological studies
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55 523 are necessary to analyse e.g., tolerance of single ammonia oxidizer species toward changes
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57 524 in salinity.
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Supplementary Material

Table S1. Coordinates of the sampling sites.

Table S2. Number of amplicon pyrosequencing-derived AOA *amoA* gene sequences and OTU-based analyses at 85% genetic similarity.

Table S3. Number of amplicon pyrosequencing-derived 16S rRNA gene sequences and bacterial diversity as assessed by the Shannon index at 97% genetic similarity.

Figure S1. Consensus tree of AOA *amoA* including representative sequences of OTUs affiliated to the *Nitrosopumilus* cluster. The following OTUs determined in this study represent the *Nitrosopumilus* cluster: OTU1 (Ho, 14.0 ± 3.8%; La: 54.0 ± 9.7%; No: 69.3 ± 6.8%) and OTU8 (Ho, 0.00000%; La: 0.00004 ± 0.00004%; No: 0.00025 ± 0.00010%). Sequences were added to the consensus tree for AOA *amoA* phylogeny constructed by Pester *et al*. (2012) as described in the Materials and Methods section. This tree was then pruned. Accession numbers and environmental sources are given in square brackets. Abbreviations: Ho, Hooge; La, Langeness; No, Nordstrandischmoor. For more details with respect to the different subclusters see Pester *et al*. (2012).

Figure S2. Consensus tree of AOA *amoA* including representative sequences of OTUs affiliated to the *Nitrososphaera* and *Nitrososphaera* sister cluster. The following OTUs determined in this study represent the *Nitrososphaera* cluster: OTU2 (Ho, 52.4 ± 14.1%; La: 25.3 ± 10.6%; No: 0.03 ± 0.02%), OTU3 (Ho: 32.8 ± 11.2%; La: 20.6 ± 6.5%; No: 29.8 ± 6.7%), OTU4 (Ho, 0.00116 ± 0.00063%; La: 0.00000%; No: 0.00000%), OTU5 (Ho, 0.00699 ± 0.00361%; La: 0.00052 ± 0.00042%; No: 0.00801 ± 0.00244%) and OTU6 (Ho, 0.00022 ± 0.00011%; La: 0.00000%; No: 0.00006 ± 0.00006%). In addition, the *Nitrososphaera* sister cluster is represented by OTU7 (Ho, 0.00000%; La: 0.00009 ± 0.00006%; No: 0.00000%). Sequences were added to the consensus tree for AOA *amoA* phylogeny constructed by

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Pester *et al*. (2012) as described in the Materials and Methods section. This tree was then pruned. Accession numbers and environmental sources are given in square brackets. Abbreviations: Ho, Hooge; La, Langeness; No, Nordstrandischmoor. For more details with respect to the different subclusters see Pester *et al*. (2012).

Figure S3. Rarefaction analyses of bacterial OTUs at 97% genetic similarity in Hooge, Langeness and Nordstrandischmoor soil.

Figure S4. Relative abundances of dominant bacterial genera in enrichment cultures. Soil derived from sampling sites Ho1, No1, No2 and No3 was used as inoculum. The values 30% and 50% indicate the dilution of artificial seawater used for enrichment (see Materials and Methods section). Enrichment medium exhibited a concentration of 500 µM or 12.5 mM NH_4Cl.

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Tables

Table 1. Characteristics of soil samples from Hooge, Langeness and Nordstrandischmoor.

Soil property	Study sites			ANOVA
	Hooge	Langeness	Nordstrandischmoor	*P*
Electrical conductivity (mS cm^{-1})	3.6 ± 0.4 a	4.0 ± 0.3 a	7.6 ± 0.8 b	**<0.001**
pH	6.5 ± 0.4 a	6.5 ± 0.0 a	7.6 ± 0.1 b	**0.009**
Total C (g kg^{-1})	50.0 ± 8.3 a	48.0 ± 3.0 a	46.5 ± 2.6 a	0.632
Organic C (g kg^{-1})	49.8 ± 8.3 a	48.0 ± 3.0 a	45.1 ± 2.7 a	0.547
Total N (g kg^{-1})	4.3 ± 0.7 a	3.9 ± 0.2 a	4.1 ± 0.3 a	0.589
NH_4^+-N (mg kg^{-1})	12.1 ± 2.5 a	5.2 ± 0.3 b	8.2 ± 1.0 ab	**<0.001**
NO_3^--N (mg kg^{-1})	0.6 ± 0.1 ab	0.4 ± 0.0 a	1.1 ± 0.6 b	**0.022**

Data are presented as mean and standard errors. Thirty soil samples, described in the Materials and Methods section, were considered. Significant ANOVA *P* values are shown in bold ($P < 0.05$). Figures followed by different letters indicate differences among Halligen. NO_2^- concentration was negligible and NO_x is therefore equivalent to NO_3^--N mg kg^{-1}.

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Figure legends

Figure 1. Maps depicting the German Halligen Hooge (Ho), Langeness (La), and Nordstrandischmoor (No). The locations of the three Halligen and the ten sampling sites are highlighted by black arrows and coloured crosses, respectively. Triplicate samples, 1 m apart, were taken from each site. The large scale map was generated using the Ocean Data View software version 4.7.10 (Schlitzer 2017) (http://odv.awi.de/).

Figure 2. Abundance of AOA and betaproteobacterial AOB *amoA* genes in Hooge, Langeness, and Nordstrandischmoor soils samples. Data are presented as mean and standard error of triplicate samples for each site. Abbreviations: Ho, Hooge; La, Langeness; No, Nordstrandischmoor. A total of 30 soil samples was analysed.

Figure 3. Abundance of (a) AOA and (b) betaproteobacterial *amoA* genes in soil slurries incubated for 30 days. Slurries contained soil from Hooge (Ho), Langeness (La), or Nordstrandischmoor (No) and artificial seawater at salinities of 5‰, 10‰, 20‰ or 30‰. Data are presented as mean and standard error of triplicate samples for each site. Asterisks indicate a significant increase (green asterisks) or decrease (red asterisks) in *amoA* abundance after incubation for 15 or 30 days (P<0.05) (the asterisks indicate changes from the initial abundance determined at day 0). A total of 108 soil slurry samples was analysed.

Figure 4. Changes in ammonium and combined nitrite plus nitrate concentrations during incubation of soil slurries for 30 days.

Figure 5. Relative abundances of dominant AOA *amoA* OTUs determined at 85% genetic similarity and *Nitrosospira* and *Nitrosomonas* 16S rRNA gene sequences. Data are presented as mean and standard error. Different letters above the bars represent significant differences in relative abundance of ammonia oxidiser subclusters or genera (P<0.05). Abundances of *Nitrosospira* and *Nitrosomonas* relative to all bacterial sequences are depited. Abbreviations: Ho, Hooge; La, Langeness; No, Nordstrandischmoor; subcl., subcluster. A total of 30 soil samples were analysed.

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Figure 6. Non-metric multidimensional scaling (NMDS) of AOA and bacterial community composition based on weighted Unifrac. Abbreviations: Ho, Hooge; La, Langeness; No, Nordstrandischmoor.

Figure 7. Phylogenetic tree based on 16S rRNA gene sequences affiliated to selected OTUs identified in this study, uncultured *Gammaproteobacteria* closely related to these OTUs, *Chromatiaceae*, and *Acidithiobacillus*. Bootstrap values ≥50% are indicated at the corresponding notes. The bar represents 0.02 changes per nucleotide position. Abundances of OTUs relative to all bacterial sequences are depicted for Hooge (Ho), Langeness (La) and Nordstrandischmoor (No) soil. Some of the considered OTUs could only be detected in soil derived from one or two Halligen. Accession numbers and environmental sources are given in square brackets. The 16S rRNA gene sequence of *Nitrosococcus wardiae* D1FHS (Wang *et al*. 2017) has been provided by Drs. Lin Wang and Martin G. Klotz, UNC Charlotte.

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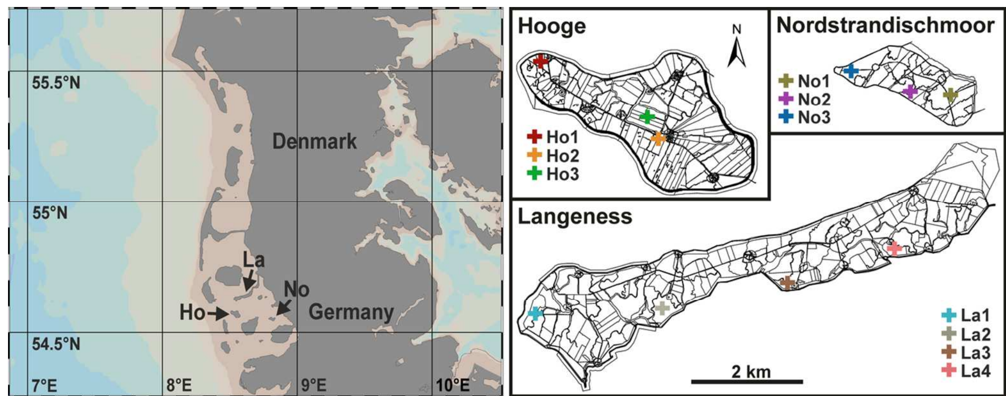

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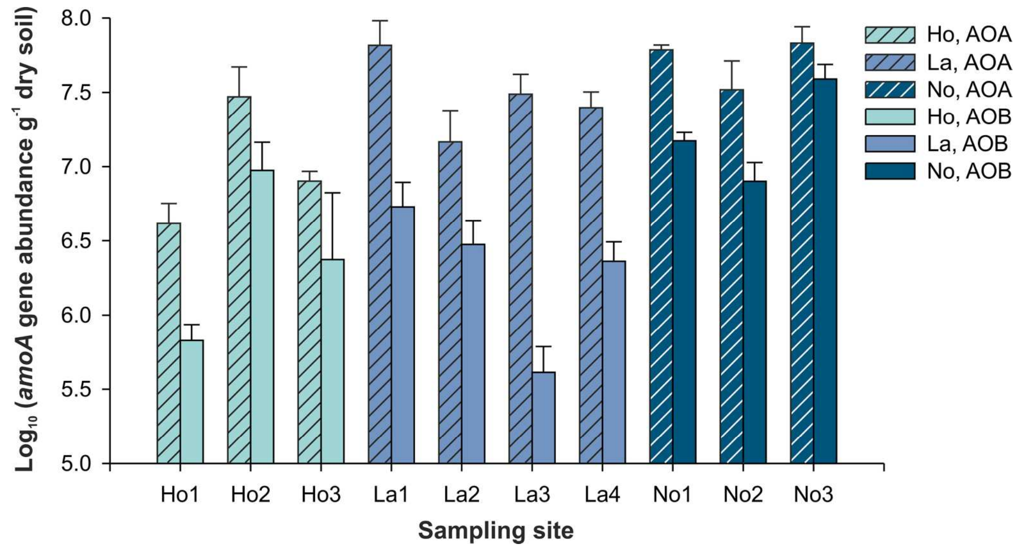

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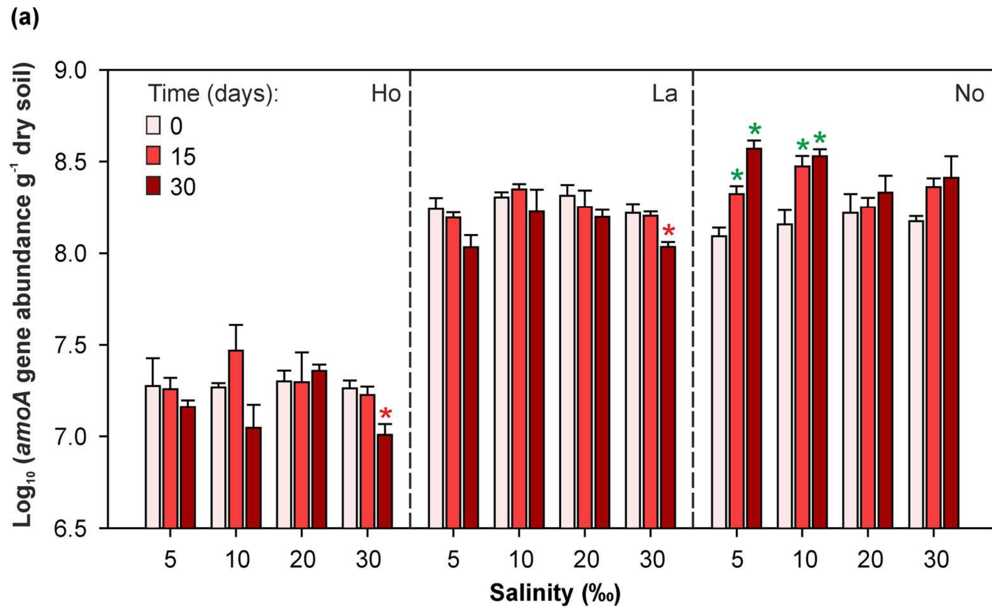

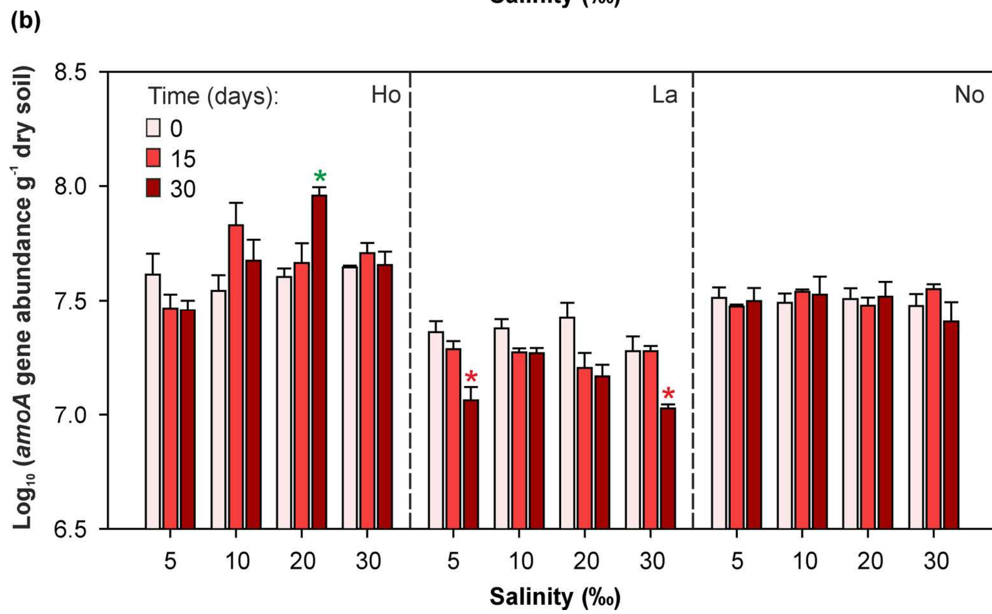

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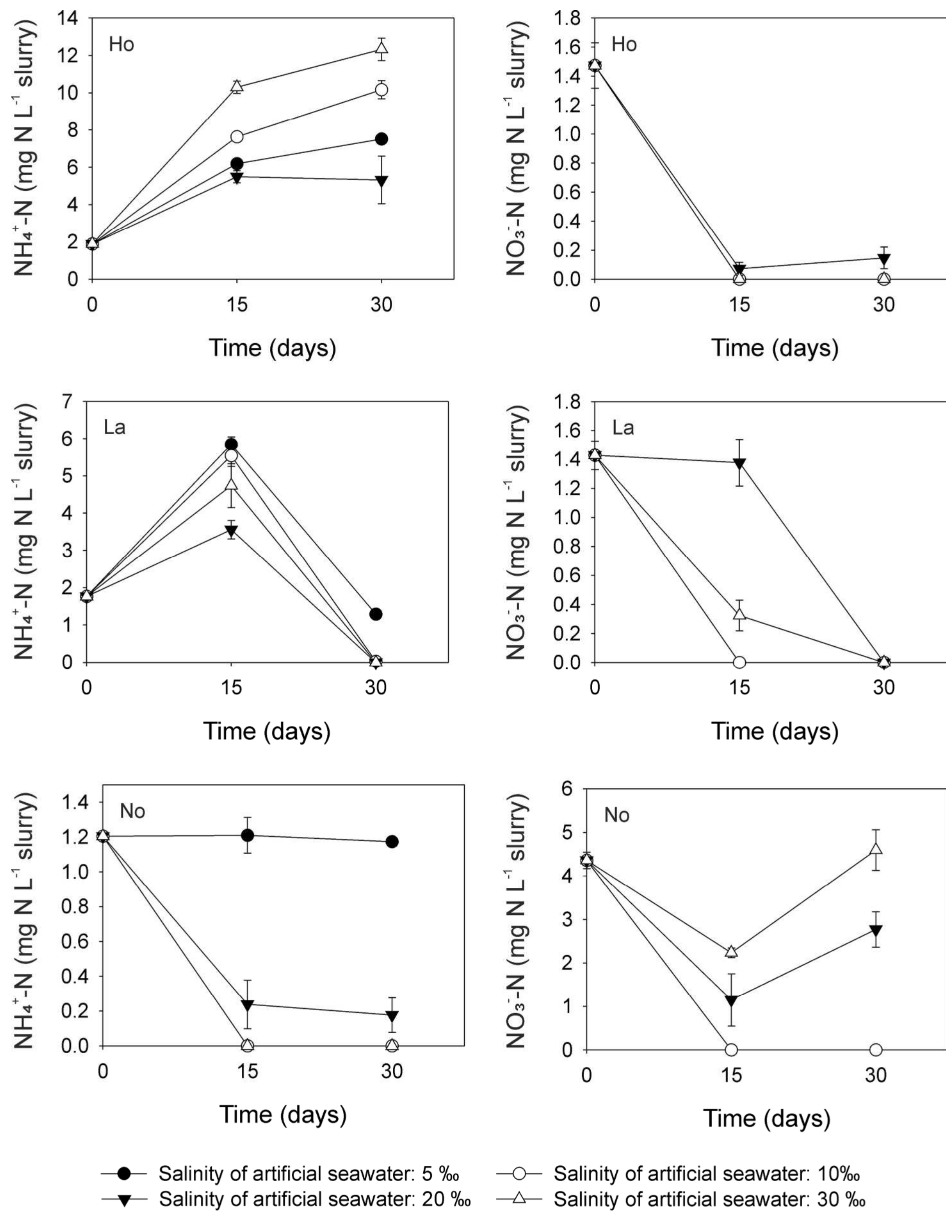

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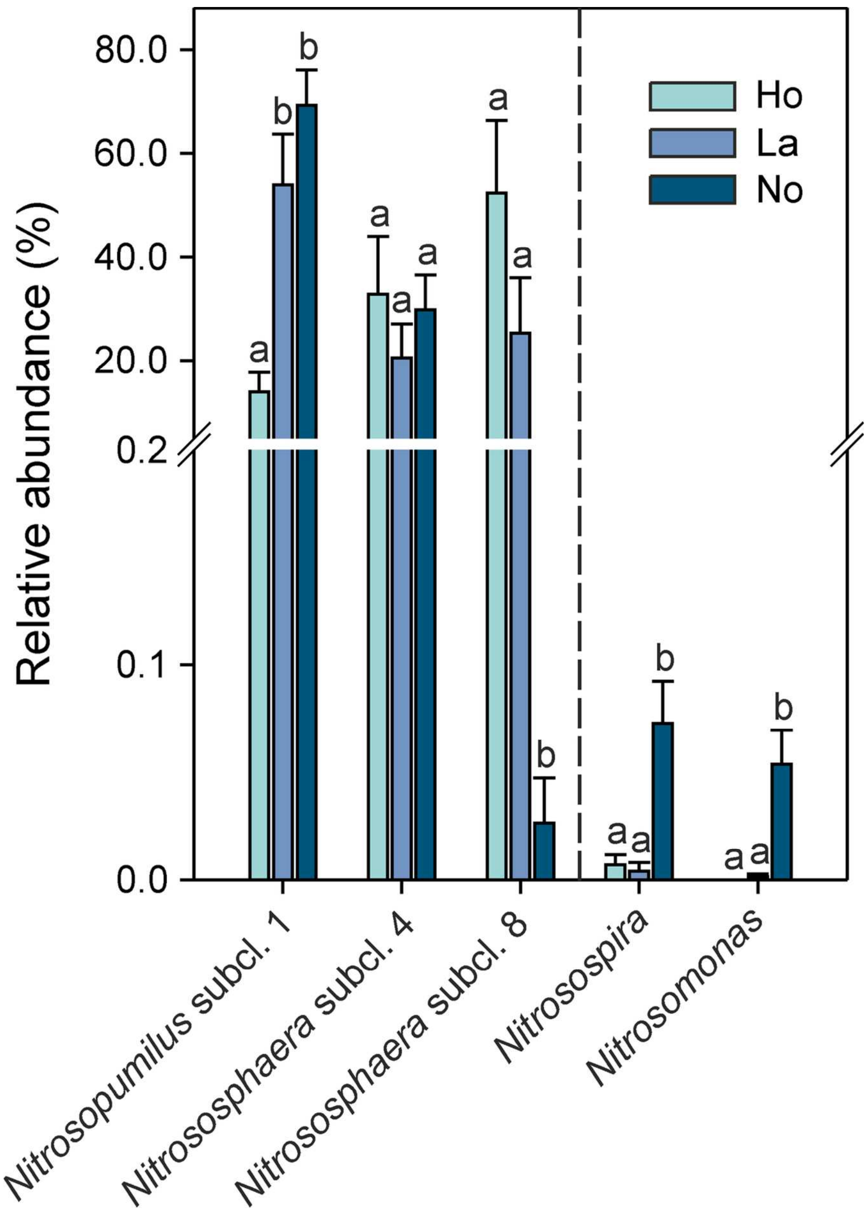

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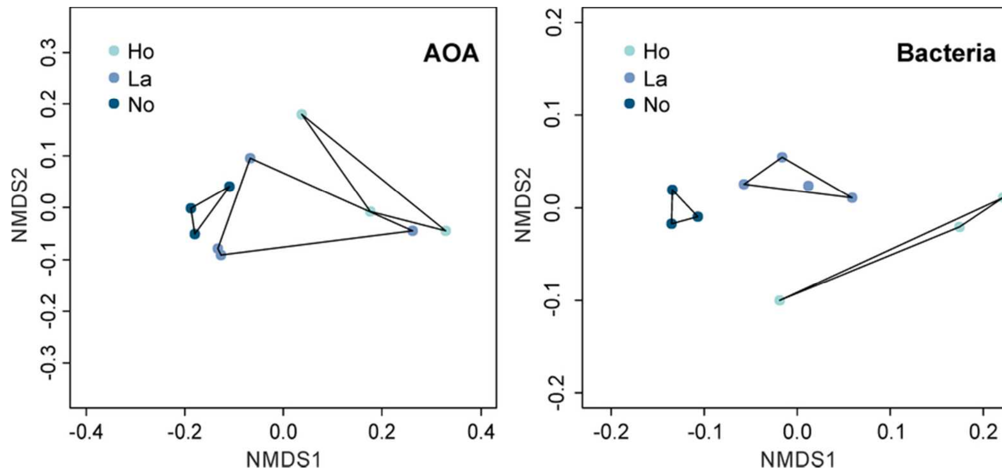

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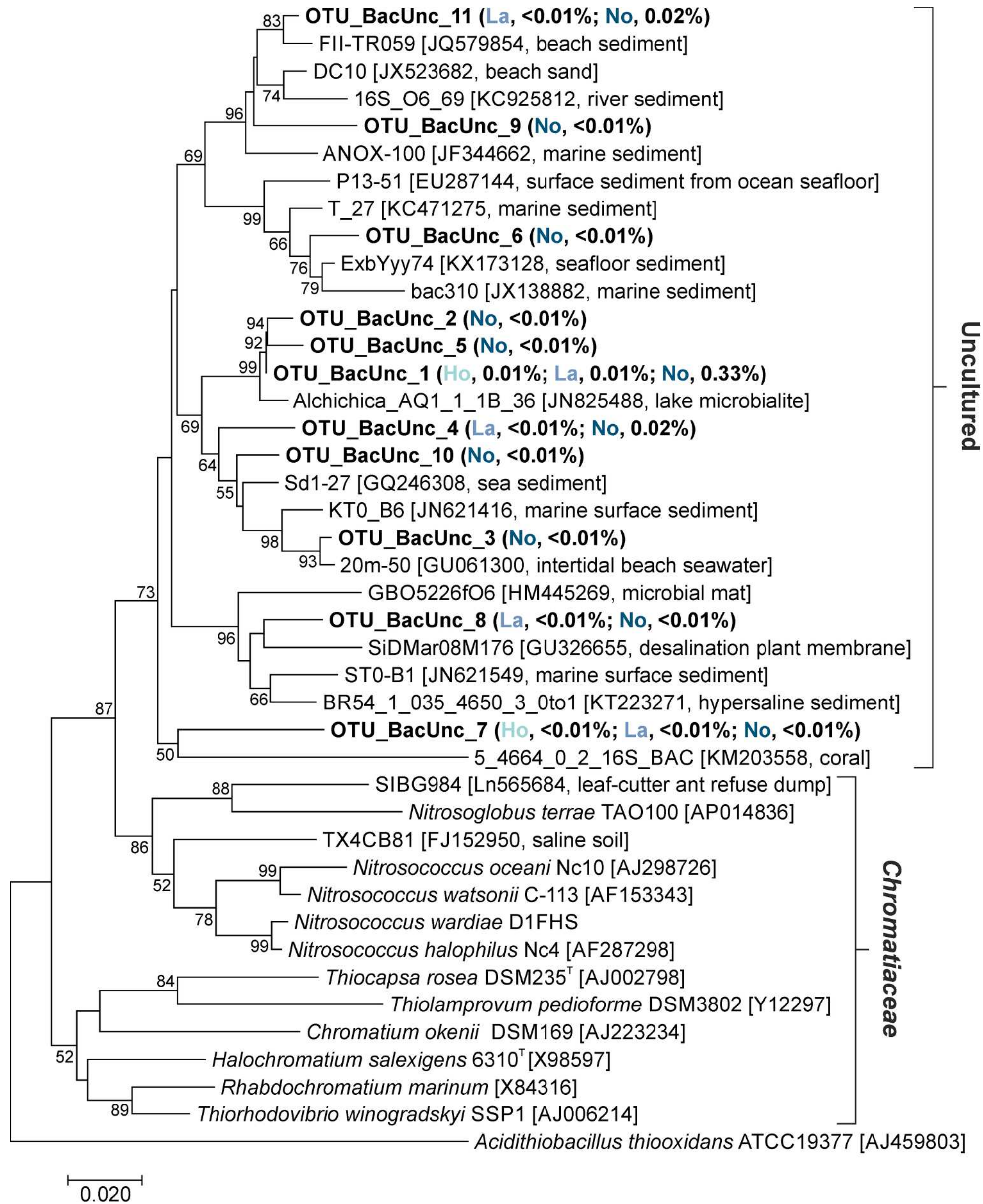

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Table S1. Coordinates of the sampling sites.

Hallig	Sampling site	Latitude (ºN)	Longitude (ºE)
Hooge	Ho1	54.57817	8.51445
Hooge	Ho2	54.56572	8.54817
Hooge	Ho3	54.56888	8.54468
Langeness	La1	54.62908	8.53368
Langeness	La2	54.63002	8.57032
Langeness	La3	54.63443	8.60470
Langeness	La4	54.63967	8.63360
Nordstrandischmoor	No1	54.54867	8.82168
Nordstrandischmoor	No2	54.54857	8.80983
Nordstrandischmoor	No3	54.55162	8.79447

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Table S2. Number of amplicon pyrosequencing-derived AOA *amoA* gene sequences and OTU-based analyses at 85% genetic similarity.

Sample	Number of raw sequences	Number of pre-processed sequences	Number of observed OTUs	Chao1-based OTU number prediction	Shannon index
Ho1a	2,795	1,760	4	4	0.72
Ho1b	2,429	1,512	5	5	0.56
Ho1c	2,598	1,428	5	5	0.64
Ho2a	2,550	1,945	4	4	0.07
Ho2b	2,437	1,788	4	4	0.20
Ho2c	2,125	1,646	3	3	0.03
Ho3a	2,374	1,728	5	5	0.98
Ho3b	2,326	1,777	5	6	0.97
Ho3c	2,261	1,729	5	6	0.98
La1a	2,886	2,023	5	5	0.60
La1b	2,282	1,540	3	3	0.78
La1c	2,458	1,781	3	3	0.80
La2a	2,686	1,953	5	5	0.56
La2b	2,550	1,766	4	4	0.32
La2c	2,493	1,863	3	3	0.93
La3a	2,627	2,055	3	3	0.47
La3b	2,748	2,053	3	3	0.65
La3c	1,697	1,218	3	3	0.33
La4a	2,594	1,704	4	4	0.24
La4b	2,775	1,847	3	3	0.40
La4c	2,706	1,890	3	3	0.78
No1a	2,269	1,588	5	6	0.51
No1b	2,497	1,868	3	3	0.64
No1c	2,775	1,766	4	4	0.56
No2a	2,801	2,055	4	4	0.46
No2b	2,572	1,864	3	3	0.61
No2c	2,789	2,015	5	5	0.58
No3a	2,847	2,011	3	3	0.68
No3b	3,107	2,131	3	3	0.75
No3c	2,373	1,818	4	4	0.31
All samples	76,427	54,122	8	8	1.08

OTU analysis of single samples was performed with the same number of sequences (1,200 randomly selected high-quality sequences per sample).

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Table S3. Number of amplicon pyrosequencing-derived 16S rRNA gene sequences and bacterial diversity as assessed by the Shannon index at 97% genetic similarity.

Sample	Number of raw sequences	Number of preprocessed sequences	Shannon index
Ho1a	9,628	8,418	6.39
Ho1b	12,062	10,285	6.54
Ho1c	7,310	6,408	6.56
Ho2a	10,104	8,797	5.65
Ho2b	10,402	9,155	5.62
Ho2c	10,015	8,329	5.50
Ho3a	12,550	11,057	5.57
Ho3b	10,805	9,386	5.40
Ho3c	9,788	8,732	5.45
La1a	12,400	10,483	6.15
La1b	8,347	6,977	6.31
La1c	9,104	7,526	6.42
La2a	10,793	9,383	6.09
La2b	9,386	8,285	6.31
La2c	9,752	8,577	6.10
La3a	8,926	7,623	5.92
La3b	7,457	6,526	5.90
La3c	8,771	7,384	6.07
La4a	9,748	8,300	6.16
La4b	10,668	9,246	6.00
La4c	8,248	7,303	6.03
No1a	11,541	10,092	6.24
No1b	11,021	9,173	6.12
No1c	10,797	9,078	6.15
No2a	10,836	9,474	6.26
No2b	10,386	8,704	6.40
No2c	9,963	8,698	6.30
No3a	10,472	9,082	6.30
No3b	9,984	8,701	6.37
No3c	11,131	9,686	6.32

Calculation of the Shannon index was performed with the same number of sequences (6,400 randomly selected high-quality sequences per sample).

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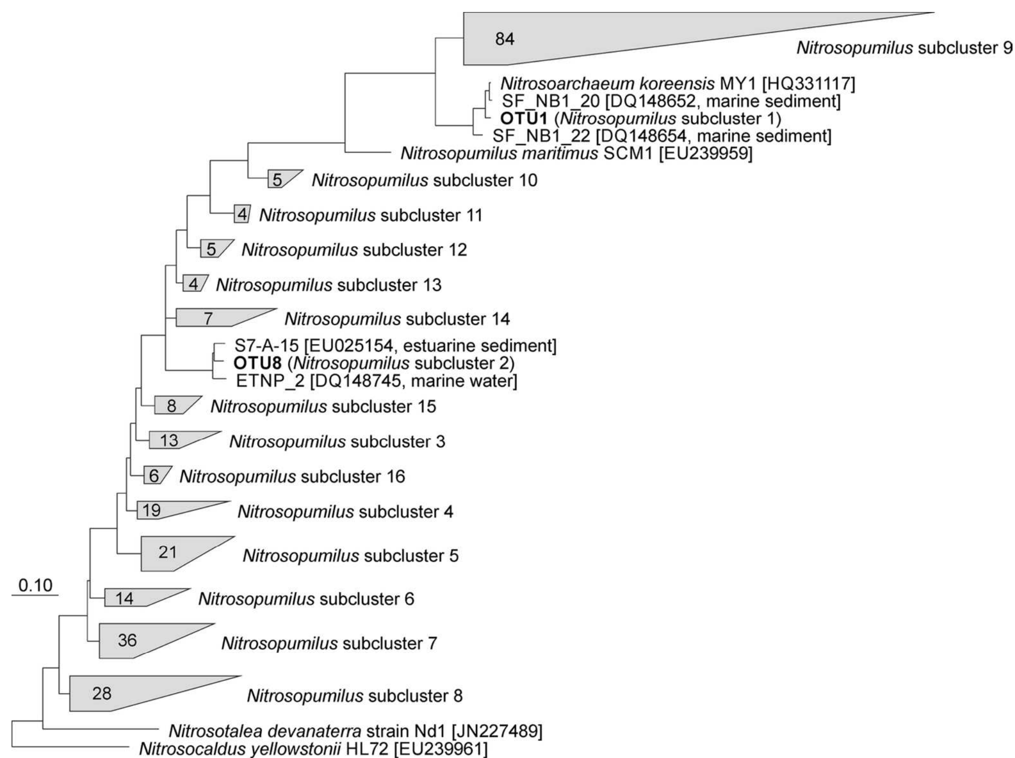

134x100mm (300 x 300 DPI)

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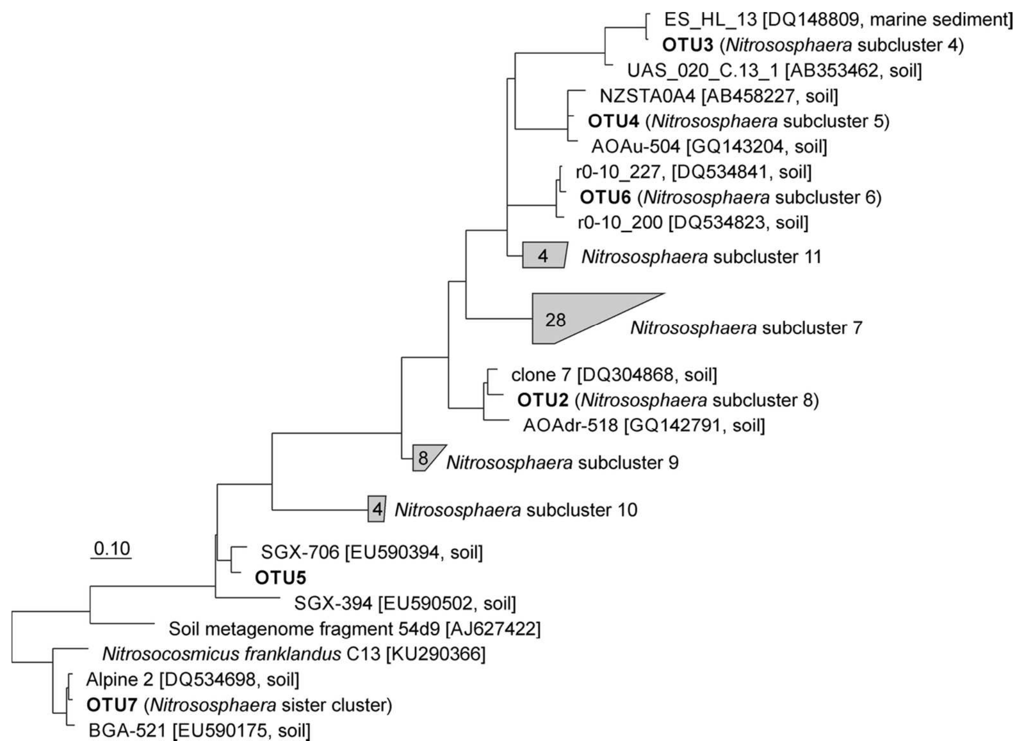

123x90mm (300 x 300 DPI)

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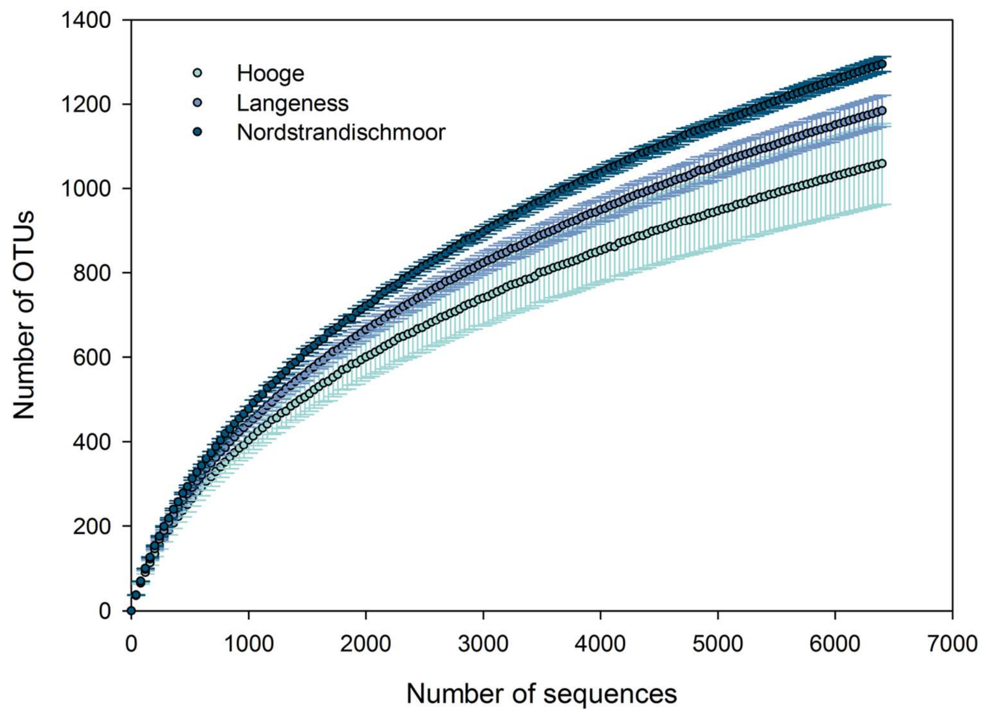

119x94mm (300 x 300 DPI)

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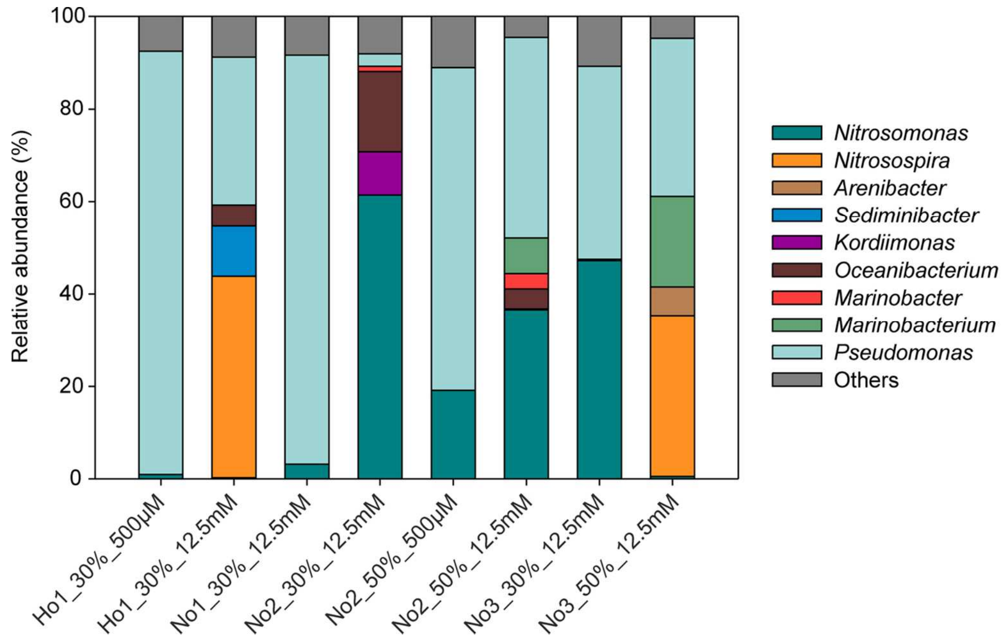

121x76mm (300 x 300 DPI)

Review